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SHORT COMMUNICATION

Formation of *N*-Methylnicotinamide in the Brain from a Dihydropyridine-type Prodrug

EFFECT ON BRAIN CHOLINE

Christina Erb, Albrecht Seidel,† Heinz Frank,† Karl L. Platt,† Franz Oesch† and Jochen Klein*‡*

DEPARTMENTS OF *PHARMACOLOGY AND †TOXICOLOGY, UNIVERSITY OF MAINZ, D-55101 MAINZ, GERMANY

ABSTRACT. The enhancement of brain choline levels is a possible therapeutic option in neurodegenerative diseases; however, brain choline levels are held within narrow limits by homeostatic mechanisms including the rapid clearance of excess choline from the brain. The present study tests whether *N*-methylnicotinamide (NMN), an inhibitor of the outward transport of choline from the brain, can elevate brain choline levels *in vivo*. As NMN does not cross the blood–brain barrier, we synthesized and administered the brain-permeable prodrug, 1,4-dihydro-*N*-methyl-nicotinamide (DNMN), and tested its effect on the levels of NMN and choline in brain extracellular fluid, using the microdialysis procedure. Administration of DNMN (1 mmol/kg s.c.) caused a 4- and 9-fold increase in plasma and liver NMN levels, respectively, as determined by HPLC. Concomitantly, the brain tissue levels of NMN were increased by a factor of twenty. In brain extracellular fluid, the injection of DNMN (1–3 mmol/kg s.c.) elevated NMN levels by 3- to 10-fold to maximum levels of >10 μM. In spite of these enhanced NMN levels, the choline concentrations in the brain extracellular fluid and in the cerebrospinal fluid (4.7 μM) remained unchanged or were even slightly decreased. Microsomal incubations of DNMN indicated that cytochrome P-450 3A isoforms may be involved in NMN formation in the liver, but not in the brain. We conclude that DNMN, a brain-permeable prodrug of NMN, is efficiently oxidized to NMN in the brain, but a 10-fold increase in extracellular NMN levels is not sufficient to reduce the clearance of choline from the brain. BIOCHEM PHARMACOL **57**;6:681–684, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. choline; microdialysis; nicotinamide; *N*-methylnicotinamide; hippocampus; brain targeting

The enhancement of brain choline is a potential therapeutic approach in neurodegenerative diseases [1]. Experimental studies have demonstrated that, under conditions of increased turnover, choline is a rate-limiting factor for the synthesis of brain phospholipids, specifically phosphatidylcholine [2], and for the synthesis of the neurotransmitter acetylcholine [3, 4]; both phosphatidylcholine and acetylcholine are reduced, for example, in Alzheimer's disease [5, 6]. However, the concentration of choline in the brain cannot be effectively enhanced by administration of exogenous choline because excess choline is rapidly removed from the brain. We have recently shown that several conditions which enhance choline levels in the brain (e.g. hypoxia and a choline-rich diet) result in an increased release of choline from the brain, which was reflected in increased CSF§ choline concentrations and in a strongly negative arteriovenous difference of brain choline [7]. Therefore, we mused that the inhibition of choline transport from the brain could be an effective way of increasing brain choline concentration.

The clearance of choline from the brain seems to involve at least two mechanisms. Choline is transported out of the brain via the arachnoid villi and via the choroid plexus. Remarkably, the transport of choline out of the brain extracellular space seems to occur by a transporter with a relatively high affinity (K_t: 16 μM), while the entrance of choline into the brain occurs via a low-affinity carrier at the blood–brain barrier (K_t > 400 μM) [8, 9]. NMN resembles choline as a quaternary cation and interferes with the transport of choline in the kidney [10] and in the brain [8, 11]. In millimolar concentrations, NMN decreased the clearance of choline during ventriculocisternal perfusion in rabbits [8]; lower concentrations were not tested in this study. NMN also inhibits the uptake of choline by the choroid plexus in a competitive manner, with a K_i value of 720 μM [11]. Thus, in order to test whether NMN would enhance brain extracellular choline levels *in vivo*, we looked for means to efficiently increase the concentration of NMN in the brain extracellular space. NMN itself does not penetrate through the blood–brain barrier, and we recently showed that the administration of nicotinamide does not efficiently increase the levels of NMN in the brain [12]. For the present study, we synthesized the prodrug,

‡ Corresponding author: Dr. Jochen Klein, Department of Pharmacology, University of Mainz, Obere Zahlbacher Str. 67, D-55101 Mainz, Germany. Tel. (49)-6131-174393; FAX (49)-6131-176611; E-mail: JKLEIN@MAIL.UNI-MAINZ.DE

§ *Abbreviations:* CSF, cerebrospinal fluid; DNMN, 1,4-dihydro-*N*-methylnicotinamide; and NMN, *N*-methylnicotinamide.

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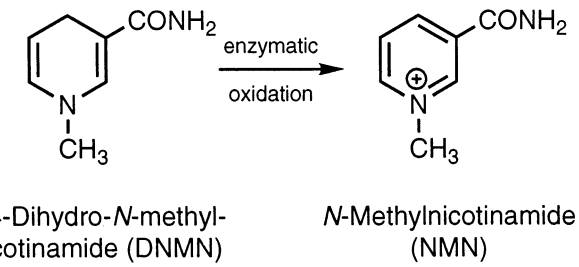

FIG. 1. Formation of *N*-methylnicotinamide (NMN), a quaternary cation, from the brain-permeable prodrug, 1,4-dihydro-*N*-methylnicotinamide (DNMN).

DNMN. In analogy to previous studies with dihydropyridines [13], we expected that DNMN, an uncharged substance, would permeate the blood–brain barrier and be enzymatically converted to NMN in the brain (Fig. 1). NMN, a charged and highly hydrophilic compound, would be trapped in the brain and, possibly, enhance brain choline levels by inhibition of choline clearance.

MATERIALS AND METHODS

Synthesis of DNMN

DNMN was synthesized from NMN (Sigma) by reduction with sodium dithionite essentially as described [14–16], and the structure was confirmed by high resolution proton NMR spectroscopy [400 MHz ($CDCl_3$) d 6.96 (1H, s, H-2) 5.64 (1H, q-d, H-6, $J_{5,6}$ = 8.0 Hz, $J_{4a,6}$ = 1.5 Hz, $J_{4b,6}$ = 3.3 Hz) 5.38 (2H, br-s, -NH_2) 4.69 (1H, m, H-5, $J_{4a,5}$ = $J_{4b,5}$ = 3.4 Hz) 3.10 (2H, s, H-4a,4b) 2.90 (3H, s, -CH_3)]. The accompanying 1,2-isomer was removed by several crystallizations from ethyl acetate until the purity of DNMN was ≥98% (based on NMR analysis).

Animal Treatments and Sample Analysis

Adult male Wistar rats of the Crl:(WI)BR strain (300 g; Charles River Co.) were treated with saline or DNMN by subcutaneous injection. Two hours later, the animals were anaesthetized with pentobarbital (40–80 mg/kg i.p.), and samples of CSF (50–100 μL) were obtained by puncture of the cisterna magna. Immediately afterwards, the animals were decapitated, and rump blood, brain, and liver samples were obtained. Blood plasma and CSF were mixed with twice the volume of ice-cold 96% ethanol and centrifuged, whereas brain and liver tissues were extracted with chloroform/methanol essentially as described [17]. Aliquots of the upper phases were evaporated to dryness and used for the determination of DNMN, NMN, and choline. Choline was determined by HPLC as described previously [4]. NMN and DNMN were separated on a 5-μm Hypersil RP18 column (Shandon; 250 × 4.6 mm) and detected by UV absorption at 265 nm. The mobile phase consisted of 20 mM trimethylamine, 5 mM KH_2PO_4, 7 mM hexane sulfonic acid, and 0.1 mM ascorbic acid (pH 3.0) [18]. The retention times were 7.8 min for DNMN and 16.7 min for NMN; the assay was linear from 0.3–30 nmol.

Microdialysis

I-shaped, concentric dialysis probes with an outside diameter of 0.24 mm and an exchange length of 4 mm (Filtral AN-69 HF; Hospal) were manufactured as previously described [4] and implanted into the right ventral hippocampus (coordinates from lambda: AP +3.5; L −4.6; DV −7.5 mm). The experiments were carried out on conscious animals on the first and second day after surgery. The microdialysis probe was perfused at 2 μL/min with Ringer's solution (concentration in mM: NaCl 147; KCl 4; $CaCl_2$ 1.2; $MgCl_2$ 1.2). DNMN or saline was administered by s.c. injection, the perfusate was collected in 15-min intervals, and samples were analyzed by HPLC without further workup.

Oxidation of DNMN by Subcellular Fractions

Liver and brain were homogenized in Tris buffer (20 mM Tris buffer pH 7.4, 1 mM EDTA, 0.2 mM dithiothreitol containing 0.25 M sucrose), and the homogenates were centrifuged at 600 g to remove cell debris. The supernatant was centrifuged at 9000 *g* and at 100,000 *g*, and the pellet of the last centrifugation was used as microsomal fraction [19]. Incubations with DNMN were carried out in 0.6 mL Hepes buffer (50 mM, pH 7.4) containing 1 mM NADPH, 10 mM $MgCl_2$, and microsomes (1 mg protein/mL). When enzyme inhibitors (1 mM, dissolved in ethanol) were present, the reaction mixture was preincubated with the inhibitors for 2 min (5 min in the case of troleandomycin). The reaction was started by the addition of 0.1 mM DNMN, proceeded at 37° for 10 min, and was stopped by the addition of two volumes of ethanol. After centrifugation, aliquots of the supernatants were evaporated by a Speed Vac concentrator, taken up in mobile phase and analyzed for DNMN and NMN by HPLC as described above. The reaction rates were corrected for spontaneous reactions determined in the absence of microsomal protein.

RESULTS AND DISCUSSION

Formation of NMN in the Periphery

As summarized in Fig. 2, DNMN administration (1 mmol/kg) led to a massive increase in the NMN levels *in vivo*; the basal concentrations of NMN in plasma and in liver homogenates were increased by a factor of 4 (plasma) and 9 (liver), respectively. In liver microsomal preparations from untreated rats, DNMN was oxidized enzymatically with a rate of 1.52 ± 0.21 nmol/min/mg protein. This rate was reduced by inhibitors of cytochrome P-450, metyrapone (−51%) and piperonyl butoxide (−98%); in addition, naringenin and troleandomycin, two specific inhibitors of the cytochrome P-450 isoform 3A [20], also reduced the enzymatic oxidation of DNMN by 84 and 76%, respectively. The 3A isoforms are prominent isoforms of P-450 in rat and human liver and are responsible for the oxidation of dihydropyridines such as nifedipine ("nifedipine oxidase")

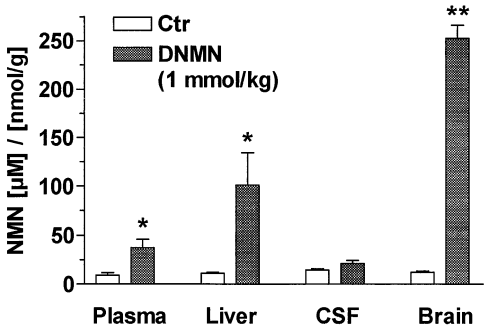

FIG. 2. Concentrations of NMN in blood plasma and CSF (in µM), and in liver and brain homogenate (in nmol/g) 2 hr after administration of DNMN (1 mmol/kg s.c.). Data are means ± SEM from 4–6 experiments. Statistical significance: *$P < 0.05$. **$P < 0.01$.

[21]; thus, the enzymatic activity of the P-450 3A isoforms may explain the efficient oxidation of DNMN in peripheral tissues.

Formation of NMN in the Brain

In total brain tissue, the concentration of NMN was increased 20-fold after administration of 1 mmol/kg DNMN (Fig. 2); however, the concentration of NMN in the CSF was only increased by 45% (from 14.6 µM to 21.1 µM; N = 5, $P > 0.1$). Concomitantly, DNMN was found in the CSF in concentrations of 45.9 ± 9.8 µM (N = 5; not illustrated), confirming the facile diffusion of this compound through the blood–brain barrier. The level of DNMN in the brain homogenate could not be determined due to interfering peaks in the HPLC chromatogram. *In vitro*, brain microsomes oxidized DNMN at a rate approximately 5-fold lower than liver microsomes (0.38 nmol/min/mg protein); however, the mechanism of DNMN oxidation in the brain remains unknown. The levels of cytochrome P-450 in the brain are low [22], and, in our hands, DNMN oxidation was unaffected by the P-450 inhibitors described above (not illustrated). Thus, DNMN may be oxidized in the brain by NADH transhydrogenases, as speculated by Bodor and Brewster [13].

In the following experiments, we used the microdialysis technique to monitor the concentrations of NMN (and choline, see below) in the brain extracellular fluid. The basal concentration of NMN in the dialysate was 0.9 ± 0.2 µM (N = 5). Figure 3A illustrates that the extracellular concentration of NMN dose dependently increased after DNMN administration. Maximum extracellular NMN concentrations were reached after 2 to 3 hr; 3- and 10-fold increases were noted after the injection of 1 and 3 mmol/kg DNMN, respectively (Fig. 3A). After 3 mmol/kg DNMN, i.e. when the extracellular NMN concentration was 11 µM (Fig. 3A), we recorded an extracellular DNMN concentration of 30 µM (not illustrated). In a single experiment, the administration of 10 mmol/kg DNMN led to high extracellular levels of DNMN (>80 µM) and NMN (40 µM); however, this dose caused severe convulsions of the animal and was not repeated.

The present data demonstrate that DNMN was efficiently taken up and oxidized in the brain. This is illustrated by the high concentrations of DNMN in the CSF and in the microdialysate, and by the strong (20-fold) increase in the NMN levels in total brain homogenate (Fig. 2). As shown by microdialysis, the concentration of NMN in the extracellular fluid was also increased after DNMN administration; however, these changes were less prominent than those in brain homogenate. After application of 1 mmol/kg DNMN, the NMN concentration in the extracellular fluid of the hippocampus was increased 3-fold (Fig. 3A), and in the CSF by only 1.5-fold (Fig. 2). Assuming that the intravasal (plasma) and extracellular (CSF) spaces represent 5 and 15% of the brain mass, respectively, and intracellular water 55% (550 µL per g brain) [23], the intracellular concentration of NMN can be calculated from the data shown in Fig. 2 as 17 µM under control conditions and 450 µM 2 hr after administration of 1 mmol/kg DNMN. These data clearly show that DNMN is efficiently oxidized in the intracellular compartment, but that the quaternary NMN leaves the brain cells in a delayed fashion, thereby leading to limited increases in brain extracellular NMN levels.

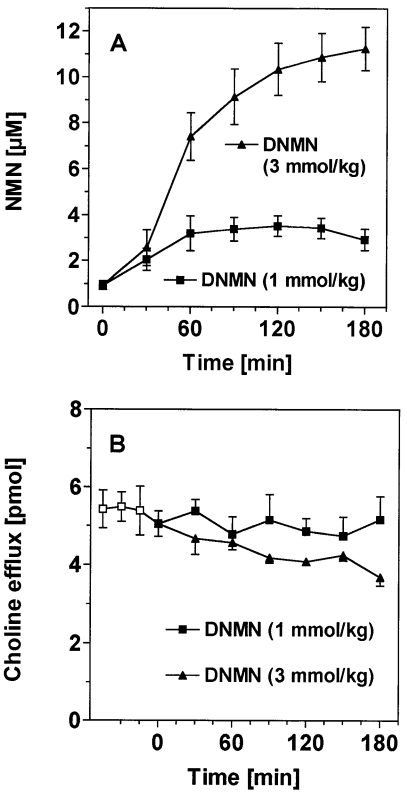

FIG. 3. Effects of the administration of DNMN (1 and 3 mmol/kg s.c.) at time zero on the extracellular concentrations of NMN (A) and choline (B) in rat hippocampus, sampled by microdialysis and measured by HPLC as described in the Methods section. Data are means ± SEM from 4–6 experiments.

Effects of NMN on Brain Extracellular Choline

Plasma and CSF concentrations of free choline in saline-treated animals were 11.6 ± 1.4 μM and 4.7 ± 0.4 μM, respectively. Two hours after s.c. injection of 1 mmol/kg DNMN, there was no significant change in the choline levels in plasma (12.1 ± 0.9 μM, N = 5) or CSF (4.1 ± 0.4 μM, N = 5) (not illustrated). In agreement with these results, the level of extracellular choline in the rat hippocampus, determined by microdialysis, did not increase, but even decreased slightly after administration of 1 and 3 mmol/kg DNMN (Fig. 3B); it decreased even more strongly after 10 mmol/kg DNMN (single experiment; not illustrated). Thus, NMN concentrations in the micromolar range do not lead to an elevation in extracellular choline levels. These data do not contradict previous findings on the inhibition of choline transport by NMN [8, 11], which were obtained at much higher NMN concentrations (>500 μM); however, higher concentrations of NMN could not be obtained in our *in vivo* experiments due to intolerable side-effects of the high DNMN dose (convulsions). Thus, we conclude that DNMN is an effective prodrug of NMN formation in the brain; however, extracellular NMN levels in the micromolar range are insufficient to elevate the concentration of free choline. A possible reason for this failure is the unknown, but probably limited affinity of NMN for the choline transporter (see Introduction); chemical analogues of NMN with high affinity may be more effective. Further experimental work would be facilitated if the carriers which are responsible for choline transport could be characterized at the protein level.

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