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Bioavailability of Reduced Nicotinamide-adenin-dinucleotide (NADH) in the Central Nervous System of the Anaesthetized Rat Measured by Laser-Induced Fluorescence Spectroscopy

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Abstract: Drugs intended to increase wellness or quality of life ("lifestyle drugs") have gained popularity and/or importance over recent years. Biogenic substances like nicotinamide adenine dinucleotide (NADH) are supposed to increase the physical and intellectual performance without side-effects. NADH is an energy-delivering co-substrate in the respiratory chain. Clinical studies showed positive effects of peripherally given NADH in Morbus Parkinson and major depression. NADH can be measured by its fluorescence. In this study a pulsed N_2-laser combined with a fibre-optic probe and photomultipliers was used to induce and measure NADH fluorescence in the rat cortex. The aims of the study were to assess the suitability of the laser-induced spectroscopy for *in vivo* and on-line measurement of NADH in neuroscience and the assessment of the central availability of NADH after peripheral administration. NADH (50 mg/kg) but not the precursor nicotinamide caused a significant rise of the NADH fluorescence intensity indicating an increase of the NADH concentration in the rat cortex. In conclusion, the results suggest that NADH given orally or intraperitoneally increases the amounts of NADH in the brain. The results may thus help to explain the clinical effects reported.

Nicotinamide adenine dinucleotide (NADH) plays a central role as a co-substrate for energy transfer in the mitochondrial oxidative phosphorylation, which supplies more than 95% of the total energy requirement in the cells (Erecinska & Wilson 1982). In most cells NADH is the main source of reducing equivalents and NADH is therefore an important factor of cellular metabolism. The vitamin B3 nicotinamide is the main precursor for the synthesis of NADH molecules in eucariotic cells.

There is evidence that an additionally supply of co-factors or substrates could have beneficial effects in the diseases of the central nervous system. NADH as an energy-supplying substrate could also support enzymatic reactions. Clinical studies demonstrate the positive effect of peripherally administered NADH on serious diseases such as Parkinson's disease, chronic fatigue syndrom and major depression (Birkmayer & Birkmayer 1991; Birkmayer *et al.* 1993; Forsyth *et al.* 1999).

In connection with these clinical studies which show central effects after peripheral administration, we wanted to investigate whether peripherally administered NADH might change the central concentration of NADH.

Selective determination of NADH *in vivo* is possible due to its spectroscopic properties. NADH and the corresponding oxidized molecule NAD+ are natural fluorophores, which start to fluoresce after stimulation. The reduced and energy-rich form NADH has two absorption peaks at (λ= 260 nm and 340 nm), the oxidized form has only one absorption peak at (λ=260 nm). Stimulation of the molecules with light at wavelengths around 340 nm (ultraviolet-spectrum) leads to generation of fluorescence signals (maximum at 465 nm) that start only from the reduced molecules. The strength of the NADH fluorescence signal is proportional to the concentration of the NADH molecules. Various fluorometric methods have been developed with high powered light sources such as xenon lamps, tungsten-halogen lamps, mercury arc lamps or lasers (Renault *et al.* 1984; Ince *et al.* 1992). In this study a nitrogen-laser-fluorescence detector with spectral filters has been used to induce and measure NADH fluorescence in the cortex of rats. Inclusion of a time-gate allows the suppression of long-lifetime fluorescence in the detection range (e.g. collagen fibres). The concentrations of NADPH with NADH-like fluorescence-properties are very low and do not interfere with the determination of NADH.

The aim of our study was application of the laser-induced fluorescence spectroscopy for *in vivo* and on-line measurement of NADH in neuroscience and determination of the central availability of NADH after peripheral administration.

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Materials and Methods

Animals. Male Wistar rats (Schönwalde, Germany) 220±25 g, group-housed under a 12 hr light-dark schedule were used. All ani-

mal experiments were carried out following the principles of laboratory animal care and the German Law on Protection of Animals and were approved by the animal protection board of the State of Berlin "Landesamt für Arbeitsschutz, Gesundheitsschutz und technische Sicherheit Berlin, Fachgruppe Veterinärwesen, Lebensmittelwesen und Gentechnik".

Drugs. The following substances were used: Nicotinamide-adenin-dinucleotide reduced (NADH), nicotinamide-adenin-dinucleotide oxidized (NAD+), nicotinamide (all obtained from Sigma-Aldrich) and vehicle (sodium bicarbonate buffer as stabilizer for NADH). All drugs were dissolved in the vehicle prior to use. The injection volume was 1 ml/kg body weight. For oral application NADH, nicotinamide and vehicle in tablet form were used, all delivered from Birkmayer Laboratories (Austria).

Apparatus. For the determination of changes in NADH concentrations a fluorescence detector (Laser Fluoroscope 302, IOM, Berlin, Germany) in combination with data acquisition software and a standard personal computer were used (Rex *et al.* 1999a). The detector uses a pulsed nitrogen laser (λ=337 nm, P=30 µJ, t=350 ps) as light source. The laser beam is coupled into an optical fibre (fused silica, ∅=100 µm) and applied to the cortex. A second fibre of the same diameter, positioned directly beside the 'stimulating' fibre, collects part of the fluorescence and randomly back-scattered light to lead it back to the detection unit. Temporal and spectral gating isolates the NADH fluorescence. Changes of the NADH fluorescence were determined with a high spatial solution, at least for an *in vivo* method, by the small diameter of the optical fibre and the penetrating depth of the laser light (0.5 mm) into the brain.

Testing procedure. Oral application of the tablets was performed immediately before anaesthesia with an adapted applicator for small animals (Kruuse, Denmark) about 60 min. before the start of the NADH fluorescence measurement.

Then the rats were anaesthetized with chloralhydrate (Sigma-Aldrich, 400 mg/kg intraperitoneally) and afterwards a catheter was placed in the vena femoralis. The drugs were injected at a rate of 50 µl/min.

The head of the anaesthetized animal was fixed in a stereotaxic frame (David Kopf, USA), the skull trepanated and the optic probe slowly inserted (4 mm posterior and 2 mm lateral from the intersection of the coronal and sagittal sutures of the skull (bregma), 2 mm deep, according to the stereotaxic brain atlas by Paxinos & Watson 1998). After the insertion of the optical probe the fluorescence was measured continuously. Due to the operative trauma of the brain tissue fluorescence data were used for the evaluation after reaching relatively stable levels (approximately after 120 min.). To secure steady experimental conditions, a continuous humidification of the cortical surface with oxygenised artificial cerebrospinal-fluid (CSF, pH 7.4) containing: 5 mM glucose, 125 mM NaCl, 27 mM $NaHCO_3$, 2.5 mM KCl, 0.5 mM NaH_2PO_4, 1.2 mM Na_2HPO_4, 0.5 mM $NaSO_4$, 1 mM $MgCl_2$ and 1 mM $CaCl_2$ was maintained.

At the end of the experiment the animals were killed quickly by an overdose of the anaesthetic administered intravenously. Following death, the utilization of NADH in the respiratory chain stopped and the intensity of NADH fluorescence increased rapidly (within the next 2 min.) and reached a stable plateau for 15 min. (fig. 1). The mean (n=10 animals) of this maximal increase in the NADH fluorescence was used as the reference point for the pharmacological manipulations and set to 100 per cent.

All animals, which did not show this typical *post mortem* increase in the NADH fluorescence (Mottin *et al.* 1997), were excluded from the study.

Data handling. The fluorescence data represent the changes in per cent of the maximally observed changes in the fluorescence intensity (overdose of the anaesthetic). The basal level of fluorescence measured before a pharmacological manipulation was set to zero.

For the minimization of haemodynamic or tissue-modulated effects on the fluorescence measured, a compensation method based on the measurement of the back-scattered light can be used to detect changes in the NADH fluorescence closely related to metabolic changes. We used a compensation method gained by subtraction of the scattered light from the NADH fluorescence (F): ($F^{450\ nm}$ - $SL^{337\ nm}$) (Harbig *et al.* 1976; Mayevsky 1984).

The intensity of the fluorescence depends on the intensity of the excitation light. To exclude artefacts induced by changes in the excitation intensity by the laser energy, the fluorescence measured was set in relation to the power of the laser.

Statistics. The data were analysed using an one-way ANOVA on repeated measures. A difference of the means with an error probability of $P<0.05$ was considered significant. Data are presented as mean±S.E.M. To ensure clarity in fig. 2 and 4 data are presented as mean and the range of the S.E.M. is stated in the legends to figures.

Results

Following insertion of the optical probe into the cortex the intensity of the NADH fluorescence declined for the first 120 min. and then reached a stable level with a variation of the fluorescence less than two percent in untreated controls.

Application of NADH (50 mg/kg) intraperitoneally induced an increase in the intensity of the cortical NADH fluorescence of about 18±3.8% for approximately 30 min. compared to the fluorescence intensity in the control group, while a lower dose of NADH (10 mg/kg intraperitoneally) did not show any effect (fig. 2).

Neither NAD+ (10 mg/kg, 50 mg/kg) nor nicotinamide (10 mg/kg, 50 mg/kg) had a significant effect on the NADH fluorescence in the cortex during the entire measurement (120 min. after the last manipulation) (fig. 2).

Following oral application of NADH (2 tablets ENADA of 5 mg NADH=51±1.1 mg/kg) the cortical fluorescence intensity at the beginning of the measurements (60 min. after application) was increased compared to the NADH

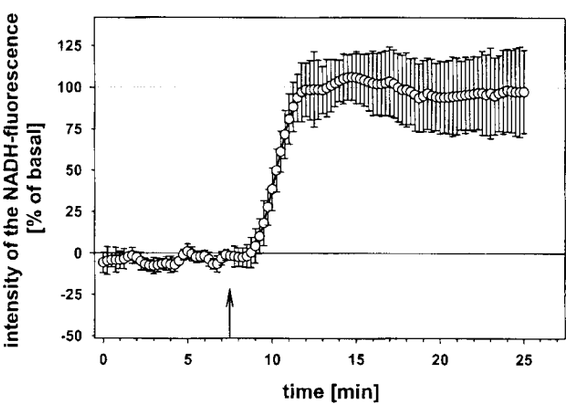

Fig. 1. Change in the intensity of the cortical NADH fluorescence following administration of an overdose of chloralhydrate compared to basal levels. The maximal increase of NADH fluorescence was set to 100 per cent. Data are corrected for scattered light and expressed as means±S.E.M. (n=10).

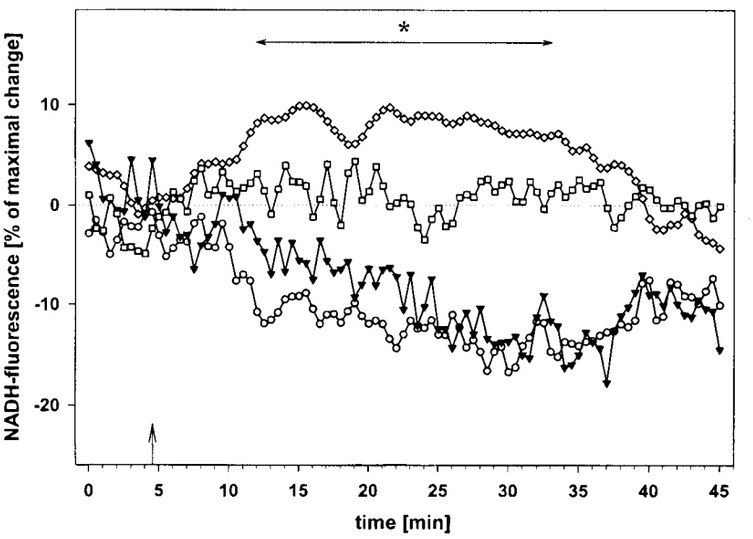

Fig. 2. Effects of NADH (◆, 50 mg/kg, intraperitoneally, S.E.M.±4.1%), NAD+ (▽, 50 mg/kg, intraperitoneally, S.E.M.±7.8%) and nicotinamide (□, 50 mg/kg, intraperitoneally, S.E.M.± 4.6%) on the cortical NADH fluorescence. Data are expressed as mean (n=10). (ONE WAY-ANOVA plus Dunnett's test, *P<0.05, compared to controls, ○, S.E.M.±8.2%).

fluorescence in the vehicle-treated controls (fig. 3). Administration of nicotinamide had no effect on the intensity of the measured NADH fluorescence (fig. 3), however, there was no alteration in the general course of the fluorescence intensity over the time the NADH fluorescence was measured, comparing the different treatments.

Intravenous application of NADH (10 mg/kg) induced an immediate rise (17±4.6% of the maximal increase) in the intensity of the cortical NADH fluorescence for about 2.5 min. Following application of a higher dose NADH (50 mg/kg) this increase in the fluorescence intensity (19±5.1% of the maximal increase) was prolonged to about 9 min. (fig. 4).

After intravenous application of NAD+ (10 mg/kg) a rise in the intensity of NADH fluorescence (16±3.9% of the maximal increase) could be observed for about 3 min. Application of 50 mg/kg NAD+ induced an increase of NADH fluorescence (20±4.8% of the maximal increase) for about 9 min. comparable to the effect of NADH (fig. 4).

Discussion

Biochemical and histochemical methods for the determination of NADH *in vitro* are routine (Bergmeyer & Bernt 1974). In addition to the already established techniques, spectroscopic procedures for measurement of NADH concentrations *in vivo* were developed (Chance *et al.* 1962; Ince *et al.* 1992; Mayevski *et al.* 1996). For selective determination of the NADH fluorescence in our study, laser-induced fluorescence spectroscopy was used (Beuthan *et al.* 1990; Fink *et al.* 1993).

In a previous study we have shown that the measurement of changes in NADH fluorescence with laser technique allows determination of changes in CNS metabolism with high regional selectivity and time resolution *in vivo* (Rex *et al.* 1999a). In the study sodium cyanide, a potent blocker of the respiratory chain, induced an increase in the cortical NADH fluorescence comparable to increased intensity following administration of an overdose of the anaesthetic chloralhydrate. Similar changes could be demonstrated in *in vitro* experiments showing inhibition of NADH consumption after treatment with potassium cyanide (Gille & Nohl 2000). Pathophysiological manipulations as the induction of a cortical spreading depression (Rex *et al.* 1999a) or pharmacological treatment with the serotonin agonist 8-

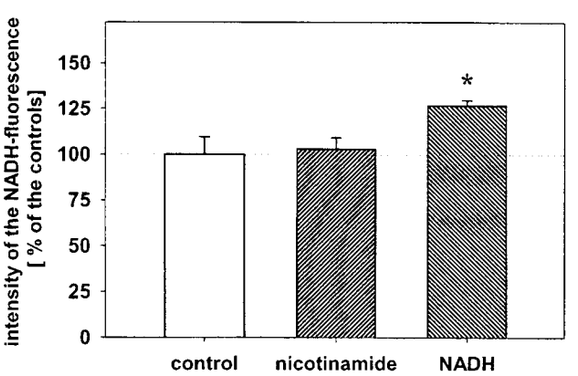

Fig. 3. Effects of NADH (50 mg/kg orally) and nicotinamide (50 mg/kg orally) on the cortical NADH fluorescence directly after implantation of the optical probe into the cortex (60 min. after drug application) compared to the NADH fluorescence of the controls. Data are expressed as mean±S.E.M. (n=10) (*P<0.05, compared to controls).

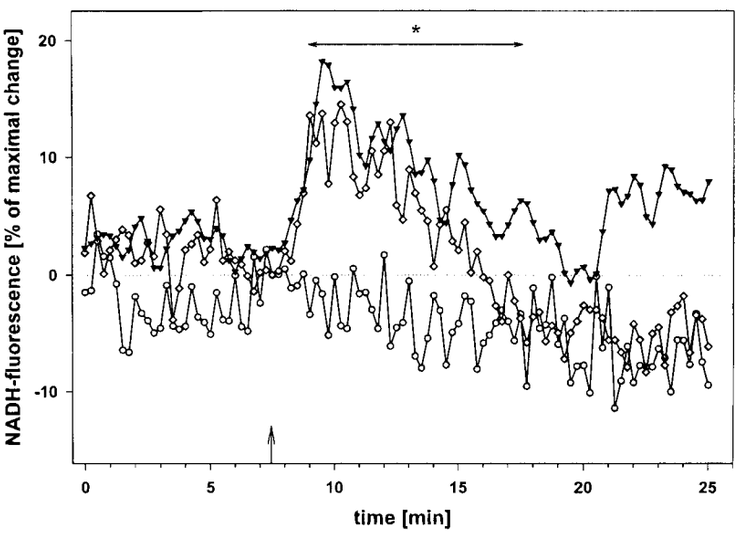

Fig. 4. Effects of NADH (◆, 50 mg/kg, intravenously, S.E.M.±5.6) and NAD+ (▽, 50 mg/kg, intravenously, S.E.M.±6.3) on the cortical NADH fluorescence. Data are expressed as mean (n=10) (*P<0.05, compared to controls, ○, S.E.M.±5.2).

OH-DPAT induces smaller changes in the intensity of the NADH fluorescence (Rex *et al.* 1999b). However, these relatively small changes may be important for the metabolism and function of tissues.

Assessment of the bioavailability of NADH shows a possible application of the laser fluorescence spectroscopy in neuropharmacological studies.

The results of the present study show a significant rise of the measured NADH fluorescence intensity in the cortex of rats after peripheral application of NADH.

To reach the central nervous system, the NADH molecules have to pass two membranes in the processes of absorption and distribution: The membranes in the gastrointestinal tract and the blood-brain barrier between the cerebral vessels and the brain tissue. To our knowledge there are no experimental results on the passage of NADH through the blood-brain barrier.

To show possible central effects of a drug, intravenous administration offers one advantage. The distribution of the drug into the brain is hindered only by the blood-brain barrier. An expected effect of the substance in the brain is not affected by reduced absorption and/or breakdown in the periphery.

The rapid increase in NADH fluorescence as observed after intravenous administration of NADH and as shown in fig. 4 suggests that NADH leaves the blood circulation and reaches the brain tissue.

It could be argued, however, that the NADH measured remains in the blood vessels in the brain, but the following arguments are in favour for passage through the blood-brain barrier.

It is known that haemoglobin absorbs both the excitation light as well as the emitted NADH fluorescence, hence NADH fluorescence measurement in blood vessels is not possible. Measurement of NADH fluorescence can be done only in vessel-free or vessel-poor areas. In addition, blood-vessel restricted rise of the NADH concentration can be excluded since an increase in the dosage given intravenously with the same injection volume leads to prolonged increase in fluorescence, but has no effect on the intensity of the fluorescence. If the increase of NADH is limited to the vessels, only the strength of the fluorescence signal would increase.

An increase in the NADH fluorescence following intraperitoneal and oral administration suggests that NADH molecules might be also absorbed from peritoneum and to a smaller degree also from the intestine as shown in the fig. 2 and 3, respectively.

The intraperitoneal injection is a standard administration of drugs to rodents in experimental pharmacology. The big surface and the good vascularization of the peritoneum ensure a good absorption of substances through the peritoneal membrane (Scheler 1989). Encouraged by the intestine movements, NADH injected into the peritoneal cavity spreads fast in the peritoneum and thus might be absorbed quickly. Intraperitoneal injection avoids degradation of the NADH through enzymes located in the digestion tract.

Absorption of NADH after oral application could be assumed by the clearly raised NADH fluorescence at the beginning of the experiment, as seen in fig. 3. The long period between the application and the measurement of the NADH fluorescence can be responsible for the relatively small changes in the fluorescence intensity of NADH. This interval however, is mandatory due to the duration of the surgery (approximately 60 min.).

The slow absorption from the gastrointestinal tract and subsequently lower concentrations in the portal vein (same amount of drug over a longer timespan) could also enhance

the effects of metabolizing enzymes in the liver leading to a relatively small bioavailability of NADH after oral application.

However, it has not been verified that the NADH molecules that cause the increased fluorescence in the cortex are identical with the injected NADH molecules. The occurrence of NADH in the periphery could also induce a change in the lactate/pyruvate ratio, since the Krebs cycle is under only minor control by NADH (Lance *et al.* 1985). Pyruvate and lactate can pass the blood-brain barrier and induce an increase in the NADH formation in the CNS.

The oxidized pyridine nucleotide NAD+ is converted within cells from nicotinamide. The pyrimidine ring in the NAD+ molecule accepts a hydrogen atom, and is able to stabilize the accepted electron. Hence, NAD+ can be reduced to NADH, which can proceed in reactions by carrying an electron and a hydrogen ion.

Intravenous administration of NAD+ causes a rise in the intensity of the NADH fluorescence. This increase in NADH fluorescence after the intravenous injection may point to a steady-state existing in idle status between the low-energy form NAD+ and the high-energy form NADH in the tissue so that a rapid and one-sided supply of NAD+ might lead to a partial reduction of NAD+ to NADH.

After intraperitoneal injection of the oxidized form NAD+, no modification of the NADH fluorescence in the cortex could be observed. This may be attributed to an impaired absorption of the positively loaded molecule.

Nicotinamide (vitamine B3) is a precursor of NADH in the biochemical pathway and is an integral component of the NADH molecule.

Since we know that nicotinamide is absorbed rapidly and completely following oral application (Stratford *et al.* 1996), intravenous administration of nicotinamide was not needed. Nicotinamide did not have any effect on the cortical NADH fluorescence. One possible explanation for the lack of effect of nicotinamide in our investigations could be either the pretreatment time and/or the single administration given. This interpretation would be in line with a study where a single injection of nicotinamide (500 mg/kg intraperitoneally) did not increase the cortical concentration of NADH in the CNS of rats (Klaidman *et al.* 2001). It is possible that chronic treatment with nicotinamide could increase the synthesis of NADH, but to our knowledge no data are available.

Our results stand in accordance with clinical studies, in which beneficial central effects of oral NADH in patients with Parkinson's disease and major depression were verified (Birkmayer *et al.* 1993; Forsyth *et al.* 1999).

The fact that the fluorescence measurements were carried out in anaesthetized animals with reduced metabolic activity should not be neglected. These animals have smaller NADH consumption and higher NADH pool. In principle measurement of NADH in awake and freely moving animals is possible and should be further investigated.

In conclusion, the results prove that the applied method for *in vivo* measurement of NADH fluorescence by laser-induced fluorescence spectroscopy is a useful method for *in vivo* and on-line measurement of NADH in neuroscience and could be a suitable tool for pharmacological and pharmaceutical studies.

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