

COCAINE TOXICITY IN FRESHLY ISOLATED RAT HEPATOCYTES – ROLE OF CYP 2B AND CYP 3A

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Abstract

Cocaine is a psychoactive compound that undergoes extensive hepatic biotransformation leading to formation of toxic metabolites responsible for its hepatotoxicity. The objectives of this study were to investigate the influence of induction and inhibition on cocaine hepatotoxicity examined in freshly isolated rat hepatocytes. For investigating the role of induction male Wistar rats have been treated *in vivo* with phenobarbital ($75\text{mg}\cdot\text{kg}^{-1}$, 4 days) and then isolated hepatocytes were exposed to cocaine ($50\mu\text{mol}\cdot\text{L}^{-1}$ for one hour). Compared to control non-treated hepatocytes phenobarbital induction resulted in greater cocaine hepatotoxicity, witnessed by decrease in cell viability by 52%, increase in lactate dehydrogenase (LDH) leakage into the medium by 120%, depletion of reduced glutathione (GSH) levels by 84% and increase in malondialdehyde (MDA) quantity by 74%. Experimental data in the literature reported two major cytochrome P 450 isoforms CYP 3A and CYP 2B to be involved in cocaine biotransformation and toxicity. In order to trace the relative contribution of these isoforms to cocaine hepatotoxicity in rats, isolated hepatocytes were pre-incubated for 15 min with amiodarone ($15\mu\text{mol}\cdot\text{L}^{-1}$) and with chloramphenicol ($100\mu\text{mol}\cdot\text{L}^{-1}$) inhibitors of CYP 3A and CYP 2B, respectively. Pre-incubation with either of the inhibitors ameliorated cocaine hepatotoxicity. Our results prove the role of both isoforms CYP 3A and CYP 2B in cocaine biotransformation in rats.

Rezumat

Cocaina este un compus psihoactiv biotransformat la nivel hepatic, conducând la formarea de metaboliți toxici responsabili de hepatotoxicitate acesteia. Obiectivele acestui studiu au fost de a investiga influența inducției și inhibiției hepatotoxicității cocainei, examinând hepatocitele proaspăt izolate de șobolan. Pentru investigarea rolului inducției, șobolanii Wistar masculi au fost tratați *in vivo* cu fenobarbital ($75\text{mg}\cdot\text{kg}^{-1}$, 4 zile) și apoi hepatocitele izolate au fost tratate cu cocaină ($50\mu\text{mol}\cdot\text{L}^{-1}$ timp de o oră). Comparativ cu hepatocite netratate, inducția fenobarbitalului este semnificativ mărită în cazul hepatocitelor expuse cocainei, însoțită de o scădere a viabilității celulelor cu 52%, creșterea nivelului lactat dehidrogenazei (LDH) în mediu cu 120%, depleția nivelelor glutatationului redus (GSH) cu 84% și creșterea malondialdehidei (MDA) cu 74%. Datele experimentale din literatura de specialitate au raportat implicarea în biotransformarea și toxicitatea cocainei a

două mari izoforme ale citocromului P 450, CYP 3A și CYP 2B. În scopul de a urmări contribuția relativă a acestor izoforme la hepatotoxicitatea cocainei la șobolani, hepatocite izolate au fost pretratate timp de 15 min cu amiodaronă ($15\mu\text{mol}\cdot\text{L}^{-1}$) și cu cloramfenicol ($100\mu\text{mol}\cdot\text{L}^{-1}$), inhibitori ai CYP 3A și respectiv CYP 2B. Pretratarea cu oricare dintre inhibitori scade hepatotoxicitatea indusă de cocaină. Rezultatele noastre justifică implicarea izoformelor CYP 3A și CYP 2B în biotransformarea cocainei la șobolani.

Keywords: cocaine, hepatocytes, CYP 3A, CYP 2B.

Introduction

Cocaine is a well-known and wide spread psychostimulant that causes behavioural activation, tolerance and development of dependence. As a result of its potent inhibitory effects on presynaptic dopamine and noradrenaline re-uptake in the brain reward system, cocaine abuse is related to intense feeling of euphoria, empathy, hyperactivity and friendliness. Cocaine misuse, however, may cause severe toxic effects, including neurotoxicity, cardiotoxicity and hepatotoxicity. Even though cocaine cardiotoxicity is more often reported and it is life-threatening, cocaine-induced liver damage should not be underestimated. Cocaine liver injury has been broadly discussed in experimental animals [7] and humans [13] alike. The proposed mechanism involves formation of reactive oxygen species (ROS) that are generated during cocaine bioactivation to norcocaine through N-demethylation by cytochrome P 450 and flavin adenine dinucleotide containing monooxygenases [8].

Two major cytochrome P 450 isoforms are reported to be involved in this process: CYP 3A - in humans and mice and CYP 2B - in rats [14]. In our earlier *in vivo* studies in rats we demonstrated that cocaine administered to phenobarbital-induced rats exerted higher toxicity compared to cocaine-only treated rats [15]. Phenobarbital is a well-known inducer of a number of cytochrome P 450 isoforms, including CYP 3A and CYP 2B [5]. We discussed that its induction of cocaine bioactivation was responsible for the more pronounced toxic effects of the compound.

On the basis of these results we designed this study with two objectives: first to assess whether cocaine hepatotoxicity *in vitro* would be potentiated in rat hepatocytes isolated from phenobarbital-induced rats and second, by applying inhibitors of CYP 3A and CYP 2B to determine the relative contribution of each isoform in cocaine bioactivation and respective toxicity in rat isolated hepatocytes.

Materials and Methods

Animals

Male Wistar rats (body weight 200–250 g) were used. The rats were housed in plexiglass cages (3 *per* cage) in a 12/12 light/dark cycle, under standard laboratory conditions (ambient temperature $20^{\circ}\text{C} \pm 2^{\circ}\text{C}$ and humidity $72\% \pm 4\%$) with free access to water and standard pelleted rat food 53-3, produced according to ISO 9001:2008. Animals were purchased from the National Breeding Center, Sofia, Bulgaria. A minimum of 7 days acclimatization was allowed before the commencement of the study and their health status was monitored regularly by a veterinary physician. 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) [3] were strictly followed throughout the experiment.

Experimental design

The animals were divided into two groups ($n=4$): control treated intraperitoneally (i.p.) with saline (0.9% NaCl) once a day for four days, and a group treated similarly with $75\text{mg}\cdot\text{kg}^{-1}$ phenobarbital, an inducer of drug metabolizing enzymes [12].

Drugs and chemicals

All the reagents used were of analytical grade. Cocaine, phenobarbital, amiodarone, chloramphenicol, as well as other chemicals, Collagenase, 1-chloro-2,4-dinitrobenzene, beta – Nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt (NADPH), ethylenediaminetetraacetic acid (EDTA), 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 (Darmsstadt).

Isolation and incubation of hepatocytes

Rats from both groups (control and phenobarbital-induced) were anesthetized with 0.24 M sodium pentobarbital (0.2 mL/100 g). *In situ* liver perfusion and cell isolation were performed as described by Fau *et al.* [4], with modifications [11]. Cells were counted under the microscope and cell viability was assessed by Trypan blue exclusion (0.05%) [4]. Initial viability averaged 89%.

Incubation of hepatocytes

In order to choose the appropriate cocaine concentration for *in vitro* experiments, hepatocytes were incubated for one hour with cocaine at four consequently increasing concentrations: $10\mu\text{mol}\cdot\text{L}^{-1}$, $50\mu\text{mol}\cdot\text{L}^{-1}$, $100\mu\text{mol}\cdot\text{L}^{-1}$ and $200\mu\text{mol}\cdot\text{L}^{-1}$. On the basis of the tested parameters: cell viability, LDH leakage into the medium, GSH levels and MDA quantity, the

average toxic cocaine concentration (TC_{50}) was determined to be approximately $50\mu\text{mol}\cdot\text{L}^{-1}$ (4.44×10^{-5}). Hepatocytes, isolated from the animals from both groups were incubated either with cocaine at $50\mu\text{mol}\cdot\text{L}^{-1}$ (TC_{50}) for one hour or pre-incubated for 15 min with inhibitors of its metabolism and then incubated with cocaine for one hour. Amiodarone, an inhibitor of CYP3A [17] at a concentration of $15\mu\text{mol}\cdot\text{L}^{-1}$ [2] and chloramphenicol, an inhibitor of CYP 2B at a concentration of $100\mu\text{mol}\cdot\text{L}^{-1}$ [10] were used.

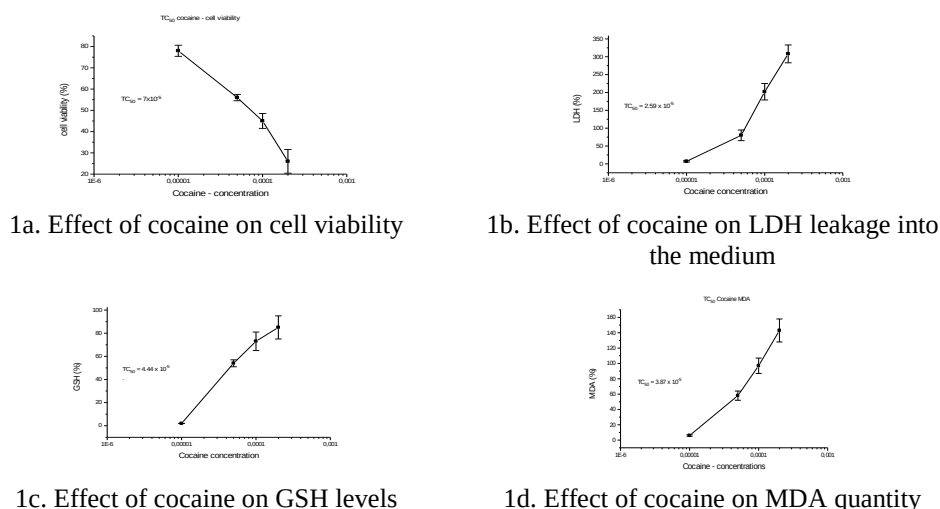
The following parameters were measured to assess the functional status of hepatocytes: cell viability, lactate dehydrogenase (LDH) leakage into the medium, reduced glutathione (GSH) levels and malondialdehyde (MDA) quantity. Cell viability was assessed by Trypan blue exclusion method [4]. The dye was used at a final concentration of 0.05% and cells were counted using a light microscope (x 100). At the end of incubation, the cells were recovered *via* centrifugation at $400 \times g$ at 4°C . The supernatant was used for LDH and MDA assessment as described by Bergmeyer *et al.* [1] and Fau at al. [4], respectively. GSH measurement following the method used by Fau at al. [4] was assessed in the sediment.

Statistical analysis

Statistical programme 'MEDCALC' was used for analysis of the data. The results are expressed as mean $\pm$ SEM of four animals per group and for each of the examined parameters, three parallel samples were used. The significance of the data was assessed using the nonparametric Mann–Whitney test, values of $p \leq 0.05$ were considered statistically significant.

Results and Discussion

In the first series of experiments, in order to choose up the appropriate cocaine concentration for our further study we incubated the hepatocytes with four consequently increasing cocaine concentrations. The data are shown in Figure 1. Cocaine showed concentration-dependent effect which is the most prominent at the highest concentration of $200\mu\text{mol}\cdot\text{L}^{-1}$. The TC_{50} of the compound for each of the tested parameters is as follows: cell viability - $TC_{50}=70\mu\text{mol}\cdot\text{L}^{-1}$ (7.3×10^{-5}) (Figure 1a); LDH leakage into the medium - $TC_{50}=26\mu\text{mol}\cdot\text{L}^{-1}$ (2.59×10^{-5}) (Figure 1b), GSH levels - $TC_{50}=45\mu\text{mol}\cdot\text{L}^{-1}$ (4.44×10^{-5}) (Figure 1c), MDA quantity - $TC_{50}=39\mu\text{mol}\cdot\text{L}^{-1}$ (3.87×10^{-5}) (Figure 1d). On the basis of these results we used an average TC_{50} of $50\mu\text{mol}\cdot\text{L}^{-1}$ for our further experiments.

**Figure 1.**

Effect of cocaine ($10\mu\text{mol}\cdot\text{L}^{-1}$, $50\mu\text{mol}\cdot\text{L}^{-1}$, $100\mu\text{mol}\cdot\text{L}^{-1}$ and $200\mu\text{mol}\cdot\text{L}^{-1}$) on isolated rat hepatocytes

The effect of cocaine $50\mu\text{mol}\cdot\text{L}^{-1}$ on cell viability, LDH leakage into the medium, GSH levels and MDA quantity in hepatocytes isolated from non-induced and phenobarbital-induced rats is shown on Table I.

Table I

Effect of $50\mu\text{mol}\cdot\text{L}^{-1}$ cocaine on cell viability, LDH leakage into the medium, GSH levels and MDA quantity in hepatocytes isolated from non-induced and phenobarbital-induced rats

Sample	Cell viability %	LDH / 10^6 cells / $\text{nmol L}^{-1} / \text{min}$	GSH levels / 10^6 cells / nmol	MDA quantity / 10^6 cells / nmol
<i>Non treated rats</i>				
Control hepatocytes	84 ± 2	0.147 ± 0.01	24 ± 1.17	0.203 ± 0.01
Hepatocytes incubated with cocaine	$56 \pm 3.9^*$	$0.270 \pm 0.03^*$	$18 \pm 1.17^*$	$0.320 \pm 0.05^*$
<i>Phenobarbital-induced rats</i>				
Control hepatocytes	$75 \pm 2.1^*$	$0.214 \pm 0.01^*$	$18 \pm 1.2^*$	0.207 ± 0.02
Hepatocytes incubated with cocaine	$36 \pm 5^{* \#}$	$0.470 \pm 0.03^{* \#}$	$2.95 \pm 0.5^{* \#}$	$0.360 \pm 0.05^+$

Data are expressed as mean $\pm$ SEM of four different experiments.

* Significant difference from hepatocytes from non-treated rats (Mann-Whitney test, $p < 0.05$).

+ Significant difference from hepatocytes from phenobarbital-induced rats (Mann-Whitney test, $p < 0.05$).

Significant difference from cocaine (non-induced rats) (Mann-Whitney test, $p < 0.05$).

After one hour of hepatocytes incubation, cell viability slightly, non-significantly decreased from the initial value (~89%) to 84% in the hepatocytes isolated from control, non-treated rats and to 75% ($p<0.05$) in the hepatocytes from phenobarbital-induced rats. It also has to be noticed that compared to non-treated hepatocytes, in the hepatocytes isolated from phenobarbital-treated rats a statistically significant ($p<0.05$) increase in LDH leakage by 48% and decreased levels of GSH by 25% were detected. The initial MDA quantity is equal in both control groups. Cocaine administered *in vitro* at a concentration of $50\mu\text{mol}\cdot\text{L}^{-1}$ affected all tested parameters in both types of hepatocytes. Cocaine incubation of hepatocytes from non-treated rats led to a statistically significant ($p<0.05$) decrease of cell viability by 33%, an increase of LDH leakage by 84%, the depletion of GSH by 25% and an increase in MDA quantity by 58%. Phenobarbital treatment potentiated cocaine toxicity as follows: cell viability decreased by 52% ($p<0.05$) which is 35% ($p<0.05$) higher compared to cocaine treated hepatocytes isolated from non-induced rats; LDH leakage increased by 120% ($p<0.05$) and MDA quantity – by 74% ($p<0.05$) which are respectively 74% ($p<0.05$) and 13% ($p<0.05$) higher than the effect of cocaine on hepatocytes isolated from non-treated rats. GSH levels decreased by 84% ($p<0.05$) which is comparable with the effect of cocaine on cells isolated from not-induced rats.

In a third series of experiments the influence of inhibitors of cocaine metabolism on its hepatotoxic activity was traced. The effects of cocaine alone and after pre-incubation with amiodarone and chloramphenicol are given in Table II.

Table II

Effect of cocaine ($50\mu\text{mol}\cdot\text{L}^{-1}$) alone and after pre-incubation with amiodarone ($15\mu\text{mol}\cdot\text{L}^{-1}$) and chloramphenicol ($100\mu\text{mol}\cdot\text{L}^{-1}$)

Sample	Cell viability %	LDH / 10^6 cells / nmol L^{-1} / min	GSH levels / 10^6 cells / nmol	MDA quantity / 10^6 cells / nmol
<i>Non-treated rats</i>				
Control	84 ± 2	0.147 ± 0.01	24 ± 1.17	0.203 ± 0.01
Cocaine	$56 \pm 3.9^*$	$0.270 \pm 0.03^*$	$18 \pm 1.17^*$	$0.320 \pm 0.05^*$
Chloramphenicol	$41 \pm 7^*$	$0.250 \pm 0.05^*$	$6.8 \pm 1.5^*$	$0.304 \pm 0.05^*$
Chloramphenicol + Cocaine	$67 \pm 5^{*+}$	$0.176 \pm 0.01^+$	$19 \pm 0.25^{*+}$	$0.258 \pm 0.01^{*+}$
Amiodarone	$50 \pm 4^*$	$0.206 \pm 0.02^*$	$11 \pm 1.3^*$	$0.283 \pm 0.04^*$
Amiodarone + Cocaine	$62 \pm 5^*$	$0.185 \pm 0.03^{*+}$	$22 \pm 2.34^+$	$0.234 \pm 0.01^{*+}$

Data are expressed as mean $\pm$ SEM of four different experiments.

* Significant difference from control values (Mann-Whitney test, $p<0.05$).

+ Significant difference from cocaine only incubated hepatocytes (Mann-Whitney test, $p<0.05$)

Both inhibitors, administered alone showed statistically significant ($p < 0.05$) toxic effects in hepatocytes, witnessed by decreases in cell viability, increases in LDH leakage, GSH depletion and increased production of MDA. Amiodarone hepatotoxicity was less pronounced. However, pre-incubation of the hepatocytes with chloramphenicol and amiodarone ameliorated cocaine hepatic damage, observed by preserved cell viability and restored LDH activity, MDA quantity and GSH levels.

Conclusions

Cocaine is a psychostimulant with high addictive potential. Its multiple administration leads to development of tolerance and moderate physical and psychotic dependence. Along with the behavioural changes observed, cocaine abuse leads to serious injuries of the central nervous system, the cardiovascular system, the neuromuscular system and the liver [16].

Cocaine also undergoes extensive hepatic biotransformation, both non-microsomal and microsomal [9]. Even though the microsomal pathway is quantitatively less important (10%) it is directly related to cocaine bioactivation to toxic metabolites and its hepatotoxicity [6].

In this study *in vitro* hepatotoxic effect of cocaine in hepatocytes, isolated from control and phenobarbital-induced rats, as well as the role of CYP 3A and CYP 2B have been examined. The latter has been investigated using amiodarone, an inhibitor of CYP 3A and chloramphenicol – an inhibitor of CYP 2B, which are the two major isoforms responsible for cocaine N-demethylation to norcocaine. *In vivo* induction with phenobarbital before hepatocytes isolation contributed to an increase in cocaine hepatotoxicity *in vitro*, witnessed by an additional decrease in cell viability, increase in LDH leakage into the medium, decrease in cell GSH levels and increase in MDA (see Table I). These results confirmed once again the role of cytochrome P 450 metabolism in cocaine liver damage and are in good correlation with our previous *in vivo* data [15] and also with the data of Poet *et al.* [14] that observed similar effects in isolated rat hepatocytes. In order to investigate the role of CYP 3A and CYP 2B in cocaine hepatotoxicity, we used inhibitors of these isoforms to determine their role. Pre-incubation of the hepatocytes with either amiodarone (CYP 3A inhibitor) or chloramphenicol (CYP 2B inhibitor) greatly attenuated the extent of cocaine toxicity (see Table II). However, the result that has to be noted concerns the levels of cell glutathione. Both inhibitors restored depleted by cocaine GSH levels; however amiodarone pre-incubation resulted in a more complete restoration, the levels being comparable to the

control (Table II). Even though there are literature data suggesting that in cocaine hepatotoxicity in rats the main role is attributed to CYP 2B [14], on the basis of our results we can conclude that along with CYP 2B, CYP 3A also plays a role in cocaine biotransformation in rats.

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