Medicinal Chemistry of Nicotinamide in the Treatment of Ischemia and Reperfusion

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Abstract: Nicotinamide can facilitate DNA repair by inhibiting poly(ADP-ribose) polymerase, increasing NAD levels and adjusting other related enzyme activities. This review will summarize recent work on the design of poly(ADP-ribose) polymerase inhibitors, poly(ADP-ribose) glycohydrolase inhibitors and will discuss the possible use of drugs that interact with NAD synthetic enzymes.

INTRODUCTION

Ischemic stroke is a major health care problem worldwide for which the mechanism is not completely defined. In the past few years, the identification of new mechanisms involved in neuronal death and particularly in apoptosis has shed light on the development of neuroprotective therapy. Glutamate neurotoxicity is involved in the cell death associated with stroke, since glutamate is released during ischemia-reperfusion. Glycine site antagonism of N-methyl-D-aspartate (NMDA) receptors may offer a means to block glutamate neurotoxicity. Inflammatory processes executed by some proinflammatory molecules contribute to secondary brain injury associated with stroke. The neutral protease calpain is capable of degrading critical cytoskeletal and regulatory proteins, mainly causing postischemic neuronal necrosis. Caspases, a family of cysteine proteases, are at the heart of the apoptotic pathway and degrade critical enzymes involved in DNA repair. Severe DNA damage induced by oxidative stress or apoptotic stimuli activates poly (ADP-ribose) polymerase (PARP, EC 2.4.2.30), causing a rapid depletion of nuclear NAD pools, cellular energy, and thiols. Inhibition of these various enzymes may be expected to achieve effective neuroprotection without serious side effects, because the mechanisms seem relatively unimportant in normal neurotransmission [1].

Based on these new molecular mechanisms, novel therapeutic strategies for stroke are being investigated. There are various candidate drugs to promote cell survival and synaptic plasticity such as anticoagulants, thrombolytic agents, and neuroprotectants [2,3]. Among them, nicotinamide is one well known neuroprotective agent that can prevent necrosis and apoptosis effectively [4]. Inhibition of DNA fragmentation, or rapid repair of DNA fragmentation, is a crucial factor in the inhibition of apoptosis and necrosis by nicotinamide.

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MEDICINAL CHEMISTRY OF NICOTINAMIDE

Nicotinamide is a soluble B group vitamin, which has been used since 1938 to treat nicotinamide deficiency (pellagra) that involves dermatitis, diarrhea and dementia. Prior to nicotinamide therapy, there were as many as 10,000 cases of pellagra in the USA each year. Many of the cases were boys between the ages of 10 and 15. Pellagra killed more than 3,000 people every year due to the neurodegeneration caused by nicotinamide deficiency. However, with improvement of dietary habits and the introduction of nicotinamide, pellagra is not a common disease. Death rates from pellagra have dropped from 30% to 1% or less. Pellagra can still be found in some poor areas, in alcoholics, other malnourished people and some elderly people [5,6]. It has been reported that epidemic optic neuropathy in Cuba was associated with niacin deficiency [7]. Niacin is converted to nicotinamide (niacinamide) in the body. Both niacin and nicotinamide are called vitamin B_3.

The biochemical activity of nicotinamide or niacin in the body is based on the conversion into nicotinamide adenine dinucleotide (NAD). There are several important enzymes such as nicotinamidase, nicotinamide phosphoribosyl transferase, NMN adenyl transferase, nicotinic acid phosphoribosyl transferase, NMN adenyl transferase and NAD synthetase involved in this biosynthetic pathway [8]. NAD is a substrate for PARP that attaches ADP-ribose usually to glutamate residues of enzymes and synthesizes poly (ADP-ribose). These polymers are generally 40-50 branching residues and are synthesized onto a number of different enzymes, including PARP, in order to alter their activities. DNA damage after ischemic insults activates PARP [8]. PARP is required for repair of many forms of DNA damage [8]. ADP-ribose polymer synthesis comes at a very high cost of energy. It has been found that excessive activation of PARP may result in NAD rundown and subsequently ATP depletion [9,10]. Further depletion of energy stores under already ischemic conditions can lead to necrosis [11] and apoptosis [12] of neurons.

Nicotinamide has two important actions in the nucleus, it is required for NAD synthesis and it can weakly inhibit PARP. Administration of nicotinamide leads to increased brain levels of NAD and ATP [13,14]. Nicotinamide, by

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providing NAD to PARP, can increase the repair of DNA damage during oxidative stress caused by reperfusion [13,14]. This prevents the induction of apoptosis and necrosis in the brain. Nicotinamide inhibition of the catalytic site of PARP inhibits the use of NAD by the enzyme, but may not interfere with the DNA nick binding of the zinc fingers of PARP. Therefore, nicotinamide may prevent the depletion of NAD, yet not prevent the stabilization and repair of DNA.

EFFECTS OF NICOTINAMIDE ON PARP

PARP, an abundant nuclear protein in all nucleated cells, is involved in DNA repair and participates in cell proliferation, differentiation and transformation. PARP is also found in the cytoplasms of neurons [15], which suggests that it may be translocated into the nucleus during apoptosis. However, PARP is so abundant in the nucleus that translocation may not be necessary. PARP catalyzes the covalent attachment of ADP-ribose subunits from its substrate, NAD, to numerous nuclear proteins, including PARP itself. PARP can also hydrolyze NAD. Formation of poly(ADP-ribose) is a unique post-translational modification that can be induced by DNA strand breaks caused by exposure to nitric oxide (NO) or oxygen-free radicals [16,17]. Ischemia-reperfusion induces the formation of poly(ADP-ribose) in the brain [18,19]. Both nitric oxide and reactive oxygen species are formed during ischemia-reperfusion, are known DNA-damaging agents, and are potent mediators of cell death [20]. Inhibitors of PARP, such as 3-aminobenzamide (3-AB), can partially prevent NO and reactive oxygen species cytotoxicity in neuronal cells [21,22].

PARP acts as a homodimer (M_r 2 x 113,000) when binding to and facilitating the repair of damaged DNA [23]. The dimerization process may require self ADP-ribosylation to activate PARP and cause it to dimerize. PARP automodification is required for activity [24]. However, excessive self ADP-ribosylation makes PARP anionic such that it detaches from negatively charged DNA. PARP may then compete with DNA for binding to several DNA complexing enzymes, thereby detaching them from DNA and altering their acitivity. The detachment of PARP from DNA is required for apoptosis progression. The detachment allows endonuclease activation, which is normally inhibited by polyADP-ribosylation, and gives endonuclease access to DNA to cleave DNA into fragments. The N-terminal of PARP (M_r 46,000) contains the two zinc fingers that bind DNA and the nuclear location signal [25]. The middle section of PARP (M_r 22,000) contains the automodification area. The C-terminal (M_r 54,000) contains the active site that binds NAD and is highly conserved among species.

The crystal structure of the catalytic fragment of PARP, from the C-terminal, has been published [25]. The fragment structure is made up of a five stranded antiparallel beta sheet and a four stranded mixed beta sheet. The two beta sheets connect through two hydrogen bonds. The beta sheets are surrounded by five alpha-helices, three 3_{10}-helices and by beta sheet excursions. The NAD binding site has been characterized and is very similar to the NAD binding sites of the bacterial toxins [26,27,28]. Apparently, PARP is a member of a family of ADP ribosyl transferases, which includes the bacterial toxins. However, PARP differs from these mono(ADP-ribosyl) transferases since PARP polymerizes ADP-ribose. The polymerization mechanism has been suggested based on the crystal structure [29].

The active site of PARP is made up of Glu988, Tyr896, Ala898, Lys903, His862, Ser904, Gly863 and Tyr907 [26]. The crystal structure of NAD bound to diphtheria toxin, an ADP-ribosyl transferase, has been published and may provide useful information about the binding of NAD to PARP [28]. A crystal structure for NAD binding to PARP is not available due to the instability of the complex. NAD appears to bind in a 3'-endo conformation for the ribose. The nicotinamide may be in a syn position (Figure **1**). There are several hydrogen bonds and hydrophobic interactions between PARP and NAD in the active site. It has been proposed that hydrogen bonds occur as follows: Gly876, Asp 770 and Arg878 bond to the adenine; Ser864 or His862 bond to the ribose connected to the adenine; Asp766, Gln763 and Tyr896 bond to the phosphates; gly863 or Ser904 bond to the nicotinamide; Tyr907 and Glu988 bond to the ribose connected to the nicotinamide. Hydrophobic bonds occur as follows: Leu877 and Ile872 bond to adenine; Tyr907 bonds to nicotinamide; possibly Tyr896 bonds to the ribose connected to the nicotinamide. Glu988 may polarize the NAD and the ADP-ribose acceptor through hydrogen bonding. This could increase the nucleophilicity of the acceptor and stabilize the NAD transition state. The active site binding of NAD favors the formation of a transition state ribose oxocarbenium ion of NAD leading to cleavage in an S_N2 mechanism.

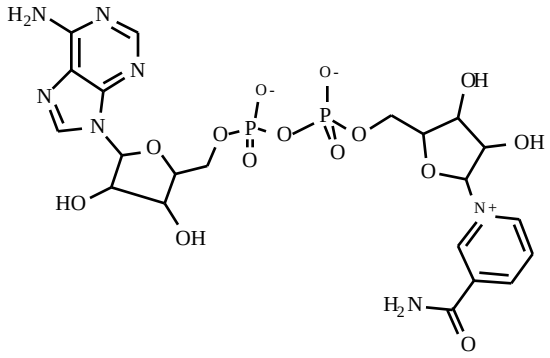

Fig. (1). NAD

Crystal structures for four PARP inhibitors have been examined [26]. These inhibitors are based on the structure of nicotinamide and bind the nicotinamide pocket of the active site. Three of the inhibitors are potent, planar ring structures, 4 - amino - 1, 8 - naphthalimide, 8 - hydroxy - 2 - methyl - 3 - hydroquinazolin - 4 - one and 3, 4 - dihydro - 5 - methylisoquinolinone. The crystal structure of PARP bound 3-methoxybenzamide was also examined. Hydrogen bonds with Gly863 (N), Gly863 (O) and Ser904 (OG) are common to all inhibitors. This demonstrates that the nicotinamide binding site is lined by amino acids 862, 863, 896, 897, 898, 903, 904, 907 and 988, which all have at least one atom within 4 angstroms of the inhibitor. The naphthalimide and quinazolinone inhibitors form additional

hydrogen bonds with Glu988 and Tyr907. New inhibitors can be synthesized perhaps designed to form hydrogen bonds with Lys903, Tyr907 and Glu988.

PARP may ADP-ribosylate various transcription factors that regulate gene transcription [30,31]. PARP is a component of positive cofactor 1 activity that regulates class II gene transcription [30]. When DNA is damaged, PARP is activated which inhibits class II gene transcription regulated by RNA polymerase II [32]. The transcriptional effects of PARP may be important in the differentiation and adaptation of cells [8]. PARP is also important in the action of p53, the tumor suppressor protein. Both PARP and p53 bind to DNA breaks. PARP can form complexes with p53 that may alter the activity of p53 [33]. Interestingly, p53 is involved in the inhibition of RNA polymerase III dependent gene transcription [34].

Recently, PARP was recognized as a substrate of activated caspase-3 (CPP-32), a mammalian homologue of the *C. elegans* ced-3 death gene, during apoptosis [35]. The role of PARP in apoptosis remains somewhat controversial. Caspase cleavage of PARP appears to facilitate apoptosis, possibly by interrupting DNA binding and repair at an earlier step than internucleosomal DNA fragmentation [36].

Cleavage decreases PARP enzymatic activity and could facilitate DNA laddering by upregulating Ca^{2+} / Mg^{2+} -dependent endonuclease as a consequence of reduced poly ADP-ribosylation [37]. Caspase cleaves the C-terminal end of PARP, which contains the catalytic site. The C-terminal fragment can bind to PARP inhibiting its homodimerization thereby decreasing PARP activity as discussed above [23].

It is clear that PARP is activated very rapidly by DNA damage that results from ischemia-reperfusion and neurotoxicity [13,18,19,38,39]. The activation of PARP is transient and quickly subsides. However, about 24 h later, when apoptosis is nearly maximal, another activation of PARP can occur [18]. The first activation of PARP is due to oxygen radical damage of DNA. The second activation may be due to endonuclease cleavage of DNA. PARP activation may be difficult to detect due to its transient nature. It may seem contradictory that endonuclease activation requires PARP deactivation, yet PARP can be reactivated by endonuclease cleavage of DNA. However, PARP is present in the nucleus in abundance, such that cleavage of some PARP by caspase may leave some PARP not cleaved. PARP bound to DNA is not a good substrate for caspase and may escape cleavage.

If a large amount of DNA damage occurs, PARP may be rapidly activated resulting in NAD and ATP depletion and necrosis within a few h [12,13]. In cells where a small amount of DNA damage has occurred, PARP activation may not deplete NAD and ATP. PARP is then deactivated, possibly by automodification or caspase. The apoptotic program may then be activated, which requires ATP. As endonuclease becomes activated at about 24 h, PARP may reactivate leading to NAD and ATP depletion [13].

Treatment with inhibitors of PARP reportedly blocks or augments apoptosis, depending on the paradigm [22,40]. It was also reported that poly ADP ribosylation contributes to ischemic cell death, and that PARP inhibition protects tissue within the ischemic territory [18,21,39,41-44]. Mice deficient in PARP are resistant to brain injury after transient focal cerebral ischemia. Infarct sparing in MCAO mice is accompanied by improved neurological scores when treated with 3-AB, suggesting that reduced PARP activity preserves neurological function [41].

There are a number of PARP inhibitors available, some of which are shown in Figure **2**. Many of these inhibitors have not been tested in stroke models. Griffin and his colleagues have synthesized a number of quinazolinones and other inhibitors [45]. Several companies are actively synthesizing possible PARP inhibitors. The structures of these inhibitors are all based on the structure of nicotinamide. However, the PARP active site contains a nicotinamide binding area and an adenine binding area. PARP inhibitors based on the structure of adenine are known [46]. These include 5-bromo-2'-deoxyuridine, caffeine, 5-bromouracil, theophylline, thymidine and other compounds. Adenine based inhibitors of PARP have not been as extensively explored as nicotinamide based inhibitors.

All inhibitors based on the structure of nicotinamide should have an electron rich aromatic ring, a substituent with a carbonyl group in the anti conformation to which is bound a nitrogen with at least one free hydrogen, and a substituent on the ring such as OH, OMe or NH_2 [47]. The effects of nicotinamide are due to a specific uptake mechanism which allows nicotinamide to penetrate into the brain rapidly, reach high levels, increase brain levels of NAD and ATP and inhibit the use of NAD by PARP by inhibiting the catalytic site of this enzyme [8]. PARP inhibitors must similarly penetrate readily into the brain and reach high levels in order to provide adequate therapy.

Delayed treatment with nicotinamide improves neurological outcome and reduces infarct volume after transient focal cerebral ischemia in Wistar rats, even when administered up to 2 hours after the onset of stroke [42,43,48]. Delayed nicotinamide treatment improves both anatomic and functional indices of brain damage [43]. Post-treatment with nicotinamide reduces the infarct volume following permanent focal cerebral ischemia in female Sprague-Dawley or Wistar rats. Its neuroprotection is more robust when administered as an intravenous bolus compared with intraperitoneal administration [49,50]. Inhibition of PARP prevents oxidative stress induced apoptosis in motoneuronal cells [21].

One kind of hydroxyl radical scavenger, (+/-)-N, N'-propylenedinicotinamide (nicaraven), attenuates ischemia-reperfusion injury in the rat neocortex after transient focal ischemia. Treatment with a dose of 60 mg/kg per h of nicaraven provides significant reductions in the volume of infarction for the pre- and posttreatment groups [51]. It was also found that the free radical scavenger, 1,2-bis (nicotinamide)-propane, significantly suppressed edema and lactate in the cold-injury trauma rat brain model [52]. These compounds point out that nicotinamide has weak radical scavenging activity [53,54]. The rate constant for nicotinamide interaction with hydroxyl radical is 1.4×10^9

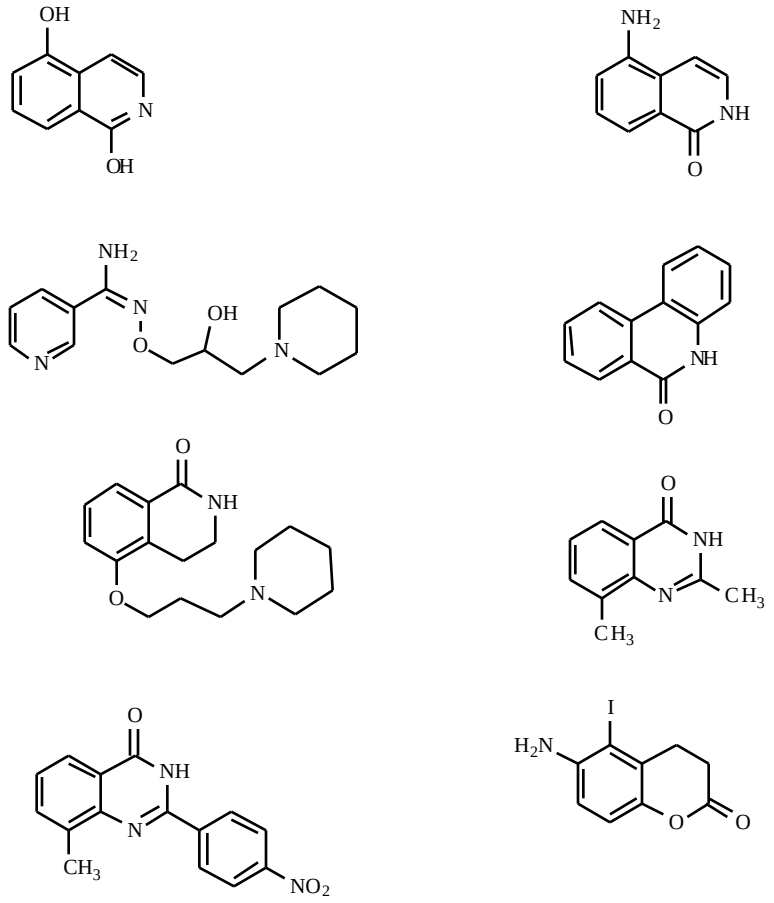

Fig. (2). Selected PARP Inhibitors – 1,5-dihydroxyisoquinoline [121], 5-aminoisoquinolin-1(2H)-one [122], O-(2-hydroxy-3-piperidinepropyl)-pyridine carbonic acid amidoxime [123], 6(5H)-phenanthridinone [124], 3,4-dihydro-5-[4-(1-piperidinyl)butoxy]-1(2H)-isoquinoline [125], 2,8-dimethyl-3-hydroquinazolin-4-one [45], 8-methyl-2-(p-nitrophenyl)-3-hydroquinazoline-4-one [45], 5-iodo-6-aminocoumarin [126].

$M^{-1}s^{-1}$ as reported by Beck [55]. The rate constant for nicotinamide interaction with superoxide is 7 x 10^9, and with singlet oxygen is 1.8 x 10^8 as reported by Beck and Kamat [54,55].

It is important to recognize that the PARP referred to above is PARP-1. There are several enzymes with PARP activity [56]. It is not known if the other enzymes, PARP-2, PARP-3, tankyrase and V-PARP, can fill in for PARP-1 when PARP inhibitors are used. Clearly, in PARP-1 knock out mice, the other PARP enzymes are still functional and can synthesize poly(ADP-ribose). It is also not known if PARP inhibitors are specific for PARP-1 or can inhibit all forms of PARP.

EFFECTS OF NICOTINAMIDE ON NAD GLYCOHYDROLASE

There is another kind of enzyme in neurons which transfers ADP-ribosyl monomers or oligomers to proteins [57]. This enzyme is NAD glycohydrolase which is contained in the cytosol and brain mitochondria and is distinct from PARP found in the nucleus and cytoplasm [58]. NAD glycohydrolase activities appear to be much higher in nonsynaptic mitochondria. NAD glycohydrolase cleaves poly ADP-ribose and NAD and may transfer ADP-ribose to proteins. However, ADP-ribose, possibly as cyclic ADP-ribose, may react on its own to alkylate proteins directly [59]. Activation of this enzyme may lead to NAD and ATP depletion in the cell and mitochondria [60]. NAD glycohydrolase also makes cyclic ADP-ribose that is involved in calcium release [61]. NAD glycohydrolase is a poorly understood enzyme, but appears to be a member of the ADP-ribosyl transferase family of enzymes. Its mechanism of action is similar to PARP and involves an oxocarbenium ion intermediate of NAD. However, NAD glycohydrolase is similar to the bacterial toxins in that it contains a Rossman fold that binds NAD in an extended conformation and may prevent it from synthesizing polymers of ADP-ribose [62]. Whether NAD glycohydrolase is identical to mono(ADP-ribosyl) transferase is not clear. Certainly, both enzymes cleave NAD and transfer ADP-ribose to proteins. However, NAD glycohydrolase is an intracellular enzyme, whereas mono(ADP-ribosyl) transferase is largely an ecto-enzyme that is membrane anchored. Mono(ADP-ribosyl) transferase is not entirely an ecto-enzyme and is found in endoplasmic reticula and bound to the cytoplasmic face of plasma membranes [63].

Nicotinamide is taken up by brain mitochondria and is used in the synthesis of NAD in mitochondria. Nicotinamide is an inhibitor of NAD glycohydrolase in brain mitochondria. The inhibitory effect of nicotinamide on NAD glycohydrolase activity appears rather stronger than its inhibitory effect on ADP-ribosyl transferase [57]. When NAD glycohydrolase is inhibited totally by nicotinamide, the transfer of ADP-ribose from NAD to mitochondria proteins still occurs. The inhibition by nicotinamide of the NAD^+ glycohydrolase bound to the plasma membrane, prepared from glial and neuronal cells, is non-competitive [64]. Using an experimental model of parkinsonism, it is established that the content of nicotinamide adenine dinucleotides and the binding of NAD by isolated brain cortical synaptic membranes are impaired. The modulative and neurotrophic effects of nicotinamide, in this model, suggest that nicotinamide acts via NAD which binds specifically with synaptic membranes [65]. NAD glycohydrolase also quickly cleaves oligomers and polymers of ADP-ribose, even those bound to proteins, such that the turnover of poly (ADP-ribose) is very rapid. Little poly(ADP-ribose) glycohydrolase activity has been found in the nucleus [58]. However, PARP has the ability to cleave NAD and perhaps poly(ADP-ribose). The inhibition of NAD glycohydrolase by nicotinamide may spare cellular NAD levels, and ATP levels, during oxidative stress. Nicotinamide also enhances mitochondrial NAD levels and should enhance mitochondrial energetics [13,66].

Inhibition of NAD glycohydrolase may have other beneficial effects including enhanced deactivation of PARP activity by automodification. This would, however, lead to decreased DNA binding of PARP and enhanced endonuclease activity, which is not beneficial. A recent study of a poly(ADP-ribose) glycohydrolase inhibitor, gallotannin from green tea, demonstrated protective effects against oxidative astrocytic death [67].

Several inhibitors of NAD glycohydrolase and mono(ADP-ribosyl) transferase are known [63,68]. These inhibitors include novobiocin, meta-iodobenzylguanidine, 3-aminobenzamide, isoniazid, bromodeoxyuridine, thymidine, theophylline, 3-methoxybenzamide, benzamide, arachidic acid, stearic acid, palmitic acid, arachidonic acid, linoleic acid, linolenic acid, palmitoleic acid, vitamin K_1, vitamin K_2, and diethylamino(benzylidineamino)guanidine [62,63,68,69]. An interesting mechanism of neuroprotection by mono(ADP-ribosyl) transferase inhibition has been suggested [69]. Activation of the NMDA receptor may involve ADP-ribosylation or poly ADP ribosylation. Therefore, use of a mono(ADP-ribosyl) transferase inhibitor may be able to block NMDA receptor activation.

EFFECTS OF NICOTINAMIDE ON NAD SYNTHESIS

NAD has a central role in energy supply during the development of stroke. The ubiquitous enzyme NAD synthetase catalyzes a key step in NAD biosynthesis, transforming deamido-NAD into NAD by a two-step reaction. Deamido-NAD accumulates during ischemia-reperfusion [13]. NAD synthetase belongs to the amidotransferase family and has been recognized as a member of the family of N-type ATP pyrophosphatases [70]. There are two metabolic pathways leading to NAD formation [71]. In the first path nicotinamide is transformed into nicotinamide mononucleotide (NMN) via catalysis by nicotinamide phosphoribosyl transferase which requires ATP. NMN is transformed into NAD via NMN adenyl transferase, which requires ATP. In the second path nicotinamide is transformed into niacin via catalysis by nicotinamidase. However, the reverse pathway, making nicotinamide from niacin is the preferred direction in most cells. Niacin can make niacin mononucleotide in the presence of nicotinic acid phosphoribosyl transferase, which requires ATP. Niacin mononucleotide is transformed into deamido-NAD via NMN adenyl transferase, which requires ATP and at last is transformed into NAD via NAD synthatase, which also requires ATP [72]. As an essential precursor of NAD, nicotinamide may increase the activity of NAD synthetase and other key enzymes. At steady state, nuclear NAD, which is in equilibrium with cytoplasmic NAD, is used to poly(ADP-ribosylate) nuclear proteins [73]. Following nicotinamide administration, NAD levels in the brain can increase within a few hours, as do ATP levels [74,75]. In fact, administration of nicotinamide results in higher brain NAD^+, NADH, $NADP^+$ and NADPH levels in some regions. The synthesis of these pyridine nucleotides is further upregulated during oxidative stress [76].

Nicotinamide administration increases brain NAD levels. Niacin it is not readily taken up into the brain, yet it can be used in place of nicotinamide in the treatment of pellagra [8]. Other compounds can be used as precursors for nicotinamide or as substrates in the NAD synthetic pathway to make NAD analogs that can be used in place of NAD [78-81]. This is an area of research that has been neglected for many years. These compounds have all been tested in nicotinamide deficiency models and found to be active. The active compounds include: ethyl nicotinate, N-ethyl nicotinamide, N,N-diethyl nicotinamide, N-methyl nicotinamide, 3-methylpyridine, N-acetyl nicotinamide (nicotinuric acid), and nicotinamide N-glucosiodide. All of these compounds may be precursors for nicotinamide. Some of them easily make nicotinamide by ester or amide hydrolysis in the body. 3-Methylpyridine (beta-picoline) may be oxidized by cytochrome P450 to make pyridyl-3-aldehyde (active) or pyridyl-3-carbinol (active) that are used in the synthesis of nicotinamide. Other compounds have been found to be active in some models of nicotinamide deficiency including 2-carboxyniacin, N-methyl nicotinamidinium, 2-carboxypyrazine and 2,3-dicarboxypyrazine. It is not clear that the pyrazines could be converted in the body to nicotinamide. They may however, make pyrazine analogs of nicotinamide that are used to make pyrazine analogs of NAD. Similarly, 2-carboxyniacin may make a 2-carboxynicotinamide analog of NAD.

EFFECTS OF NICOTINAMIDE ON OTHER ENZYMES IN OXIDATIVE STRESS

The progressive neurodegeneration in stroke is exhibited when cerebral blood flow is restored. Reactive oxygen radicals are involved in the process of brain injury that

ensues [82-84]. In focal or global cerebral ischemia, reoxygenation during spontaneous or thrombolytic reperfusion provides oxygen as a substrate for numerous enzymatic oxidation reactions in the cytosolic compartments or subcellular organelles and mitochondria [82].

It has been demonstrated that approximately 2-5% of the electron flow in isolated brain mitochondria produces superoxide radical anion ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2). These constantly produced reactive oxygen species (ROS) are scavenged by superoxide dismutase (SOD), glutathione peroxidase (GSHPx), and catalase. Small molecule antioxidants, including glutathione (GSH), ascorbic acid, and [alpha]-tocopherol, are also involved in the inactivation of free radicals. During reperfusion, endogenous antioxidative defenses are likely to be perturbed as a result of overproduction of oxygen radicals by cytosolic prooxidant enzymes and mitochondria, inactivation of detoxification systems, consumption of antioxidants, and failure to adequately replenish antioxidants in ischemic brain tissue. It has been demonstrated that ROS are directly involved in oxidative damage to cellular macromolecules such as lipids, proteins, and DNA in ischemic tissues, which leads to cell death [83]. Many prooxidant enzymes are known to participate in oxidative stress-induced brain injury. Three major classes of prooxidant enzymes can be designated: (1) nitric oxide synthase; (2) cyclooxygenase, xanthine dehydrogenase, xanthine oxidase and NADPH oxidase; and (3) myeloperoxidase and monoamine oxidase [84].

Brain mitochondria have been shown to take up nicotinamide (but not niacin) rapidly through the ATP/ADP translocator [85]. Nicotinamide can protect against inhibition of mitochondrial respiration in rat cardiomyocytes exposed to H_2O_2 or $ONOO^-$ [86]. Brain mitochondria subjected to oxidative stress suffer from extensive GSH oxidation and formation of protein-GS mixed disulfides [87]. High NADPH levels produced by nicotinamide administration allow the reduction of protein-GS mixed disulfides by thioredoxin/thioltransferase which is coupled to the reduction of GSSG by GSSG reductase [88]. Nicotinamide administration can lead to higher GSH levels in brain mitochondria, perhaps by sparing ATP that is required for GSH synthesis or by increasing NADPH levels that are used in the reduction of GSSG [76]. GSH and NAD levels remain elevated, following nicotinamide administration, even during oxidative stress induced DNA damage in mitochondria. Interestingly, thioredoxin is required for DNA synthesis and repair. Thioredoxin is involved in the reduction of ribonucleotides making deoxyribonucleotides at the expense of GSH [8].

It was found that nicotinamide inhibits xanthine oxidase and is capable of scavenging oxygen free radicals [53,75]. Studies indicate nicotinamide is a weak scavenger of oxygen radical species as discussed above. In an electron paramagnetic resonance study of nicotinamide reactivity toward $HO^{\cdot}$ radical produced from H_2O_2 + $FeSO_4$, nicotinamide diminished the $HO^{\cdot}$ radical signal slightly, perhaps due to radical scavenging by nicotinamide. In this reaction, hydroxyl radical takes an electron from nicotinamide [53].

It has been affirmed that the induction of nitric oxide synthase is inhibited by nicotinamide [89]. This implies that nicotinamide can alter the effects of excitotoxicity and other forms of neurodegeneration that involve the induction of nitric oxide synthase. Ischemia and reperfusion in the brain is known to induce NO synthase [90]. Ischemia and reperfusion, as well as NO toxicity, are known to induce DNA damage that should be ameliorated by nicotinamide. The inhibition of induction of nitric oxide synthase by nicotinamide can further promote its effective protection against stroke.

Nicotinamide has been reported to induce hypothermia that is neuroprotective [91]. The hypothermia induced by nicotinamide can last 6 hr or more. It is known that inhibition of NO synthase produces hypothermia [92]. It may imply that nicotinamide inhibition of the induction of NO synthase may be responsible for the hypothermic effects of nicotinamide. However, not all laboratories have been able to demonstrate the hypothermic effects of nicotinamide.

Though lipid peroxidation, once suggested as the most important toxic event in neurotoxicity, can occur at the onset of reperfusion, it is not as important as DNA fragmentation in oxidative stress-induced neurotoxicity. In fact, DNA is the primary target of oxidative stress in the brain [93]. Damage to nuclei is probably the consequence of DNA fragmentation. Without enough energy supply to repair DNA damage, apoptosis and necrosis result from DNA fragmentation. DNA fragmentation is a hallmark of apoptosis.

RELATED PHARMACOLOGICAL EFFECTS OF NICOTINAMIDE

Neuronal death in stroke is due to an imbalance between energy supply and demand. Vindication of this kind of balance can be achieved by either reducing the neuronal energy demands or increasing the neuronal energy reserves, such as with nicotinamide. Nicotinamide was also reported to be an anticonvulsant [94,95], anticoagulant [96], angiogenic agent [97] and an inhibitor of lipid peroxidation [95]. All these effects of nicotinamide potentially protect against injury due to focal cerebral ischemia.

Nicotinamide (350 mg/kg) administered intravenously significantly increases regional cerebral blood flow and cerebral oxygen consumption, but decreases mean arterial blood pressure and cerebral vascular resistance in dogs [98]. But a neuroprotective dose of nicotinamide (500mg/kg) administered intraperitoneally to Wistar rats has been reported to decrease regional cerebral blood flow in normal animals and does not change regional cerebral blood flow in rat brain tumors [99]. Hence, the role of nicotinamide in adjusting cerebral blood flow is yet to be decided, under different conditions.

CLINICAL USE OF NICOTINAMIDE

Nicotinamide has already been shown to offer therapeutic benefits against MELAS syndrome (mitochondria

myopathy, encephalopathy, lactic acidosis and stroke-like episodes) in human trials, where a known mutation in mitochondrial DNA exists in some patients [100-102]. Nicotinamide has also been shown to reduce blood lactate levels and increase intracellular levels of NAD in MELAS patients [100,101]. These patients experience clinical improvements associated with nicotinamide use.

Some neurodegenerative disorders associated with aging such as Parkinson's disease [103], Alzheimer's disease [104], memory loss [105] and in gerontopsychiatry [06,107] have been reportedly effectively treated with analogs of nicotinamide, niacin or NAD. Nicotinamide is released and taken up into the systemic circulation when NAD is broken down in the gut following oral administration of NAD [108]. Therefore, it is very clear that clinical studies of the use of NAD in these neurodegenetive diseases have actually been studies of nicotinamide use. It has also found, that due to dietary insufficiency, senescent patients deficient in nicotinamide are more susceptible to neurodegeneration [6].

Recent research has indicated that the secondary brain injury associated with stroke is induced by inflammatory processes. These processes are executed by many proinflammatory molecules [1]. Nicotinamide can decrease the recruitment of neutrophils to potential sites of inflammation by inhibiting PARP in neutrophils and other cells [109]. In fact, nicotinamide has been recommended for the treatment of arthritic patients since the 1940's. It was found in pilot trails that nicotinamide improved joint mobility and decreased the need for antiinflammatory medication in arthritic cases [110]. In the process of inflammation, the genes for intercellular adhesion molecule 1 and collagenase in neutrophils are activated. Neutrophils are recruited to the inflammatory sites. Nitric oxide synthase is activated and oxygen radicals, hydrogen peroxide and nitric oxide are released. These reactive species can damage cellular DNA in the area of inflammation, resulting in apoptosis, necrosis and more serious inflammation [111]. PARP has a number of functions in inflammation due to its ability to regulate gene expression [111]. Inhibition by nicotinamide of PARP leads to decreased expression of these genes and decreases the extent and severity of inflammation.

Another important application of nicotinamide is related to the prevention and treatment of insulin-dependent diabetes mellitus [112-114]. Diabetes is an autoimmune disease where auto-antibodies are made against beta cells in the islets of Langerhans. T Lymphocytes are activated as part of the inflammatory process [112,113,115]. Nicotinamide can prevent apoptosis and necrosis of beta cells by inhibiting nitric oxide production [116] and DNA fragmentation [117] that are involved in the destruction of the beta cells.

The effects of one analogue of nicotinamide, nicorandil (N-2-(hydroxyethyl)-nicotinamide nitrate), was studied in 9 stroke patients. Regional cerebral blood flow (rCBF) was measured before and after a single oral 10 mg of dose of nicorandil. Mean rCBF was significantly increased by 20.9% after administration of nicorandil ($P<0.05$). Nicorandil may produce beneficial effects on cerebral circulation in stroke patients [118]. Another analogue, N, N'-Propylenedinicotinamide, is presently being developed for the treatment of cerebral stroke including subarachnoid hemorrhage. This drug is promising because data suggest that it may have an ability to scavenge hydroxyl radical under physiological conditions, while it also has a high permeability through the blood brain barrier [119]. In another clinical survey it was found that patients with ischemic stroke show a marked deficit of thiamine, nicotinamide (vitamin PP) and vitamin C. When all these substances were used together in the treatment of stroke, the neurological deficits and general conditions of patients were improved [120].

CONCLUSION

Stroke research has progressed recently. A driving force is the increasing availability of new research tools in this field. Animal stroke models have been extensively applied to advance our understanding of the mechanisms of ischemic brain injury and to develop novel therapeutic strategies for reducing brain damage after a stroke. There is currently only one therapeutic approach to the treatment of stroke. Tissue plasminogen activator (tPA) and other thrombolytic agents are somewhat effective in a small percentage of patients, where they may serve to dissolve blood clots and help restore blood flow. Research continues on possible neuroprotective agents that can decrease the progression of neurodegeneration following stroke by inhibiting DNA fragmentation caused by oxygen free radical production associated with ischemia and reperfusion. The interest in nicotinamide and nicotinamide analogues centers around their abilities to be delivered to the brain, to increase NAD levels and to inhibit PARP.

In our latest study, a transient middle cerebral artery occlusion (MCAO) rat model was utilized [48]. We found that to rescue the still viable but injured nerve cells, within the ischemic area, effective therapy with nicotinamide should be begun at the earliest possible time. It was very interesting to find that early injection of nicotinamide can reduce the numbers of necrotic and apoptotic neurons and elevate the ratio of apoptotic/necrotic cells in the infarct area at the same time. This effect was due to the direct protection of neurons by nicotinamide during ischemia-reperfusion. At 3 hours post-reperfusion, delayed treatment with nicotinamide could not protect neurons in the core section from necrosis but still played a key role in inhibiting apoptosis. This protective effect against apoptosis could be seen even when nicotinamide was administered 12 h after the onset of reperfusion. As a new effective means of inhibiting the development of stroke, treatment with nicotinamide could be further applied and tested in clinical trails.

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ABBREVIATIONS

PARP = Poly(ADP-ribose) polymerase

NMN = Nicotinamide mononucleotide

rCBF = Regional cerebral blood flow

tPA = Tissue plasminogen activator, protein

GS = Protein GSSG mixed disulfides

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