EXPERIMENTAL STUDY ON THE EFFECTS OF 7-NITROINDAZOL, A SELECTIVE INHIBITOR OF NEURONAL NITRIC OXIDE SYNTHASE (nNOS), ON SOME BRAIN AND HEPATIC BIOCHEMICAL PARAMETERS

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Abstract

The objective of the present study was to investigate the effects of selective neuronal nitric oxide inhibitor 7- nitroindazole (7-NI) on rat brain and liver, after multiple administrations. Male Wistar rats were treated with 7-NI (25 mg/kg b.w. i.p.) for 5 days. 24 hours after the last administration brains and livers were processed for the biochemical assay. Being an inhibitor of neuronal nitric oxide synthase (nNOS), 7-NI significantly decreased the enzyme activity by 41%.

The production of thiobarbituric acid reactive substances (TBARS) and reduced glutathione (GSH) levels both in liver and brain were measured to assess a possible toxic effect of the compound. Multiple administration of 7-NI did not affect the TBARS levels, neither in the brain, nor in the liver but GSH levels were depleated in both tissues, which suggests a toxic effect of 7-NI. The cytochrome P 450 quantity and the activity of ethylmorphine-N-demethylase (EMND) and anilinehydroxilase (AH), were also measured. 7-NI decreased P 450 quantity by 30 % and AH activity by 26 %. At the same time EMND activity remained unchanged.

On the basis of these results we proved a hepatic metabolism of 7-NI that might be responsible for the detected GSH depletion in the liver and could be regarded as a precondition for hepatic drug interactions.

Rezumat

Lucrarea prezinta studii experimentale privind efectele 7-nitroindazolului (7-NI), un inhibitor selectiv al biosintezei oxidului nitric la nivelul ţesuturilor cerebrale şi hepatice, provenite de la şobolani. Au fost evaluate atât concentraţia TBARS (*thiobarbituric acid reactive substances*) cât şi concentraţia glutationului total.

A fost evaluat cantitativ citocromul P450, precum şi activitatea enzimatică a etilmorfin-N-demetilazei (EMND) şi a anilinhidroxilazei (AH).

Keywords: 7-nitroindazole, nNOS, rat, metabolism, ethylmorphine-N-demethylase (EMND), anilinehydroxilase (AH)

Introduction

Nitric oxide (NO) has been shown to be involved in multiple processes in the central nervous system, including the development of

tolerance to centrally acting compounds such as opioids [10], psychostimulants [6], ethanol [18] etc. The role of NO in the processes of drug tolerance and dependence development has been elucidated by using selective inhibitors of neuronal nitric oxide synthase (nNOS). The heterocyclic compound 7-nitroindazole, which inhibits NOS by competing with both L-arginine and tetrahydrobiopterine [19] has been used extensively as selective inhibitor of nNOS [7,1]. Several studies have indicated that 7-NI affects different physical processes and behaviors, related to drug abuse, such as tolerance, withdrawal, neurotoxicity, psychomotor stimulation and reward. In one of their studies Itzhak and Ali [6] proved that administration of 7-NI reduced the hyperactivity and attenuated the induction of behavioral sensitisation to cocaine and amphetamines. Lue *et al*. [10] demonstrated that 7-NI blocked the development of morphine tolerance in rat hippocampal slices and Uzbay *et al.* [18] showed that the inhibitor alleviated the signs of ethanol withdrawal. However, there are insufficient data about 7-NI toxicity and hepatic biotransformation. The chemical structure of the compound, benzpyrazole, is a part of the structure of such substrates of cytochrome P 450 as anthelmintic agents [16, 12]. Its structure suggests that 7-NI might undergo metabolism in the liver and might be involved in drug interactions.

On the basis of these data, the objective of the present study was to investigate a possible toxicity and hepatic metabolism of 7-NI after multiple administrations.

Materials and Methods

Drugs and Chemicals

All the reagents used were of analytical grade. 7-nitroindazole, as well as other chemicals, sucrose, Tris, DL-dithiotreitol, phenylmethyl-sulfonyl fluoride, potassium phosphate, calcium chloratum ($CaCl_2$), magnesium chloratum ($MgCl_2$), L-arginine, L-valine, bovine hemoglobin, beta – nicotinamide adenine dinucleotide 2`-phosphate reduced tetrasodium salt (NADPH), ethylenediaminetetraacetic acid (EDTA), bovine serum albumin (fraction V) were purchased from Sigma Chemical Co (Germany). 2,2'-dinitro-5,5'dithiodibenzoic acid (DTNB) was obtained from MERCK (Germany).

Animals

Male Wistar rats (weighing 200 g ± 10 g) were housed under standard laboratory conditions at 20°C, with 12 h alternating light/dark cycles and free access to food and water. The animals were purchased from

the National Breeding Centre, Slivnitza, Bulgaria. All experiments were performed after at least one week of adaptation to this environment. All performed procedures were approved by the Institutional Animal Care Committee and the principles stated in the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS 123) [4] were strictly followed throughout the experiment.

The animals were divided into two groups: treated with 7-NI 25 mg/kg b.w. i.p. for 5 days [1] and control animals treated with saline solution i.p. in the same conditions.

The animals from both groups were sacrificed on the sixth day of the experiment, brains and livers were used for the biochemical assays.

Assessment of nNOS activity

nNOS activity was measured spectrophotometrically using the oxidation of oxyhemoglobin to methemoglobin by NO [8]. The change in the difference in absorbance at 401 nm and 421 nm was monitored with a double split beam spectrophotometer (Spectro UV-VIS Split), at 37 °C. The activity of the enzyme was expressed in nmol/min/mg, using the milimolar extinction coefficient of methemoglobin 77.2 $M^{-1}cm^{-1}$.

Preparation of liver microsomes for biochemical assay

The liver microsomes were isolated following the procedure of Guengerich [5]. Rats were decapitated and the livers were excised, perfused with 0.15 M KCl and minced. Then 1g of liver was homogenized with 3 volumes of 1.17% KCl solution in a glass homogenizer. The liver homogenates were then centrifugated at 10 000 x g for 30 min. The supernatant fractions were centrifugated at 105 000 x g for 60 min. The resulting microsomal pellets were stored at -20°C until assayed.

Evaluation of Phase I of biotransformation

Assay of aniline 4-hydroxilase activity (3) – 4-hydroxilation of aniline to 4-aminophenol, that is chemically converted to a phenol-indophenol complex with an absorption maximum at 630 nm. Enzyme activity was expressed as nmol/min/mg.

Assay of EMND activity (3) - the enzyme activity was evaluated by the formation of formaldehyde, trapped in the solution as semicarbazone and measured by the colorimetric procedure of Nash, at 415 nm. Enzyme activity was expressed as nmol/min/mg.

Assessment of cytochrome P450 quantity, [13] - On the day of the assay the microsomal pellets were resuspended and diluted in phosphate

buffer + EDTA (pH=7.4). Liver protein concentration was measured, using the method of Lowry [9] and was adjusted to 10 mg/mL. Cyt P450 quantity was quantified spectrophotometrically as a complex with CO, at 450nm.

Lipid peroxidation in brain and liver homogenate

Lipid peroxidation was determined by measuring the rate of production of thiobarbituric acid reactive substances (TBARS) (expressed as malondialdehyde equivalents) described by Polizio and Pena [14] with slight modifications. Briefly one volume of homogenate was mixed with one volume 25 % trichloracetic acid (TCA) and one volume 0.67 % thiobarbituric acid (TBA). Samples were then mixed thoroughly, heated for 20 min in a boiling water bath, cooled and centrifuged at 4000 rpm for 20 min. The absorbance of supernatants was measured at 535 nm against a blank that contained all the reagents except the tissue homogenate. Malondialdehyde concentration was calculated using a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} and expressed in nmol/g wet tissue.

GSH assessment in brain and liver homogenate

GSH was assessed by measuring non-protein sulfhydryls after precipitation of proteins with TCA, using the method described by Bump et al. [2]. Briefly, tissues were homogenized in 5 % trichloracetic acid (TCA) and centrifugated for 20 min at 4 000 x g. The reaction mixture contained 0.05 mL supernatant, 3 mL 0.05 M phosphate buffer (pH = 8) and 0.02 mL DTNB reagent. The absorbance was determined at 412 nm and the results expressed as nmol/g wet tissue

Statistical analysis

Statistical analysis was performed using the statistical programme "MEDCALC". Results are expressed as mean ± SEM for six rats in each group. The significance of the data was assessed using the non-parametric Mann-Whitney test. Values of $p \leq 0.05$ were considered statistically significant.

Results and Discussion

In our study the effect of multiple administration of 7-NI on some brain and hepatic biochemical parameters was investigated. At brain level, being an inhibitor of nNOS, 7-NI led to a significant decrease in the enzyme activity by 41 % ($p<0.05$) (Table I). These data are supported by the results, obtained by Ali and Itzhak [1] that also detected an enzyme inhibition by 7-NI, administered alone.

Table I

Effects of multiple administration of 7-NI on rat brain. Data are expressed as mean ± SEM

Parameter	Control	7-NI
nNOS (nmol/ mg)	0.622 ± 0.05	0.353 ± 0.06*
MDA (nmol/g)	3.30 ± 0.18	3.65 ± 0.15
GSH (nmol/g)	1.72 ± 0.14	1.13 ± 0.05*

*$p < 0.05$ *vs* control group

In one of his studies Marletta [11] discussed the structure similarity between nNOS and cytochrome P 450. On the basis of this information, as well as regarding the structure of 7-NI, benzpyrazole, which is a part of the structure of such substrates of cytochrome P 450 as anthelmintic agents [16, 12], we hypothesized that along with the inhibition of nNOS activity, 7-NI might also influence the activities of some CYP isoenzymes in the liver. Moreover, there are no data in the literature about its hepatic biotransformation. Thus we measured the activities of EMND (CYP 3A) and AH (CYP 2E1) and the total quantity of cytochrome P 450. The results are shown in Table II.

Table II

Effects of multiple administration of 7-NI on rat liver. Data are expressed as mean ± SEM

Parameter	Control	7-NI
P450 (nmol/ mg)	0.329 ± 0.03	0.237 ± 0.03*
EMND(nmol//min/mg)	0.390 ± 0.02	0.363 ± 0.03
AH (nmol//min/mg)	0.038 ± 0.002	0.028 ± 0.002*
MDA (nmol/g)	1.59 ± 0.05	1.71 ± 0.05
GSH (nmol/g)	5.72 ± 0.20	4.15 ± 0.11*

*$p< 0.05$ *vs* control group

7-NI statistically significant decreased the cytochrome P 450 quantity and the AH activity by 30 % ($p<0.05$) and by 26 % ($p<0.05$), respectively. The EMND activity remained unchanged. Along with this, 7-NI showed a hepatotoxic effect manifested by significant depletion of the cell protector GSH (table II). Aniline hydroxylation in humans, as well as in rats is catalyzed by CYP 2E1 [15]. This enzyme is responsible for the benzene ring hydroxylation and its derivatives to epoxide. Epoxides are generally reactive intermediates which may exert direct toxic effect, due to covalent bindings with cell structures or being nucleophiles conjugate with cell glutathione and cause its depletion [17].

Conclusions

Considering the results of this study, we could conclude that the nNOS inhibitor 7-NI undergoes hepatic metabolism that might be responsible for the detected hepatotoxicity. On the other hand, due to its hepatic biotransformation, 7-NI might be involved in drug metabolic interactions.

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Manuscript received: March 2nd 2011