Effects of NADH on Dopamine Release in Rat Striatum

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ABSTRACT Nicotinamide adenine dinucleotide (NADH) may be utilized for the synthesis and regeneration of tetrahydrobiopterin (BH4), which in turn is an essential cofactor for tyrosine hydroxylase, the rate-limiting enzyme in the synthesis of dopamine (DA). NADH has been reported to relieve some of the symptoms of Parkinson’s disease, presumably by altering dopaminergic function. The present study examines the efficacy of NADH in influencing DA activity in the rat striatum. In striatal slices, NADH (350 μM) significantly increased basal DA and DOPAC efflux and caused a 2-fold increase in the DA overflow evoked by high KCl (25 mM). Tissue levels of BH4, basal BH4 efflux, and KCl-evoked BH4 overflow were unaffected by NADH, as was [3H]DA uptake into striatal synaptosomes. In contrast to the effects of NADH on DA function in vitro, no effects were observed when NADH was administered systemically. NADH (10 or 100 mg/kg, s.c.) did not influence the tissue content of DA, 5-HT, or their metabolites in the midbrain or striatum, nor did it alter DA extracellular concentrations. These results indicate that NADH can increase DA release from striatal slices, although we are as yet unable to detect this effect in vivo. Synapse 36:95–101, 2000.

INTRODUCTION Parkinson’s disease (PD) is characterized by a progressive degeneration of dopaminergic terminals in the nigrostriatal pathway. Postmortem analysis has shown that tyrosine hydroxylase (TH), the rate-limiting enzyme of DA synthesis, is depleted in the striatum of patients with this disorder (McGeer et al., 1971). TH requires the cofactor 5,6,7,8-tetrahydrobiopterin (BH4) (Brenneman and Kaufman, 1964), which also is decreased in PD (Nagatsu et al., 1981). BH4 has been shown to increase intracellular DA stores in PC12 cells (Anastasiadis et al., 1994), enhance DA release from rat striatal slices (Liang and Kaufman, 1998), and increase the in vivo synthesis and release of DA in rat striatum (Koshimura et al., 1995; Tsukada et al., 1994). Although it is generally assumed that the effects of BH4 can be attributed to an increase in TH activity, some effects may result from a direct stimulation of DA release (Koshimura et al., 1990, 1995).

Because nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH) may be utilized for BH4 synthesis and regeneration, it has been proposed that these agents may be a useful therapeutic intervention for PD patients. Indeed, when given to such patients, NADH has been shown to improve some symptoms (Birkmayer and Birkmayer, 1989b; Birkmayer et al., 1993; Kuhn et al., 1996) and to increase plasma levels of L-DOPA (Kuhn et al., 1996), although a lack of effectiveness of NADH also has been reported (Dizdar et al., 1994). NADH also has been found to increase DA synthesis and efflux in PC-12 cells (Vrecko et al., 1993, 1997).

In this report we have examined the effects of NADH on dopaminergic function in the striatum. First, we used an in vitro superfusion technique to measure how NADH influences basal and evoked striatal DA efflux. Second, we measured the synthesis and quantity of DA, 5-HT, and their metabolites in the midbrain and striatum after systemic NADH. Third, we measured BH4 levels in tissue and superfusate to determine if they are elevated in the presence of NADH. Fourth, using striatal synaptosomes, we examined the possibility that NADH influences DA uptake. Finally, we used
in vivo microdialysis to assess the effects of systemically administered NADH on extracellular DA levels in the striatum. Our results are consistent with an ability of NADH to stimulate DA release. However, these results derive entirely from an in vitro preparation of striatal slices and we have as yet found no such effects in vivo.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (Zivic Miller Laboratories, Allison Park, PA or Hilltop Laboratories, Scottsdale, PA) weighing 300–500 g were used for all experiments. Animals were housed in a temperature-controlled room (22°C) with the lights on from 7 AM to 7 PM daily. The animals were given either Rodent Lab Chow 5001 (Ralston-Purina, St. Louis, MO) or ProLab 3000 (PMI Feeds, St. Louis, MO) food pellets, and water ad libitum. All procedures were conducted during the light portion of the light/dark cycle.

Chemicals

The following chemicals were purchased from Fisher Scientific Co. (Pittsburgh, PA): Dextrose, KCl, MgCl2, methanol, NaCl, sodium bisulfite, and HClO4. Chemicals acquired from Sigma Chemical Co. (St. Louis, MO) included: activated alumina, cocaine HCl, choline chloride, and diethylene-triaminepentaacetic acid (DATA-PAC), 3-hydroxytryptamine HCl (DA), 3,4-dihydroxybenzylamine HBr (DHBA), 3,4-dihydroxyphenylacetic acid (DOPAC), 4-hydroxy-3-methoxy-phenylacetic acid (HVA), 5-hydroxyindole-3-acetic acid (5-HIAA), and 5-hydroxytryptamine HCl (5-HT). Chemicals purchased from Fluka (Buchs, Switzerland) included ethylenediamine-tetraacetic acid (EDTA), sodium acetate, L-ascorbic acid, NaHCO3, NaH2PO4, and octyl sodium sulfate. β-Nicotinamide adenine dinucleotide (NADH) disodium salt and tris-(hydroxymethyl)-aminomethane (TRIS) were purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA). CaCl2 was obtained from EM Science (Cherry Hill, NJ). [3H]DA was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO) with a specific activity of 24 Ci/mmol at a concentration of 0.1 mCi/ml.

In Vitro superfusion

The in vitro efflux of DA and BH4 was measured from striatal slices as previously described (Stachowiak et al., 1987). Immediately after rats were decapitated, the striatum was dissected out of the brain on ice, and coronal slices prepared (350 μm) with a McIlwain-Brinkmann Tissue Chopper (Brinkmann, Instruments, Westbury, NY). Slices were placed into an ice-cold, oxygenated, Krebs-bicarbonate buffer containing the following (in mM): 117 NaCl, 25 NaHCO3, 11.5 dextrose, 4.7 KCl, 1.25 CaCl2, 1.2 MgCl2, 1.2 NaH2PO4, and 0.86 L-ascorbic acid, pH 7.4. Slices were loaded into low-volume tissue chambers of the Brandel Multichannel Superfusion Instrument (Brandel Research and Development Laboratories, Inc., Gaithersburg, MD). Chambers were filled with Sephadex G-25 resin to minimize dead space. Slices were immediately superfused at 200 μl/min with a continually gassed (95% O2, 5% CO2) Krebs-bicarbonate buffer. All slices were given 60 minutes to equilibrate in the presence or absence of NADH (175–2,000 μM) before fractions were collected. Superfusate fractions were collected in 3-minute intervals into tubes containing 50 μl of 1.1 N HClO4 with 2.2 mM sodium bisulfite. In order to depolarize the tissue, the KCl concentration in the superfusion buffer was increased to 25 mM for 3 minutes. When the KCl concentration was elevated to 25 mM, the concentration of NaCl was subsequently decreased by an equiosmolar amount. Collected fractions were frozen immediately on dry ice prior to analysis.

At the end of the superfusion experiment, slices were collected into tubes containing 500 μl 0.1 N HClO4 plus 0.2 mM sodium bisulfite, homogenized, and centrifuged at 18,000g for 10 minutes at 4°C. The supernatant was frozen on dry ice for later analysis of BH4, biogenic amines, and their metabolites. The pellet was resuspended in 200 μl 0.1 M NaOH for analysis of protein content (Bradford, 1976).

Sample purification

Superfusate samples and their corresponding tissue samples were extracted and concentrated over alumina according to modifications of previously published methods (Snyder et al., 1990). Catecholamines were eluted with 50 μl HPLC mobile phase (pH 2.0) containing 0.05 M NaH2PO4, 15% methanol, 75 μM EDTA, and 0.8 mM octyl sulfate sodium salt. This method resulted in approximately a 70% recovery for DA and 85% for DOPAC. Samples were analyzed by HPLC with electrochemical detection. The HPLC consisted of an ESA Coulochem Model 5100A detector with ESA microdialysis cell 5014B (ESA Inc., Chelmsford, MA), a Waters 712 WISP autosampler, either a Waters 501 pump or a Shimadzu LC-10AD pump (Shimadzu Corp., Columbia, MD), and a Waters Symmetry C18 column (3.9 × 150 mm, 5 μ, Waters Corporation, Milford, MA). Electrochemical detection was accomplished with the first electrode (oxidative) set at 0.26 V, the second electrode (quantitating) set at 0.28 V, and the guard cell set at 0.40 V. Quantitation was achieved using a Waters 740 or HPChemStation Integrator (Hewlett-Packard Co., Wilmington, DE) which allowed for the comparison of unknown samples to standards of DOPAC, DHBA, and DA. The mobile phase was delivered at 0.8 ml/minute. The limit of detection of this HPLC system was approximately 0.01 pmol.
Systemic NADH and tissue amines

NADH (10 or 100 mg/kg) was freshly dissolved in sterile 0.9% saline and administered subcutaneously. Rats were decapitated 60 minutes later, and the striatum and midbrain were dissected on ice and homogenized in 1.3 or 0.65 μl of 0.1 N HClO₄, respectively. The resulting homogenate was centrifuged at 18,000 g for 10 minutes. The supernatant was removed for analysis of DA, DOPAC, HVA, 5-HT, and 5-HIAA as described below. In order to evaluate if NADH might influence midbrain and striatal levels of BH₄, aliquots were saved for analysis of BH₄ using HPLC reversed-phase and fluorescence detection as detailed below.

In vivo microdialysis

The concentration of DA in the extracellular fluid of the striatum was measured using previously published methods (Abercrombie et al., 1990). Approximately two weeks after arrival, rats were removed from the colony room and anesthetized with Equithesin (2.8 ml/kg, i.p.) for implantation of a dialysis probe. Vertical, concentric dialysis probes were constructed by using a dialysis membrane (Spectra Por; Spectrum, Los Angeles, CA) with a 13,000 molecular weight cutoff and an exterior diameter of 250 μm. The dialyzing length of the probes was 4 mm. Before and after implantation, probes were perfused at a rate of 1.8 μl/min with a solution consisting (in mM) of 147 NaCl, 2.7 KCl, 1.0 MgCl₂, and 1.2 CaCl₂. Probes were implanted vertically into the neostriatum (AP +0.5; ML −2.5; DV −7.0 from dura). The inlet line of the probe was connected to a swivel (Spaulding Medical Products, Birmingham, AL) to allow for free movement of the animal. After surgery, the animals were placed in a cylindrical Plexiglas cage (27 cm diameter, 39 cm height) and given approximately 18 hours to recover.

Analysis of DA content in dialysate and ex vivo tissue samples

Dialysate and tissue samples were assayed using HPLC with electrochemical detection. The chromatography system consisted of a pump (model 510; Waters, Milford, MA) operating at a rate of 0.7–0.8 ml/min, a Brownlee Velosep RP-18 column (Applied Biosystems, San Jose, CA), and an amperometric detector (Waters 460 electrochemical detector) set to a potential of +0.60 V vs. Ag/AgCl. The mobile phase consisted of 100 mM sodium acetate, 1.6 mM octyl sodium sulfate, 0.1 mM EDTA, and 6% methanol. The detector output was connected to a HPChemStation data analysis system (Hewlett Packard, Pittsburgh, PA) and compounds were quantified based on peak heights compared with standards. The limit of the sensitivity of the assay for DA detection was approximately 1 pg/20 μl.

Synaptosomal uptake

Rat striatal synaptosomes were prepared as previously described (Berman et al., 1997). Synaptosomal aliquots (100 μl, 60–80 μg protein) were equilibrated for 30 or 60 minutes in the presence or absence of NADH (350 μM) at 37°C in a Krebs bicarbonate buffer composed of the following (in mM): 117 NaCl, 25 NaHCO₃, 11.5 glucose, 4.7 KCl, 1.25 CaCl₂, 1.2 NaH₂PO₄, 1.2 MgCl₂, and 1 DATAPAC, pH 7.4. Uptake was initiated by the addition of [3H]DA (specific activity ~4,500 dpm/pmol) at a concentration (250 nM) near the Kt for high affinity DA uptake (Yi and Johnson, 1990). Uptake was terminated 3 minutes later by putting the samples immediately on ice and adding 2 ml ice-cold Krebs buffer containing choline chloride instead of NaCl. Synaptosomes were harvested via rapid vacuum filtration (Brandel Research and Development Laboratories) onto GF/B filters. Synaptosomes then were washed 3 times with ice-cold choline buffer and the filters were mixed with 5 ml Cytoscint (ICN, Costa Mesa, CA). Radioactivity was assessed 24 hours later using liquid scintillation spectroscopy (LS 6500, Beckmann Laboratories, Inc., Fullerton, CA). High affinity [3H]DA uptake was calculated by subtracting the nonspecific uptake measured by the addition of 500 μM cocaine. In these experiments, nonspecific uptake was <16%.

BH₄ analysis

BH₄ from striatal tissue and superfusate samples was oxidized under acidic conditions and quantified by reverse-phase HPLC with fluorescence detection according to methods previously described (Fukushima and Nixon, 1980; Kapatos et al., 1982).

Statistical analysis

In vitro superfusion data were analyzed by 2-way ANOVA (group × time) with repeated measures of time. All other data were analyzed by independent Student’s t-tests. Basal efflux was defined as the average efflux of the three superfusate fractions collected during the 12 minutes prior to the application of KCl. Overflow was defined as the increase in efflux that occurred during the three minute exposure to KCl. A P < 0.05 was considered statistically significant.

RESULTS

Effect of NADH on DA efflux and overflow in striatal slices

Striatal slices exhibited a low level of DA and DOPAC efflux, consistent with previous reports (Snyder et al., 1990). When the concentration of KCl in the superfusion was increased to 25 mM, DA efflux was increased by 0.056 ng/mg protein/min. Previous studies have indicated that basal DA efflux is Ca²⁺-independent (Snyder and Zigmond, 1990), whereas KCl-in-
duced overflow reflects Ca\(^{2+}\)-dependent exocytosis (Nomura et al., 1981; Schwarz et al., 1980).

In the presence of NADH (350 \(\mu\)M), we observed a significant increase in the basal rate of DA and DOPAC efflux from striatal slices (t-test \(P < 0.02\) for 350 \(\mu\)M vs. control for DA efflux; t-test \(P < 0.05\) for 350 \(\mu\)M vs. control for DOPAC efflux; Table I). There were no significant changes in the concentration of DA or DOPAC taken immediately after superfusion from the remaining superfused striatal slices (Table I). The addition of 350 or 700 \(\mu\)M NADH increased KCl-evoked DA efflux by an average of 66 \(\pm\) 21\% (ANOVA \(P < 0.02\)) and 67 \(\pm\) 3\% (ANOVA \(P < 0.03\)), respectively (Fig. 1). Lower (175 \(\mu\)M) and higher (2 mM) concentrations of NADH did not, however, potentiate DA overflow above that caused by KCl alone (Fig. 2).

**Effect of NADH on DA uptake**

Next, we examined the possibility that the NADH-potentiated increase in KCl-evoked DA overflow in striatal slices was due to the inhibition of DA reuptake rather than increased DA release. Striatal synaptosomes were preincubated with NADH (350 \(\mu\)M) for 30 or 60 minutes and then high-affinity DA uptake was assessed. Specific uptake of \(^{3}H\)DA into synaptosomes was 95 \(\pm\) 5\% and 96 \(\pm\) 4\% of control uptake following 30 and 60 minutes exposure to NADH, respectively. Thus, NADH does not appear to alter DA reuptake and the effects observed in striatal slices appear to be due to an increase in DA release.

**Effect of NADH on BH\(_{4}\) in superfusate and tissue**

It has been suggested that NADH will be effective in alleviating symptoms of PD due to its ability to increase the availability of BH\(_{4}\), which in turn should enhance TH activity. Thus, we investigated the effects of NADH on basal BH\(_{4}\) efflux in striatal slices and on the tissue levels of this cofactor. However, we observed no effect of either 350 or 700 \(\mu\)M NADH (Table II) on these measures, although there was a trend for the 350 \(\mu\)M group to be lower in each instance. Furthermore, since we observed that NADH enhanced KCl-evoked DA overflow, we examined the possibility that NADH may alter BH\(_{4}\), but only in the presence of KCl. However, we failed to observe any change in BH\(_{4}\) levels in response to KCl even when NADH was present (Table II).

**Effect of systemic NADH on striatal DA levels in extracellular fluid and in tissue**

For NADH to be an effective treatment for PD, it must be able to produce its effects when administered systemically. To examine this possibility, NADH (100 mg/kg, s.c.) was administered to freely moving animals and extracellular DA was monitored in the striatum by microdialysis. However, we observed no change in the extracellular levels of DA as a result of this treatment. Moreover, the extracellular levels of DOPAC, HVA, and 5HIAA also were not altered by NADH (Table III). In addition, NADH (10 or 100 mg/kg, s.c.), administered 60 minutes prior to decapitation did not influence the concentration of DA in either the striatum or midbrain. 5-HT, another transmitter whose synthesis is dependent on BH\(_{4}\) also was unaffected, as were the concentrations of their major metabolites (Table IV).
DISCUSSION

Effects of NADH on striatal DA overflow

In the present study, NADH increased basal DA and DOPAC efflux, and caused a 2-fold increase in KCl-evoked DA overflow from striatal slices. The latter effect was highly concentration-dependent; although with 350 and 700 μM NADH, no effect was seen with either a lower (175 μM) or higher (2 mM) concentration of the cofactor.

There are several mechanisms by which NADH might act to produce this effect. The most likely explanation might seem to be that NADH acts by increasing the availability of BH₄. However, NADH did not alter either tissue levels of BH₄ or the rate at which BH₄ appeared in the superfusate. A second possibility is that NADH increases DA availability by increasing DA synthesis via a mechanism independent of alterations in BH₄. Some evidence of this was observed as NADH acted to increase DA release independent of an increase in synthesis, or even to produce its apparent therapeutic effects through a non-dopaminergic mechanism. For
example, NADH might influence 5-HT release since NADH is a necessary cofactor for the synthesis of 5-HT by tryptophan hydroxylase. It has been shown that exogenous 5-HT can elevate extracellular DA levels in the ventral striatum (Parsons and Justice, 1993), and we have recently observed a similar phenomenon in the dorsal striatum (G.D. Stanwood and M.J. Zigmond, unpublished observations). Moreover, 5-HT is reduced in PD (see Birkmayer and Riederer, 1983). Thus, it is possible that NADH may increase DA release via an increase in the availability of 5-HT. However, in our studies NADH did not alter tissue 5-HT or 5HIAA, nor did it affect extracellular 5HIAA. This reduces the likelihood of a role for 5-HT in the actions of NADH.

Additional possible routes of NADH actions

At least three other possibilities exist through which NADH might exert a clinically effective action, either via increased DA release or in some other manner. First, NADH might influence glutamate, which in turn could modulate dopaminergic activity or influence the symptoms of PD in some other manner. It has been demonstrated that exogenous glutamate increases DA overflow in striatal slices (Lonart and Zigmond, 1991; Roberts and Sharif, 1978). Furthermore, striatal levels of NMDA receptors are reported to be elevated in PD (Weihmuller et al., 1992). Because NADH is involved in the conversion of alpha-ketoglutarate into glutamate, it is possible that NADH alters glutamate synthesis and/or transmission, which may then influence DA activity.

Second, NADH may be clinically useful but only as a supplement to ongoing therapy with L-Dopa. It has been suggested, for example, that NADH may affect the pharmacokinetics of L-Dopa such that the DA precursor persists for longer periods in the brain (Swerdlow, 1998). This could explain the findings that NADH improves PD disability scores when administered with L-Dopa (Birkmayer and Birkmayer, 1989a; Birkmayer et al., 1993; Kuhn et al., 1996), an observation not easily interpreted in terms of changes in tyrosine hydroxylation. Third, NADH may play a neuroprotective role in PD. NADH is a strong reducing agent and as such may have antioxidant properties. Thus, NADH may promote a healthier cellular environment for the remaining DA neurons, allowing for enhanced DA activity and possible alleviation of PD symptoms. In support of this hypothesis, NADH has been shown to enhance the survival of PC12 cells in the absence of serum and growth factors (Koshimura et al., 1998), and to reduce oxidative cell death in PC12 cells when combined with BH4, horseradish peroxidase, and dihydroypteridine reductase (Shen and Zhang, 1991). However, the relatively rapid improvements reported by parkinsonian patients given NADH (Birkmayer and Birkmayer, 1989b; Birkmayer et al., 1993; Kuhn et al., 1996) cannot easily be explained by a mechanism involving neuroprotection. The relevance of each of these hypotheses to our data and to the clinical efficacy of NADH remains to be examined.

SUMMARY AND CONCLUSIONS

In summary, NADH enhanced basal DA and DOPAC efflux and caused a 2-fold increase in KCl-evoked DA overflow from striatal slices. It is too early to draw any firm conclusions from these results regarding the use of NADH in the treatment of Parkinson's disease. First, we were unable to detect any in vivo effects of NADH on extracellular levels of striatal DA. It is possible of course that, given the narrow dose range within which NADH was effective in vitro, and the possible limited ability of NADH to reach the brain, we simply failed to provide an effective dose. Second, we have thus far only examined tissue from intact animals and it is possible that partial loss of dopaminergic neurons as occurs in PD will alter the response to NADH. Such lesions have been shown to increase DA synthesis within the remaining DA neurons, and DA release from these residual elements seems to be increased as well (Agid et al., 1973; Hefti et al., 1980; Zigmond and Stricker, 1977; Zigmond et al., 1984). Thus, further studies of NADH seem warranted.

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