

## NIFEDIPINE LOWERS COCAINE-INDUCED BRAIN AND LIVER ENZYME ACTIVITY AND COCAINE URINARY EXCRETION IN RATS

Vessela VITCHEVA<sup>1</sup>, Rumyana SIMEONOVA<sup>1</sup>, Dima KAROVA<sup>2</sup>, and Mitka MITCHEVA<sup>1</sup>

*Department of Pharmacology, Pharmacotherapy and Toxicology, Faculty of Pharmacy, Medical University Sofia<sup>1</sup>,  
Doping Control Laboratory, State Agency for Youth and Sport<sup>2</sup>, Sofia, Bulgaria*

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The aim of this study was to see how nifedipine counters the effects of cocaine on hepatic and brain enzymatic activity in rats and whether it affects urinary excretion of cocaine. Male Wistar rats were divided in four groups of six: control, nifedipine group (5 mg kg<sup>-1</sup> *i.p.* a day for five days); cocaine group (15 mg kg<sup>-1</sup> *i.p.* a day for five days), and the nifedipine+cocaine group. Twenty-four hours after the last administration, we measured neuronal nitric oxide synthase (nNOS) activity in the brain and cytochrome P450 quantity, ethylmorphine-N-demethylase, and anilinehydroxylase activity in the liver. Urine samples were collected 24 h after the last cocaine and cocaine+nifedipine administration. Urinary cocaine concentration was determined using the GC/MS method.

Cocaine administration increased brain nNOS activity by 55 % ( $p < 0.05$ ) in respect to control, which indicates the development of tolerance and dependence. In the combination group, nifedipine decreased the nNOS activity in respect to the cocaine-only group.

In the liver, cocaine significantly decreased and nifedipine significantly increased cytochrome P450, ethylmorphine-N-demethylase, and anilinehydroxylase in respect to control. In combination, nifedipine successfully countered cocaine effects on these enzymes.

Urine cocaine excretion in the cocaine+nifedipine group significantly dropped (by 35 %) compared to the cocaine-only group.

Our results have confirmed the effects of nifedipine against cocaine tolerance and development of dependence, most likely due to metabolic interactions between them.

**KEY WORDS:** *anilinehydroxylase, cytochrome P450, ethylmorphine-N-demethylase, GSH, nNOS*

Multiple cocaine administration leads to behavioural activation, tolerance, and development of dependence in animals and humans (2). These adaptive changes in the central nervous system (CNS) are largely mediated by L-type calcium channels (3). One of the mechanisms underlying drug tolerance and dependence, and the related withdrawal syndrome, is a change in calcium homeostasis and the consequent activation of N-methyl-D-aspartate/nitric oxide (NMDA/NO) cascade (4). Changes in Ca<sup>2+</sup> homeostasis

seem to be induced by calcium channel blockers of the 1,4-dihydropyridine type.

In our earlier studies (5-6), we have demonstrated that nifedipine, co-administered with morphine, attenuates withdrawal symptoms, which correlate with changes in the activity of neuronal nitric oxide synthase (nNOS) (5). Interactions between morphine and nifedipine in the liver and on the level of urine excretion were also observed, probably due to a metabolic interaction between these compounds (6).

Nifedipine is known to induce one of the most abundant cytochrome P450 isoform CYP 3A and other isoenzymes, including CYP 2B (7). In rats, these two enzymes mediate partial N-demethylation of cocaine to norcocaine (8). Data on cocaine effects on drug-metabolising enzymes are controversial (9-11). In our earlier studies, we investigated the effect of cocaine on brain and liver cytochrome P450 after multiple administration in rats and found that the cumulative intraperitoneal (*i.p.*) dose of 15 mg kg<sup>-1</sup> led to a significant increase in the quantity of cytochrome P450 (12) and in ethylmorphine-N-demethylase (CYP 3A) (13) and anilinehydroxylase (CYP 2E1) activity (14). These findings suggest that cocaine and nifedipine interact in the brain and liver. The aim of this study was to see to which extent and also to see if nifedipine affects urinary excretion of cocaine in Wistar rats.

## MATERIAL AND METHODS

### *Animals*

To avoid the influence of hormonal fluctuations during the oestrus cycle, this study included only male Wistar rats (b. w. 200 g to 230 g). Many have observed sex-related differences in the behavioural response to and toxicity of cocaine (15, 16).

The animals were housed in plexiglass cages (three per cage) at (20±2) °C and 12 h light/dark cycles. Food and tap water were provided *ad libitum*. The animals were purchased from the National Breeding Centre, Slivnitsa, Bulgaria. All experiments were performed after at least one week of adaptation to this environment. All procedures were approved by the Institutional Animal Care Committee and observed the principles of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 123) (17).

### *Experimental design*

The animals were randomised into four groups of six. The first group received *i.p.* doses of 5 mg kg<sup>-1</sup> nifedipine once daily for five days (18). The second group received 15 mg kg<sup>-1</sup> of cocaine *i.p.* once daily for five days (19). The third group received nifedipine (5 mg kg<sup>-1</sup>) followed by cocaine (15 mg kg<sup>-1</sup>) 30 min later. This combination was also administered once daily for five days. The selection of nifedipine and cocaine doses was based on our earlier study (12) and

on doses commonly used by other laboratories (18, 19). Our earlier study (12) demonstrated that 5 mg kg<sup>-1</sup> of nifedipine, administered intraperitoneally to male Wistar rats once daily for five days, led to increases in cytochrome P450. The fourth group consisted of control animals, which were treated with saline, involved in the experiment from the very beginning, and housed under the same standard laboratory conditions as the treated animals.

Twenty-four hour urine was collected from animals from the second and third group, which were transferred to stainless steel metabolism cages 40 min after the last injection of cocaine and/or nifedipine.

Twenty-four hours after the last injection, all animals were killed by decapitation, brain and liver removed, washed with ice-cold saline (0.9 % NaCl), blotted dry, weighed, and stored on ice. Liver was then divided into three pieces of one gram each to determine cytochrome P450, glutathione (GSH), and enzyme activity.

### *Chemicals and reagents*

All reagents used were of analytical grade. Cocaine hydrochloride, nifedipine hydrochloride, sucrose, Tris, DL-dithiotreitol, phenylmethylsulfonyl fluoride, potassium phosphate, calcium chloratum (CaCl<sub>2</sub>), magnesium chloratum (MgCl<sub>2</sub>), L-arginine, L-valine, haemoglobin bovine, beta-Nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt (beta-NADPH), ethylenediaminetetraacetic acid (EDTA), and bovine serum albumin (fraction V) were purchased from Sigma Chemical Co. (Taufkirchen, Germany). 2,2'-dinitro-5,5'-dithiodibenzoic acid (DTNB) was obtained from Merck (Darmstadt, Germany).

### *Preparation of brain tissue extracts and assessment of nNOS activity*

Rats were decapitated and the brains minced and homogenised in 9 mL of a buffer containing 320 mmol L<sup>-1</sup> sucrose, 50 mmol L<sup>-1</sup> Tris, 1 mmol L<sup>-1</sup> DL-dithiotreitol, 100 µg L<sup>-1</sup> phenylmethylsulfonyl fluoride (pH=7.2), according to the method used by Knowels and Moncada (20). The homogenates were then centrifuged at 17,000 rpm for 60 min. Protein content was measured using the method of Lowry (21) with bovine serum albumin as a standard.

nNOS activity was measured spectrophotometrically, using the oxidation of oxyhaemoglobin to methaemoglobin by NO (20) with slight modifications.

The incubation medium contained 40 mmol L<sup>-1</sup> potassium phosphate buffer, pH=7.2, 200 µmol L<sup>-1</sup> CaCl<sub>2</sub>, 1 mmol L<sup>-1</sup> MgCl<sub>2</sub>, 100 µmol L<sup>-1</sup> L-arginine, 50 mmol L<sup>-1</sup> L-valine, 2.6 µmol L<sup>-1</sup> oxyhaemoglobin, 100 µmol L<sup>-1</sup> NADPH, and brain extract. The concentration of oxyhaemoglobin in the original method of Knowels (20) was 1.6 µmol L<sup>-1</sup>, but we adjusted it to 2.6 µmol L<sup>-1</sup> to optimise reproducibility and to obtain spectra with a higher amplitude.

Change in absorbance at 401 nm and 421 nm was monitored with a double split beam spectrophotometer (Spectro UV-VIS Split) at 37 °C. Enzyme activity was expressed in nmol mg<sup>-1</sup> per minute, using a millimolar extinction coefficient for methaemoglobin 77.2 L mol<sup>-1</sup> cm<sup>-1</sup>.

#### Urine analysis

For urine collection, animals were housed for 24 h in metabolism cages, equipped to separate urine and faeces. Food and water were available *ad libitum*.

A 1.0-mL aliquot of urine was mixed with NaHCO<sub>3</sub>:K<sub>2</sub>CO<sub>3</sub> (1:2), pH=9.6 by shaking manually in 20 mL glass tubes. Then we added Na<sub>2</sub>SO<sub>4</sub> anhydrous and 20 µL codeine as internal standard. Samples were extracted with 4 mL of tert-butylmethylether for 30 min and then centrifuged at 2,500 rpm for 5 min. To separate the organic from non-organic phase, the samples were put in cryostat at -29 °C. The separated ether phase was then evaporated to dryness under a stream of nitrogen.

The purified dried extract was added to 50 µL N-Methyl-N-trimethylsilyltrifluoroacetamide (MBTFA) and derivatised at 80 °C for 20 min. Aliquots of 1 µL were injected into a gas chromatograph/mass spectrometer (GC-MS, Agilent Technology, Santa Clara, USA) to determine cocaine concentrations.

#### Preparations of standard and internal control

Stock solution of cocaine was prepared in methanol to yield a final drug concentration of 10 µg L<sup>-1</sup>. The standard curve for cocaine was linear over the concentration range of 0.1 µg L<sup>-1</sup> to 0.4 µg L<sup>-1</sup>. Linearity was verified by adding (10, 20, 30, and 40) µL from the stock solution to drug-free urine. The concentration of the working solution of the internal standard, codeine, was 21.8 µg L<sup>-1</sup>.

#### Preparation of liver microsomes for biochemical assay

The excised liver was perfused with 0.15 mol L<sup>-1</sup> KCl, minced, and homogenised with 3 mL of 1.17 %

KCl solution in a glass homogenizer (PX-OX 2000) as described by Guengerich (22). Liver homogenates were then centrifuged at 9,000 rpm for 30 min. Supernatant fractions were further centrifuged at 38,000 rpm for 60 min. The resulting microsomal pellets were stored at -20 °C until assayed.

Ethylmorphine-N-demethylase activity was evaluated based on the measurement of formaldehyde, trapped in the solution as semicarbazone, using the colorimetric procedure of Nash (23), at 415 nm.

Aniline hydroxylase activity was evaluated colorimetrically, based on the measurement at 630 nm of a phenol-indophenol complex, which is a converted form of 4-aminophenol (24).

To quantify cytochrome P450 we resuspended the microsomal pellets and diluted them in phosphate buffer + 1 mmol L<sup>-1</sup> EDTA (pH=7.4) (25). The protein content of liver homogenate was measured using the method of Lowry (17) with bovine serum albumin as a standard. Cytochrome P450 quantity was quantified spectrophotometrically as a complex with CO at 450 nm and expressed as nmol mg<sup>-1</sup>.

To assess GSH levels, samples of liver tissue were homogenised in 5 % trichloroacetic acid (TCA) and centrifuged at 4,000 rpm for 20 minutes. GSH was assessed by measuring non-protein sulfhydryls after precipitation of proteins with TCA, followed by measurement of thiols in the supernatant by the DTNB reagent using the method described by Bump et al. (26).

#### Statistical analysis

Statistical analysis included ANOVA and Student's *t*-test. Probability values of less than 0.05 were considered significant. The results were presented as means ± SD of six animals per group. For each parameter three parallel samples were tested.

## RESULTS

Table 1 shows the effects of cocaine and nifedipine on nNOS activity in rat brain. Compared to control, cocaine administered alone increased nNOS activity by 55 % (p<0.05) while nifedipine alone did not significantly change its activity. In combination with cocaine, however, nifedipine decreased nNOS activity by 27 % (p<0.05) in respect to cocaine alone.

Table 2 shows the effects of cocaine and nifedipine, administered alone and in combination, on liver

enzymes and P450. Nifedipine given alone showed the opposite effects to cocaine, significantly increasing cytochrome P450 quantity (28 %) and ethylmorphine-N-demethylase (34 %) and anilinehydroxylase (27 %) activity in respect to control. These results are in good correlation with our earlier findings (5, 12).

Cocaine significantly depleted GSH (by 29 %;  $p < 0.05$  in respect to control), while nifedipine did not change its levels. However, in the cocaine+nifedipine group GSH level increased 20 % in respect to the cocaine-only group ( $p < 0.05$ ).

In combination, nifedipine significantly increased cytochrome P450 (by 34 %), ethylmorphine-N-demethylase (101 %) and anilinehydroxylase (41 %) activities, and increases GSH levels (20 %) in relation

to the cocaine only group. Compared to control, GSH levels dropped 15 % ( $p < 0.05$ ).

Table 3 shows the effects of nifedipine on urinary excretion of cocaine. In the nifedipine+cocaine group, cocaine excretion dropped 35 % ( $p < 0.05$ ) in comparison with the cocaine-only group.

## DISCUSSION

The significant increase in nNOS activity observed after five days of cocaine administration in our study confirms earlier findings (19, 27) and could be regarded as a sign of tolerance and development of dependence.

**Table 1** nNOS activity in rat brain after multiple administration of cocaine (15 mg kg<sup>-1</sup> per day) and nifedipine (5 mg kg<sup>-1</sup> per day), alone and in combination

Group	nNOS activity / nmol min <sup>-1</sup> mg <sup>-1</sup> of tissue	Effect vs. control / %	Effect vs. cocaine / %
Control	0.604±0.04	100	
Nifedipine	0.597±0.08	98.9	
Cocaine	0.935±0.012*	155	100
Nifedipine + Cocaine	0.683±0.10 <sup>+</sup>	113	73

Note: Data are expressed as mean±SD of six animals

\*  $p < 0.05$  vs. control group

<sup>+</sup>  $p < 0.05$  vs. cocaine treated group

**Table 2** Multiple administration of cocaine (15 mg kg<sup>-1</sup> per day), alone and along with nifedipine (5 mg kg<sup>-1</sup> per day) – effect on liver biochemical parameters

Group	Cytochrome P450 <sup>#</sup> / nmol mg <sup>-1</sup>	Ethylmorphine-N-demethylase activity as HCHO / nmol min <sup>-1</sup> mg <sup>-1</sup> of tissue	Anilinehydroxylase activity / nmol min <sup>-1</sup> mg <sup>-1</sup> of tissue	Quantity of GSH / nmol g <sup>-1</sup>
Control	0.345±0.02	0.258±0.02	0.037±0.002	5.31±0.20
Nifedipine	0.440±0.04*	0.346±0.02*	0.047±0.003*	5.18±0.13
Cocaine	0.286±0.01*	0.165±0.04*	0.031±0.003*	3.77±0.11*
Cocaine + Nifedipine	0.384±0.05 <sup>+</sup>	0.332±0.01* <sup>+</sup>	0.044±0.005* <sup>+</sup>	4.54±0.41* <sup>+</sup>

Note: Data are expressed as mean±SD of six animals

\*  $p < 0.05$  vs. control group

<sup>+</sup>  $p < 0.05$  vs. cocaine treated group

<sup>#</sup> - previously published in (12)

**Table 3** Urinary excretion of cocaine (15 mg kg<sup>-1</sup> per day) after multiple administrations alone and in combination with nifedipine (5 mg kg<sup>-1</sup> per day)

Group	Urinary concentration of cocaine / µg mL <sup>-1</sup>	24-h urinary volume / mL	Effect vs. cocaine / %
Cocaine	0.57±0.02	6±1	-
Cocaine + Nifedipine	0.37±0.09*	6.5±1.5	↓35

Note: data are expressed as mean±SD of six animals

\*  $p < 0.05$  vs. cocaine treated group

Vetulani (28) has proposed that L-type  $\text{Ca}^{2+}$  channel blockers like 1,4-dihydropyridines could be used as anti-addiction agents. Our results showed that nifedipine countered cocaine effects on nNOS activity when given in combination with it. This is in agreement with a study by Nasif et al. (29), who found that repeated cocaine administration stimulated voltage-sensitive  $\text{Ca}^{2+}$  channel function in rat mPFC pyramidal neurons as a result of the increased activity of L-type  $\text{Ca}^{2+}$  channels. The authors also showed that selective blocking of L-type  $\text{Ca}^{2+}$  channels by nifedipine not only significantly increased the threshold but also reduced the duration and amplitude of  $\text{Ca}^{2+}$  plateau potentials that resulted in a decrease of  $\text{Ca}^{2+}$  influx into the neurons.

Apart from this interaction on the level of the CNS, cocaine and nifedipine are also expected to interact in the liver, since both undergo hepatic biotransformation. Nifedipine is a substrate of CYP 3A and other isoenzymes such as CYP 2B (30, 31) which mediate cocaine's N-demethylation to N-hydroxynorcocaine in liver microsomes (32-34). Our results seem to confirm this interaction, since nifedipine successfully counteracted cocaine effects when administered in combination.

This includes lowering cocaine-induced toxic effects in the liver. According to Yu et al. (35), cocaine is oxidised by cytochrome P450 to N-hydroxynorcocaine and norcocaine nitroxyde. The latter is a free radical that induces lipid peroxidation and GSH depletion. In our study, we detected a significant decrease in GSH levels after cocaine administration. Co-administration with nifedipine, however, resulted in increased GSH levels (Table 2). Similarly, Mazumder et al. (36) suggested that nifedipine attenuated lipid peroxidation and glutathione depletion caused by sulphur mustard.

In order to confirm this metabolic interaction, we also investigated the effects of nifedipine on the urinary excretion of cocaine. In a study by Nayak et al. (38), urinary excretion of unchanged cocaine in chronically treated rats was around 1.5 % (37). In our study, we measured unchanged urinary cocaine because we expected it to increase due to nifedipine interference with its cytochrome P450-mediated metabolism. However, our results proved us wrong, as urinary cocaine excretion in the combination group was significantly lower than in the cocaine-only group (Table 3). These findings suggest that the observed metabolic interaction between cocaine and nifedipine might result in an increased activity of non-microsomal

enzymes, which may explain the decreased urinary excretion of unchanged cocaine.

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### Sažetak

#### NIFEDIPIN UBLAŽAVA DJELOVANJE KOKAINA NA ENZIMSKU AKTIVNOST U MOZGU I JETRI TE SMANJUJE NJEGOVO IZLUČIVANJE PUTEM MOKRAĆE

Cilj je ovoga istraživanja bio utvrditi kako nifedipin ublažava djelovanje kokaina na enzimsku aktivnost u mozgu i jetri Wistar štakora te utječe li na njegovo izlučivanje putem mokraće. Mužjaci su podijeljeni u četiri skupine po šest jedinki: kontrolnu skupinu, nifedipinsku skupinu koja je pet dana intraperitonealno primala nifedipin u dozi od 5 mg kg<sup>-1</sup>; skupinu koja je pet dana primala kokain u dozi od 15 mg kg<sup>-1</sup> na dan te skupinu koja je zajedno primala nifedipin i kokain u odgovarajućim dozama.

Dvadeset i četiri sata nakon posljednje doze izmjerena je enzimska aktivnost sintaze dušičnoga oksida (nNOS) u mozgu, razina citokroma P450 te aktivnosti enzima etilmorfin-N-demetilaze i anilinhidroksilaze u jetri štakora.

Uzorci mokraće prikupljeni su 24 sata nakon posljednje doze kokaina odnosno kombinacije nifedipina i kokaina. Koncentracija kokaina u mokraći izmjerena je s pomoću vezanog sustava plinske kromatografije i spektrometrije masa.

Kokain je povećao aktivnost nNOS-a u mozgu za 55 % (p<0,05) u odnosu na kontrolnu skupinu, što upućuje na stvaranje tolerancije i ovisnosti. U kombiniranoj skupini nifedipin je značajno smanjio aktivnost nNOS-a u odnosu na skupinu koja je primila samo kokain.

Kokain je značajno snizio, a nifedipin značajno povisio razinu citokroma P450 u jetri te aktivnost etilmorfin-N-demetilaze i anilinhidroksilaze u odnosu na kontrolnu skupinu. U kombiniranoj skupini nifedipin je uspješno ublažio djelovanje kokaina na aktivnost spomenutih enzima.

Izlučivanje kokaina putem mokraće u kombiniranoj skupini bilo je značajno manje (35 %) nego u skupini koja je primala samo kokain.

Ovi rezultati potvrđuju da nifedipin štiti od djelovanja kokaina i stvaranja ovisnosti, najvjerojatnije zbog interakcija u metabolizmu dvaju spojeva.

**KLJUČNE RIJEČI:** *anilinhidroksilaza, citokrom P450, etilmorfin-N-demetilaza, GSH, nNOS*

#### CORRESPONDING AUTHOR:

Vessela Vitcheva  
Department of Pharmacology, Pharmacotherapy  
and Toxicology  
Faculty of Pharmacy, Medical University Sofia  
Dunav str. 2, 1000 Sofia, Bulgaria  
E-mail: [vesselavitcheva@yahoo.com](mailto:vesselavitcheva@yahoo.com)