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Scientific Paper

D-AMPHETAMINE TOXICITY IN FRESHLY ISOLATED RAT HEPATOCYTES: A POSSIBLE ROLE OF CYP3A

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The aim of this study was to trace D-amphetamine toxicity in isolated rat hepatocytes and to elucidate a possible involvement of CYP3A in the mechanisms of its toxicity. To this end, male Wistar rats were treated with nifedipine (5 mg kg^{-1} *i.p.*, 5 days), a substrate and inducer of CYP3A. Hepatocytes isolated from nifedipine-treated and control rats were incubated with D-amphetamine at a concentration of 100 μmol L^{-1}, which was determined to be an average toxic concentration (TC_{50}) for the compound. To evaluate the possible toxic effects of D-amphetamine on freshly isolated rat hepatocytes, we assessed the following parameters: cell viability, lactate dehydrogenase (LDH) activity, and glutathione (GSH).

The results showed that nifedipine potentiated amphetamine cytotoxicity *in vitro*, as follows: cell viability dropped by 65 % ($p<0.001$), GSH by 80 % ($p<0.001$), and LDH activity increased by 190 % ($p<0.001$).

To clarify the role of nifedipine in amphetamine cytotoxicity, we used amiodarone, a substrate and an inhibitor of CYP3A. Pre-incubation of nifedipine-treated hepatocytes with amiodarone (14 μmol L^{-1}) significantly lowered amphetamine cytotoxicity.

Our results confirmed the toxicity of D-amphetamine in isolated rat hepatocytes and the involvement of CYP3A in its metabolism and hepatotoxicity.

KEY WORDS: *amiodarone, amphetamine cytotoxicity, nifedipine*

Amphetamines are potent psychostimulants and commonly used drugs of abuse. Not only does chronic amphetamine administration create tolerance and addiction, but is also associated with neurotoxicity and hepatocellular damage through oxidative stress. Amphetamine is a drug of abuse with variable interspecies biotransformation (1). In rats its aromatic ring is hydroxylated, and the urinary excretion of p-hydroxyamphetamine (81 %) is the main elimination pathway (1). Animal studies *in vitro* (2) and *in vivo* (3) have proved that D-amphetamine 4-hydroxylation in rats is favoured by CYP2D. This metabolic pathway is thought to be responsible for amphetamine hepatotoxicity. Experimental studies performed in isolated rat hepatocytes by Carvalho et al. (4) have proved that in the liver D-amphetamine metabolises into an epoxide, its toxic intermediate, which reacts with glutathione, and forms a GSH-adduct (GSH-S-yl-p-hydroxyamphetamine). This partly explains D-amphetamine hepatotoxicity. D-amphetamine is a substrate of CYP2D, but it is also known to inhibit cytochrome P450 through a nitroso metabolic intermediate, which complexes with the enzyme and causes its inhibition and possible toxic effects (5, 6). The main cytochrome P450 isoform which catalyses the formation of the nitroso intermediate is CYP3A (7).

This suggests a possible involvement of other cytochrome P450 isoenzymes in D-amphetamine metabolism and hepatotoxicity, and the objective of

this study was to elucidate a possible involvement of CYP3A in D-amphetamine hepatotoxicity in isolated rat hepatocytes.

MATERIALS AND METHODS

Animals

Male Wistar rats (body weight 200 g to 250 g) were housed in Plexiglas cages (3 per cage) at (20±2) °C and 12-h light : 12-h dark cycle. Food and water were provided *ad libitum*. The animals were purchased from the National Breeding Centre, Sofia, Bulgaria. All procedures were approved by the Institutional Animal Care Committee and performed strictly following the principles stated in the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS 123) (1991) (8).

Experimental design

The animals were divided in two groups (n=6): the control group treated intraperitoneally (*i.p.*) with saline 0.9 % twice a day for 5 days, and a group receiving intraperitonally 5 mg kg^{-1} of nifedipine, an inducer of CYP 3A, twice a day for 5 days. This nifedipine dose was based on previous experiments in which we found that multiple nifedipine administration to rats (5 mg kg^{-1}, *i.p.*, twice daily for 5 days) led to an increase in cytochrome P450 (*9*).

Chemicals and reagents

All reagents used were of analytical grade. The drugs used in this study, D-amphetamine sulphate, nifedipine, and amiodarone, as well as other chemicals, collagenase (type IV), bovine serum albumin (fraction V), 4-(2-hydroxyethyl)-1-piper-azineethanesulphonic acid (HEPES), glutathione (GSH), and [ethylenebis (oxyethylenenitrilo)] tetraacetic acid (EGTA) were purchased form Sigma Chemical Co. (Germany). 2,2'-dinitro-5,5'dithiodibenzoic acid (DTNB) was obtained from MERCK (Germany) and the Lactate Dehydrogenase Kit (EC 1.1.1.27) was obtained from Randox (United Kingdom).

Isolation of hepatocytes

Rats were anesthetised with sodium pentobarbutal (0.2 mL per 100 g b. w.).Two-stepped collagenase liver perfusion and cell isolation were performed *in situ*, as described by Fau (10) with modifications (11). After portal catheterisation, the liver was perfused with 100 mL of HEPES buffer (pH=7.85) containing 10 mmol L^{-1} HEPES, 142 mmol L^{-1} NaCl, 7 mmol L^{-1} KCl, 5 mmol L^{-1} glucose, and 0.6 mmol L^{-1} EDTA (pH=7.85), then by 200 mL of HEPES buffer (pH=7.85) alone, and finally by 200 mL of HEPES buffer containing collagenase type IV (50 mg per 200 mL) and 7 mmol L^{-1} $CaCl_2$ (pH = 7.85). The liver was removed, minced, and hepatocytes were dispersed in 60 mL Krebs-Ringer-bicarbonate (KRB) buffer (pH = 7.35), containing 1.2 mmol L^{-1} KH_2PO_4, 1 mmol L^{-1} $CaCl_2$, 1.2 mmol L^{-1} $MgSO_4$, 5 mmol L^{-1} KCl, 5 mmol L^{-1} $NaHCO_3$, 4.5 mmol L^{-1} glucose, and 1 % bovine serum albumin. After filtration, the hepatocytes were centrifuged at 500xg for 1 min and washed three times with the KRB buffer. The initial viability, assessed using the Trypan blue exclusion method (10), averaged 90 %. Cells were then diluted with KRB to make a suspension of approximately $3x10^6$ hepatocytes per mL and incubated in sterile 25 mL Erlenmeyer flasks, each containing 3 mL of the cell suspension (i.e. $9x10^6$ hepatocytes).

Incubation of hepatocytes

In order to choose appropriate amphetamine concentration for the *in vitro* experiments, hepatocytes were incubated with D-amphetamine at four concentrations (50 µmol L^{-1}, 100 µmol L^{-1}, 150 µmol L^{-1}, and 200 µmol L^{-1}) for one hour. Average toxic amphetamine concentration (TC_{50}) was determined (Figure 1) to be 100 µmol L^{-1} (TC_{50}=1.05x10^{-4} mol L^{-1}). Hepatocytes isolated from the animals of both groups were incubated with D-amphetamine at TC_{50} for one hour. Hepatocytes isolated from nifedipine-treated rats were pre-

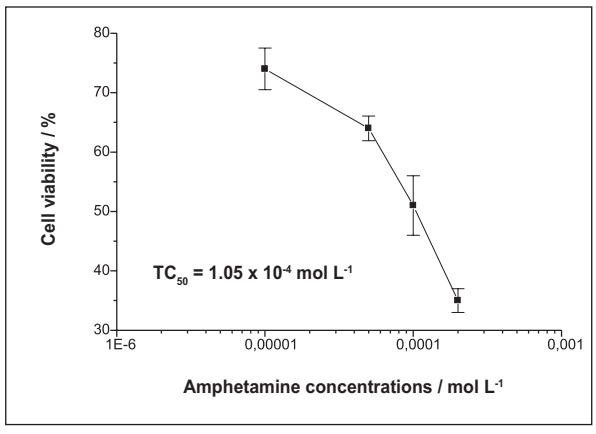

Figure 1 *The influence of D-amphetamine on cell viability in rat hepatocytes*

incubated for 15 min with amiodarone (14 μmol L^{-1}) (12) and then incubated for with D-amphetamine for one hour. Incubations were performed in a 5 % CO_2 + 95 % O_2 atmosphere (10). For both groups, non-treated hepatocytes were used as controls.

The following parameters were used to evaluate amphetamine cytotoxicity: cell viability, LDH activity, and GSH level. Cell viability was assessed using the Trypan blue exclusion method (10). The dye was used at the final concentration of 0.05 %, and approx. 300 cells were counted under a light microscope (x100). Cell samples were taken from each experimental group from three separate hepatocyte preparations at hour one of incubation. At the end of incubation, cells were recovered by centrifugation at 400xg at 4 °C. The supernatant was used for the assessment of LDH activity while the sediment to measure intracellular glutathione (GSH). The level of LDH activity was measured spectrophotometrically, as described by Bergmeyer et al. (13), using a commercially available kit (LDH opt., Randox). Briefly, the method is based on the reduction of pyruvate to lactate. During the reduction, an equimolar amount of NADH is oxidised to NAD^+, which leads to a decrease in the absorbance at 340 nm, measured at minute 1, 2, and 3. The decrease in absorbance at 340 nm is directly proportional to LDH activity in the sample. LDH activity was calculated from the absorbance of NADH (6.22 mmol), and the final results are given in mmol L^{-1} min^{-1} per 10^6 cells.

The GSH level was assessed by measuring non-protein sulfhydryls after precipitation of proteins with trichloroacetic acid (TCA), followed by measurement of thiols in the supernatant using 2,2'-dinitro-5,5'-dithiodibenzoic acid (DTNB) as reagent. The absorbance was measured at 412 nm (10), using a Spectro UV-VIS Split spectrophotometer, and the final results are expressed in nmol per 10^6 cells.

Statistical analysis

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

RESULTS

After one-hour of incubation *in vitro*, cell viability showed a slight, non-significant decrease from the initial value (~90 %) to 85 % in the hepatocytes isolated from non-treated rats and to 78 % in the hepatocytes isolated from nifedipine-treated rats (Table 1). D-amphetamine administered *in vitro* at a concentration of 100 μmol L^{-1} affected cell viability, LDH activity, and GSH levels in hepatocytes isolated from non-treated and Nifedipine-treated rats. The incubation of hepatocytes isolated from non-treated rats with D-amphetamine led to a decrease in cell viability by 40 % ($p<0.05$), increased LDH leakage by 184 % ($p<0.05$), and decreased level of cellular GSH by 54 % ($p<0.05$). Nifedipine treatment potentiated D-amphetamine cytotoxicity, and the most affected parameter was GSH level, followed by cell viability, and LDH activity. GSH was depleted by 80 %

Table 1 *The effect of D-amphetamine (100 μmol L^{-1}) administered* in vitro *on cell viability, LDH activity, and GSH level in hepatocytes isolated from non-treated and nifedipine-treated rats*

Sample	Cell viability / %	LDH activity per 10^6 cells / mmol L^{-1} min^{-1}	GSH level per 10^6 cells / nmol
Non-treated rats			
Control hepatocytes	85±3.2	0.131±0.01	19.14±1.93
Hepatocytes exposed *in vitro* to D-amphetamine	51±2.0*	0.372±0.07*	8.85±0.10*
Nifedipine-treated rats			
Control hepatocytes	78±5.1	0.165±0.03	17.00±2.67
Hepatocytes exposed *in vitro* to D-amphetamine	26±1.5$^{+\#}$	0.479±0.03$^{+\#}$	3.40±0.40$^{+\#}$

Note. Data are expressed as mean ± SD of four different experiments.
**p<0.05 vs. LDH activity hepatocytes from non-treated rats*
$^{+}$p<0.05 vs. per 10^6 cells / hepatocytes from nifedipine-treated rats
$^{\#}$p<0.05 vs. mmol $L^{-1}min^{-1}$ hepatocytes from non-treated rats exposed to D-amphetamine

($p<0.05$) which is 62 % ($p<0.05$) more than the effect of D-amphetamine on hepatocytes isolated from non-treated rats. Cell viability decreased by 67 % ($p<0.05$), which is 51 % ($p<0.05$) more than it was observed in amphetamine-treated hepatocytes isolated from non-treated rats. LDH activity increased by 190 % ($p<0.05$), which is commeasurable with the effect of amphetamine on cells isolated from non-treated rats.

Table 2 shows the influence of amiodarone (14 μmol L^{-1}) on D-amphetamine effects in hepatocytes isolated from nifedipine-treated rats. Amiodarone itself exerted a hepatotoxic effect, manifested by a decrease in cell viability and GSH level by 35 % ($p<0.05$) and 50 % ($p<0.05$), respectively, and by an increase in LDH activity by 101 % ($p<0.05$). Pre-incubation of hepatocytes with amiodarone resulted in lower cytotoxycity of D-amphetamine. Cell viability and the GSH level increased 22 % ($p<0.05$) and 79 % ($p<0.05$), respectively, while LDH activity decreased 45 % ($p<0.05$) in respect to hepatocytes treated with D-amphetamine alone.

DISCUSSION

Amphetamines belong to a class of drugs known as central nervous system indirect-acting sympathomymetics, which are commonly abused due to their physical and psychostimulating effects. Sympathetic arousal induced by amphetamine produces rapid and sometimes irregular heart rate, sweating, papillary dilation, hypertension, and increased body temperature. The psychological changes include euphoria, alertness, hypervigilance, and decreased appetite. Beside the risk of developing tolerance and addiction, chronic abusers may experience neurotoxic effects (14) and hepatocellular damage (15). The latter has increasingly been reported in humans (16, 17).

In vitro studies in rats (4) have shown that amphetamine hepatotoxicity is closely related to its bioactivation and GSH-adduct (GSH-S-yl-p-hydroxyamphetamine) formation, a process considered to be catalysed by CYP2D (4). Although the p-hydroxylation of amphetamine is considered to be the main metabolic pathway (1), it is possible that some other cytochrome P 450 dependent metabolic pathways be involved in its toxicity. There are clinical data revealing significant interactions between recreational drugs, including amphetamine, and potent inhibitors of CYP3A4 such as some of the HIV protease inhibitors (18). Antiretroviral may cause fatal amphetamine accumulation from normally safe dosages, as they inhibit amphetamine metabolism. It is therefore possible that CYP3A was involved in amphetamine metabolism and toxicity in rats.

L-type calcium channel blockers of the dihydropiridine class, such as nifedipine, nimodipine, and amlodipine have been reported to affect addiction to various types of drugs, including psychostimulants (19). At the same time, they are substrates of one of cytochrome P 450 most abundant isoenzyme CYP3A. Nifedipine is known to induce cytochrome P 450 both in rats (20) and humans (21).

To test the hypothesis of a possible involvement of CYP3A in amphetamine toxicity we used rat hepatocytes isolated from non-treated and nifedipine-

Table 2 *The influence of amiodarone (14 μmol L^{-1}) on the toxic effects of D-amphetamine in hepatocytes isolated from nifedipine-treated rats*

Sample	Cell Viability / %	LDH activity per 10^6 cells / mmol L^{-1} min^{-1}	GSH level per 10^6 cells / nmol
Control hepatocytes isolated from nifedipine-induced rats	78±5.0	0.165±0.01	17.00±1.7
Hepatocytes exposed *in vitro* to amiodarone (14 μmol L^{-1})	51±7.0*	0.334±0.03*	8.42±1.3*
Hepatocytes exposed *in vitro* to D-amphetamine (100 μmol L^{-1})	36±2.0*	0.479±0.03*	4.50±0.4*
Hepatocytes exposed *in vitro* to D-amphetamine and amiodarone	44±1.5*$^+$	0.225±0.04$^+$	8.05±1.5*$^+$

Note. Data are expressed as mean ± SD of four experiments
**p< 0.05 vs. control*
$^+$p<0.05 vs. hepatocytes treated only with D-amphetamine

treated rats, which were then incubated with amphetamine. Our results showed that nifedipine increased amphetamine cytotoxicity.

Cell GSH was the most affected (Table 1) and its significant depletion is an interesting finding. It seems that CYP2D favours the formation of p-hydroxyamphetamine, and CYP2D is known not to be inducible (22). The observed decrease in the hepatocyte GSH level caused by amphetamine in nifedipine-treated rats may therefore be a result of nifedipine induction of CYP3A. In addition, multiple nifedipine administration may induce several other CYP isoforms such as CYP2B and CYP2C (23). Literature data suggest that in addition to CYP2D, aromatic hydroxylation is catalysed by CYP2B (24), which can be induced by nifedipine, and therefore also responsible for the GSH depletion. This hypothesis, however, needs further investigations that are an object of future studies.

In order to clarify the role of nifedipine in amphetamine toxicity, hepatocytes isolated from nifedipine-treated rats were pretreated with amiodarone, a substrate and inhibitor of CYP3A (23; 12). At a concentration of 14 μmol L^{-1} amiodarone decreased cell viability and GSH, and increased LDH activity (Table 2). Pre-incubation of hepatocytes with amiodarone significantly reduced D-amphetamine cytotoxicity. The most indicative was the effect on the GSH level (Table 2). These results suggest that amiodarone may interfere with amphetamine metabolism by inhibiting amphetamine's toxic metabolites to form. However, because amiodarone is metabolised by CYP3A, a competition between the two compounds is likely. To better understand this interaction, a more specific inhibitor should be used, such as ketoconazole.

Using an inducer and inhibitor of CYP3A proved to be a reliable approach in elucidating the involvement of this isoform in amphetamine hepatotoxicity in isolated rat hepatocytes.

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Sažetak

ULOGA CYP3A-ENZIMA U TOKSIČNOSTI D-AMFETAMINA U SVJEŽE IZOLIRANIMA ŠTAKORSKIM HEPATOCITIMA

Cilj je ovoga istraživanja bio utvrditi toksičnost D-amfetamina u izoliranim štakorskim hepatocitima kako bi se razjasnila eventualna uloga enzima CYP3A u mehanizmu toksičnosti ovoga lijeka. U tu su svrhu mužjaci Wistar štakora pet dana primali nifedipin (5 mg kg^{-1} *ip.*), koji je supstrat i induktor CYP3A. Stanice izolirane iz jetre štakora koji su primali nifedipin i kontrolnih štakora inkubirane su s D-amfetaminom u njegovoj srednjoj toksičnoj koncentraciji (TC_{50}) od 100 μmol L^{-1}. Za procjenu eventualnoga toksičnoga djelovanja D-amfetamina na svježe izolirane hepatocite uporabili smo sljedeće parametre: preživljenje stanica te razine laktat dehidrogenaze (LDH) i glutationa (GSH). Rezultati su pokazali da je nifedipin pojačao toksično djelovanje amfetamina *in vitro* na sljedeći način: preživljenje stanica palo je za 65 % ($p<0,001$), GSH za 80 % ($p<0,001$), a razine LDH porasle su za 190 % ($p<0.001$). Radi pojašnjenja uloge nifedipina u citotoksičnosti amfetamina uporabili smo amiodaron, koji je supstrat i inhibitor CYP3A. Inkubacija hepatocita štakora koji su primali nifedipin s amiodaronom (14 μmol L^{-1}) prije dodavanja amfetamina značajno je smanjila njegovu citotoksičnost. Ovi rezultati potvrđuju da D-amfetamin djeluje toksično na štakorske hepatocite te da u njegovome metabolizmu i toksičnome djelovanju na jetru sudjeluje CYP3A.

KLJUČNE RIJEČI: *amfetamin, amiodaron, citotoksičnost, nifedipin*

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