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Inhibition of mitochondrial complex II affects dopamine metabolism and decreases its uptake into striatal synaptosomes

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Abstract

The mitochondrial toxin, 3-nitropropionic acid (3-NP), is a specific inhibitor of succinate dehydrogenase, complex II in the mitochondrial respiratory chain. The aim of our study was to determine the relationship between inhibition of mitochondrial complex II and dopamine (DA) metabolism and its transport into rat striatal synaptosomes after exposure to 3-NP. The study was carried out using spectrophotometric, radiochemical and HPLC methods.

Our data showed that inhibition of succinate dehydrogenase by intraperitoneal (i.p.) injection of 3-NP (cumulated dose 100 mg/kg in 4 days) significantly affected DA metabolism, leading to the accumulation of its metabolites, 3,4-dihydroxylphenylacetic acid (DOPAC) and homovanillic acid (HVA) in the rat striatum. These experimental conditions had no effect on free radical dependent lipid peroxidation in the brain. In vitro experiments revealed that DA and DOPAC significantly decrease lipid peroxidation in the brain homogenate. Moreover, 3-NP significantly inhibited [^{3}H]DA uptake into striatal synaptosomes by specific dopamine transporter (DAT). The scavengers of superoxide radical (O_2^-) Tempol and Trolox had no effect on DAT function, but the nitric oxide synthase (NOS) inhibitor Nω-nitro-L-arginine (100 μM) prevented 3-NP-evoked DAT down-regulation.

In summary, our results indicate that inhibition of mitochondrial complex II by 3-NP enhances DA degradation and decreases its uptake into synaptosomes. It is suggested that NO and energy failure are responsible for alteration of the dopaminergic system in the striatum.

Key words: *3-nitropropionic acid, dopamine, DAT, striatum, nitric oxide*

Abbreviations: *3-NP – 3-nitropropionic acid; DA – dopamine; i.p. – intraperitoneal; DOPAC – 3,4-dihydroxylphenylacetic acid; HVA – homovanillic acid; DAT – dopamine transporter; TBARS – Thiobarbituric acid reactive substances; NOS – nitric oxide synthase; MAO – monoamine oxidase; MDA – malondialdehyde; TCA – trichloroacetic acid*

Introduction

The mitochondrial toxin 3-nitropropionic acid (3-NP) is a specific inhibitor of succinate dehydrogenase, complex II in the mitochondrial respiratory chain. The striatum is significantly affected in neurodegenerative disorders such as Parkinson's and Huntington's diseases. It has been suggested that dopaminergic system perturbation is responsible for the vulnerability of this brain region to damage.

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Recent work has indicated that 3-NP increases DA concentration and turnover in the rat brain [9] and induces the loss of striatal dopaminergic terminals [3]. Removal of dopamine (DA) significantly reduces the severity of striatal lesions caused by systemic administration of 3-NP or direct intraparenchymal injection of malonate, another complex II inhibitor [12,16]. Moreover, addition of DA to cultured striatal neurons potentiates apoptotic cell death caused by methyl malonate [14]. Also 3-NP toxicity is enhanced by amphetamine, which stimulates DA release from neurons [4]. It is widely known that the autooxidation and monoamine oxidase (MAO)-mediated metabolism of DA cause free radical production. However, there are some data indicating that DA, depending on circumstances, exhibits prooxidative or antioxidative properties [8].

Recent data have indicated that inhibition of the dopamine transporter (DAT), which removes DA from the synapses, could be responsible for dopaminergic system perturbation. It has been found that DAT knock-out mice are hypersensitive to 3-NP-induced striatal damage [5] and that 3-NP inhibits [^{3}H]DA uptake into rat striatal synaptosomes [13]. The mechanism of DAT alteration is not fully understood. The transporter activity could be affected by energy disturbances or by free radical generation as a result of respiratory complex II inhibition.

The aim of this study was to determine the effect of mitochondrial complex II inhibition with 3-NP on DA and its metabolite concentration in the rat brain and to evaluate its role in DAT function. Moreover, we investigated the role of oxidative stress in dopaminergic system alteration evoked by inhibition of succinate dehydrogenase.

Material and methods

2-thiobarbituric acid was purchased from Merck KgaA (Darmstadt, Germany), [^{3}H]DA was from Amersham Biosciences, protease cocktail inhibitors were from Roche Diagnostics and all remaining chemicals were from Sigma (St Louis, MO USA).

All experiments on animals were accepted by the Polish National Ethics Committee, and were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). Male, adult Dowley Rats (200–250 g) were obtained from the Animal Breeding House of the Medical Research Centre in Warsaw (Poland). All rats were housed under standard conditions (12 hr light/dark cycle) with free access to food and water.

Animal treatment with 3-NP

Animals received four intraperitoneal injections of 3-NP (60 mg/ml, pH 7.4, 30 mg/kg) at 24 h intervals. Control animals received intraperitoneal injection of saline. Animals were killed at 24 h after the last injection of the neurotoxin.

Preparation of brain samples for HPLC analysis

Rats were killed and the brain was quickly removed. The striatum was isolated on ice and homogenized in ice-cold 50 mM TRIS-HCl, pH 7.4. For HPLC analysis brain parts were homogenized in 3.6 g/l $HClO_4$, centrifuged 15,000 x g 10 min and supernatant was filtrated by syringe Millipore 0.22 µm filter prior to injection onto a column.

Preparation of striatal synaptosomes

For preparation of striatal synaptosomes, the striatum was homogenized in 10 vol. of 0.32 M sucrose in 10 mM Tris-HCl pH 7.4 containing 1 mM EDTA, using 12 up- and down-strokes of a Dounce homogenizer. The homogenates were then centrifuged at 1000 x g for 10 min at 4°C to obtain a crude nuclear pellet (P1). Supernatants were stored at 4°C, and the pellet was resuspended in 10 vol. of homogenizing buffer and centrifuged for 10 min at 1000 x g. The two supernatants were pooled and centrifuged at 17,500 x g for 30 min at 4°C. The resulting supernatant was discarded and the final pellet resuspended in ice-cold Krebs-Ringer buffer, pH 7.6 (in mM: NaCl 120, KCl 5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, and glucose 10). Protein concentration was measured by the method of Lowry et al. [11], using bovine serum albumin as standard.

Determination of lipid peroxidation

Thiobarbituric acid reactive substances (TBARS), including malondialdehyde (MDA), were determined as an index of lipid peroxidation [2]. Brain homogenate (1 mg protein) was incubated in the presence of DA, HVA or DOPAC. Incubation was carried out for 1 h at 37°C. After incubation 1 ml of 30% TCA, 0.1 ml of 5 M HCl and 1 ml 0.75 % thiobarbituric acid in 0.1 M NaOH were added. The tubes were capped and

the mixtures were heated at 100°C for 15 min in boiling water bath. After cooling and centrifugation the optical density of the supernatant was determined at 535 nm. TBARS concentration calculation was based on MDA standard curve.

Determination of [^{3}H]DA uptake into synaptosomes

DA uptake was measured according to the method described previously [21] with slight modification. Briefly, synaptosomes were preincubated for 30 min at 37°C in 2 ml calcium-free Krebs-Ringer-glucose buffer and were added to all incubation tubes containing $CaCl_2$ (2 mM) and pargyline (100 µM). Then 3-NP (1 mM) was added to the appropriate tubes and pre-incubation in the presence or absence of nomifensine (10 µM) was further prolonged for 30 min at 37°C.

DA uptake was assayed using tritiated [^{3}H]DA and nonlabelled DA to obtain a final concentration of 400 nM. After preincubation, synaptosomes were suspended in Krebs-Ringer-glucose buffer to the required protein concentration (usually 60–100 µg protein/assay). The assay was initiated by addition of [^{3}H]DA. After 2 min of DA uptake at 37°C the reaction was stopped by addition of 3 ml of ice-cold Krebs-Ringer buffer, followed by vacuum filtration through Whatman GF/C filters. Each tube was rinsed in 2 x 3 ml of ice-cold Krebs-Ringer buffer. The filters were placed in scintillation vials containing 7 ml of Bray's scintillator and stored at room temperature overnight.

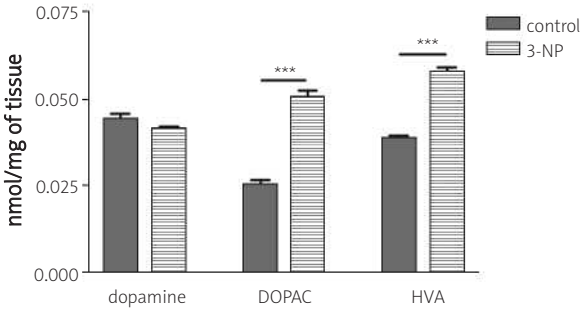

Fig. 1. DA and its metabolites concentration in the rat striatum after 3-NP chronic treatment. DA, DOPAC and HVA concentration was determined in the rat striatum by HPLC method. Data represent the mean value ±S.E.M. from 3 animals. *** – statistical significance compared to control, $p<0.001$

Radioactivity was determined by liquid scintillation spectrometry (Wallac 1409, LKB). Specific uptake was calculated by subtracting non-specific uptake, determined in the presence of 10 µM nomifensine, a specific DAT inhibitor, from total uptake.

HPLC analysis of DA, DOPAC and HVA

DA and its metabolites were separated by high-pressure liquid chromatography equipped with an electrochemical detector (Gynkotek) using Aquasil C-18 reverse phase columns (4.6 x 150 mm) at 20°C. Flow rate was adjusted to 1 ml/min; injection volume was 20 µl. Dopamine and its metabolites were eluted under isocratic conditions in a 0.1 M citric-phosphate buffer containing 0.3 mM Na-octan sulphonate, 0.1 mM EDTA and 4% acetonitril (pH=3.6). Mobile phase was filtered through a Millipore 0.22 µm filter, and degassed prior to use. Electrode potential was adjusted to +0.35 mV. To determine and quantify DA, DOPAC, and HVA the corresponding standards were used.

Statistical analysis

Statistical analyses between the two groups were conducted using a two-tailed, paired Student's t-test. Analyses among multigroup data were conducted using one-way analysis of variance (ANOVA), followed by a Newman-Keuls post-hoc test. Differences among groups were considered significant if the probability of error was less than 5%. The data represent mean ±S.E.M.

Results

The level of DA and its metabolites after chronic inhibition of succinate dehydrogenase by 3-NP were measured in the rat striatum by HPLC method. It was observed that DOPAC and HVA concentration were elevated by 50% and 34% respectively, in animals treated with 3-NP compared to control. DA concentration was unchanged (Fig. 1). The HVA/DA ratio provides an index of the rate of DA turnover in the dopaminergic nerve terminals. Our results indicated that HVA/DA ratio in 3-NP treated rats was shifted in favour of HVA, which indicates an increase in the turnover and degradation of DA.

Inhibition of mitochondrial respiratory chain decreases ATP synthesis in mitochondria which subsequently might provoke oxidative stress. However, our *in vivo* experiments demonstrated that

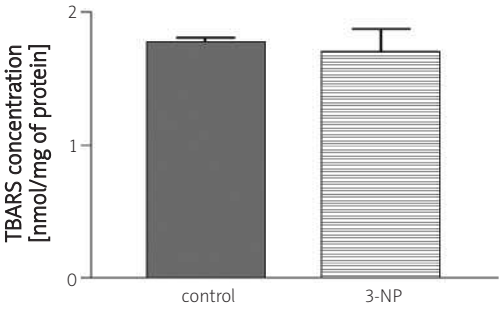

Fig. 2. Effect of 3-NP on TBARS concentration in the rat striatum *in vivo*. Data represent the mean value ±S.E.M. from 3 animals

3-NP had no effect on free radical dependent lipid peroxidation in the rat striatum (Fig. 2). Moreover, a significant decrease of TBARS concentration by DA and DOPAC was observed (Fig. 3A and 3B). HVA in high concentration (200 µM) slightly enhanced lipid peroxidation, but had no effect at 10–100 µM concentration (Fig. 3C). 3-NP decreased specific [^{3}H]DA uptake into rat striatal synaptosomes by 30% and Tempol, an agent that imitates superoxide dismutase, had no effect on DAT function altered by 3-NP (Fig. 4). However, inhibition of nitric oxide synthase (NOS) by Nω-nitro-L-arginine (NNLA, 100 µM) prevented 3-NP--evoked DAT down-regulation (Fig. 5).

Discussion

In the present study we have shown that failure of mitochondrial respiration, evoked by inhibition of succinate dehydrogenase, induced dopaminergic system disturbances, leading to enhancement of DA metabolites concentration in the rat brain striatum. These disturbances did not trigger free radical generation. This indicates that oxidative stress is not responsible for dopaminergic system impairment evoked by inhibition of succinate dehydrogenase. Moreover, DA and its metabolite DOPAC, which accumulate in the striatum in this experimental condition, exert antioxidative action *in vitro*. One possible mechanism of the observed DA turnover perturbation is impairment of DA uptake by specific DA transporter. Under our experimental conditions DA specific uptake into striatal synaptosomes was decreased and this event was unrelated to oxidative stress. Exclusively NOS inhibitor prevented DA uptake inhibition, suggesting a role of NO in dopaminergic system perturbation.

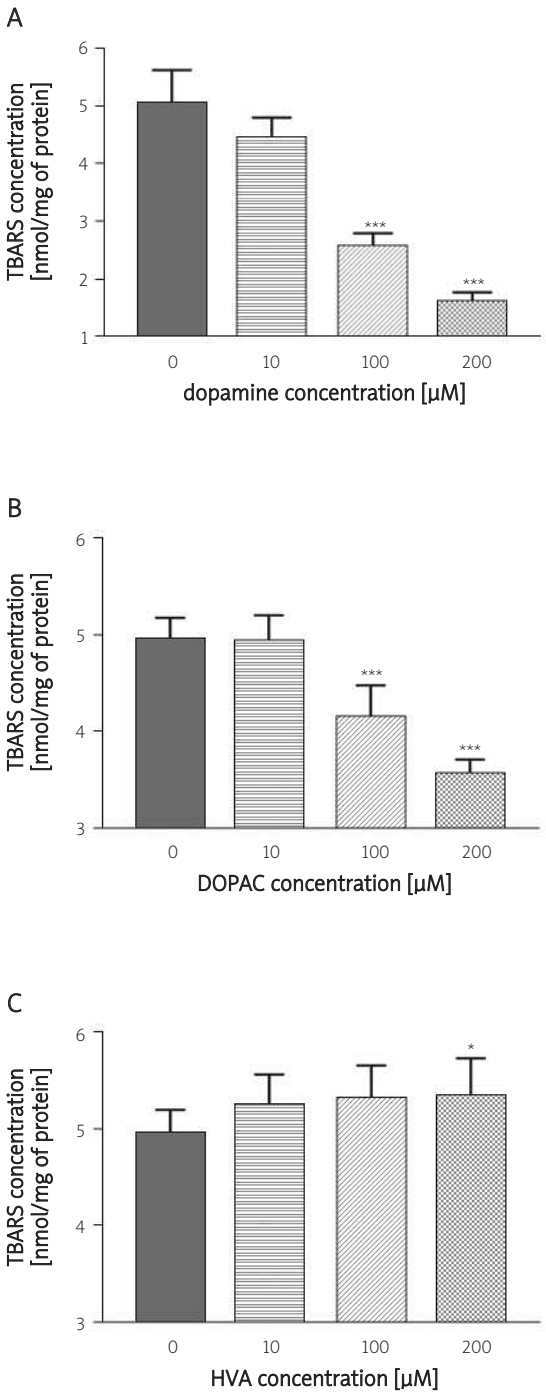

Fig. 3. Effect of DA and its metabolites on TBARS concentration in the rat brain homogenate *in vitro*. **A)** Effect of DA, **B)** effect of DOPAC, **C)** effect of HVA. Data represent the mean value ±S.E.M. from 3 animals. *, *** – statistical significance compared to control, $p<0.05$, $p<0.001$ respectively

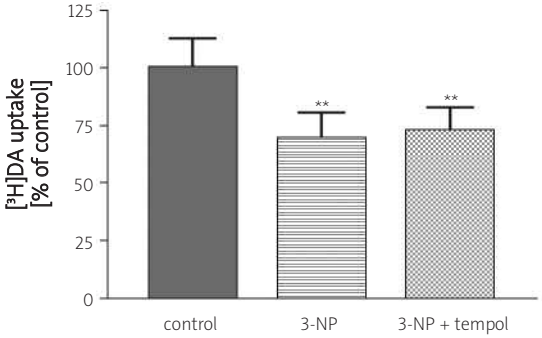

Fig. 4. Specific uptake of [³H]DA into synaptosomes from the rat striatum. Data represent the mean value ±S.E.M. from 3 separate experiments. ** – statistical significance compared to control, p<0.01

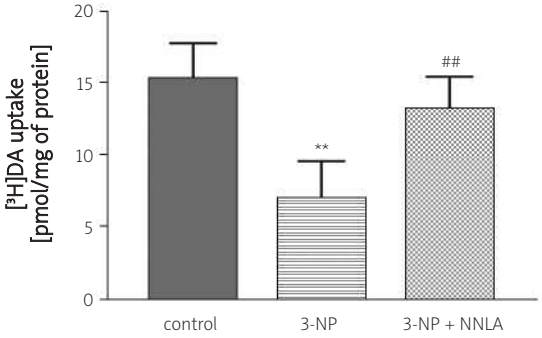

Fig. 5. NOS inhibition prevented DAT dysfunction evoked by 3-NP. DA uptake was measured after 2 min of incubation with [³H]DA. Data represent the mean value ±S.E.M. for 4 separate experiments, each carried out in duplicate. ** – statistical significance compared to control, p<0.01, ## – statistical significance compared to 3-NP, p<0.01

Previously it was observed that the concentrations of extracellular DA and DOPAC are both increased in rats exposed to 3-NP [15]. Johnson et al. [9] observed an increase of DA turnover in animals treated with 3-NP in the caudate nucleus. In our experimental conditions we have found a marked increase of DOAPC and HVA concentration without change in DA concentration. These results indicate that DA degradation is enhanced by mitochondrial complex II inhibition.

It is known that metabolism of DA by MAO results in the formation of hydrogen peroxide, which may mediate its toxicity. An increase in DA level and DA metabolism has been suggested to cause massive free radical production [7,15]. Under our experimental conditions free radical dependent lipid peroxidation was not detected. Moreover, DA and DOPAC exerted antioxidative action against lipid peroxidation in *in vitro* conditions. Our results are consistent with the data of Yen and Hsieh [22], who reported antioxidant properties for DA. Also Hermida-Ameijeiras et al. [8] found that the presence of DA significantly reduces the formation of thiobarbituric acid reagent substances (TBARS). Moreover, Li et al. [10] found that DA is able to suppress the lipid peroxidation caused by iron-ADP in brain homogenates. It was previously indicated that DA is able to enhance the production of •OH by H_2O_2/Fe^{3+}, but also inhibits the lipid peroxidation induced by iron-ascorbate in liposomes. Alteration of such a balance between the reported capacity of DA to stimulate •OH production and its ability to scavenge •OH may be responsible for this pro- or antioxidative effect [20,17,19]. DA seems to show pro- or antioxidative properties depending on circumstances.

Decrease of DAT function by inhibition of succinate dehydrogenase may play an important role in the mechanism of dopaminergic system disturbances. It has been found that DAT knock-out mice are hypersensitive to 3-NP-induced striatal damage [5] and that 3-NP inhibits [³H]DA uptake into rat striatal synaptosomes [13]. It was suggested that DAT inhibition is caused by oxidative damage to transporter macromolecules, but under our experimental conditions there were no indications of macromolecule oxidation. Moreover, Tempol (superoxide dismutase mimicking antioxidant) and Trolox (data not shown) did not prevent DA uptake inhibition. Our last data [1] showed also that Tempol had no effect on DAT down-regulation evoked by alpha-synuclein. Similar results were obtained by Maragos et al. [13], who found that addition of the reactive oxygen species spin trap alpha-phenyl-N--tert-butyl nitrone to synaptosomes exposed to either malonate or cyanide failed to prevent mitochondrial toxin-induced inhibition of DAT function. They also demonstrated that addition of either ATP or ADP plus P(i) to synaptosomes treated with 3-NP is not beneficial for DAT function, indicating that its dysfunction is not related to energy disturbances. There are some indications that excitotoxicity mediated by NMDA receptors may be involved. It was observed that MK-801, an NMDA receptor antagonist,

significantly attenuated the inhibition of DA uptake in cell cultures treated with 3-NP [23]. This observation was supported by data indicating that decortications involving dorsal and lateral frontal cortex or both frontal and parietal cortices significantly decreased the severity of striatal injury evoked by 3-NP. These results suggest that 3-NP may affect the postsynaptic sites and make the striatal neurons more vulnerable to endogenous levels of glutamate [6]. Previously it was observed that NOS inhibition protects against striatal lesions produced by systemic administration of 3-NP, and also against malonate induced decrease in ATP [18]. The present data demonstrate that NOS inhibition prevented DAT dysfunction evoked by 3-NP.

In summary, we demonstrated that inhibition of mitochondrial respiration by 3-NP enhances dopamine metabolism in the direction of its degeneration and inhibits DA uptake which causes alteration of dopaminergic neurotransmission. We suggest that nitric oxide and energy failure could be responsible for DAT dysfunction evoked by 3-NP.

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