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Involvement of mitochondrial/lysosomal toxic cross-talk in ecstasy induced liver toxicity under hyperthermic condition

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ABSTRACT

The initial objectives of this study were to evaluate the extent of 3, 4-methylenedioxymethamphetamine (MDMA) induced loss of cell viability (cytotoxicity), induction of reactive oxygen species formation and damage to sub-cellular organelles (e.g. mitochondria/lysosomes) in freshly isolated rat hepatocytes under normothermic conditions (37 °C) and to compare the results with the effects obtained under hyperthermic conditions (41 °C). MDMA induced cytotoxicity, reactive oxygen species formation, mitochondrial membrane potential decline and lysosomal membrane leakiness in isolated rat hepatocytes at 37 °C. A rise in incubation temperature from 37 °C to 41 °C had an additive/synergic effect on the oxidative stress markers. We observed variations in mitochondrial membrane potential and lysosomal membrane stability that are significantly ($P<0.05$) higher than those under normothermic conditions. Antioxidants, reactive oxygen species scavengers, lysosomal inactivators, mitochondrial permeability transition (MPT) pore sealing agents, NADPH P450 reductase inhibitor, and inhibitors of reduced CYP2E1 and CYP2D6 prevented all MDMA induced hepatocyte oxidative stress cytotoxicity markers. It is therefore suggested that metabolic reductive activation of MDMA by reduced cytochrome P450s and glutathione could lead to generation of some biological reactive intermediates which could activate reactive oxygen species generation and cause mitochondrial and lysosomal oxidative stress membrane damages. We finally concluded that hyperthermia could potentiate MDMA induced liver toxicity probably through a mitochondrial/lysosomal toxic cross-talk in freshly isolated rat hepatocytes.

1. Introduction

The amphetamine designer drug 3, 4-methylenedioxymethamphetamine (MDMA or "ecstasy") has been used as recreational drug of abuse, whose consumption appears to be spreading in many parts of the world, with a high prevalence among young people. MDMA consumption results in a plethora of systemic and organ-specific effects, including cardiac arrhythmias, serotonergic neurotoxicity, convulsions, fulminant hyperthermia, disseminated intravascular coagulation, rhabdomyolysis and acute renal failure in rodents and humans (Alves et al., 2007; Crean et al., 2006; Izco et al., 2007; Piper, 2007).

Clinical evidence has increasingly shown that the liver is also a target of MDMA toxicity. Jaundice, hepatomegaly, centrilobular necrosis, hepatitis, fibrosis and liver failure represent some of the adverse effects caused by MDMA (Green et al., 2003; Maurer et al., 2004). MDMA hepatotoxicity appears with different intensities, from mild hepatitis that resolves spontaneously, to fulminant liver failure requiring liver transplantation. The latency of MDMA induced liver toxicity is also variable, ranging from days to weeks after the intake. Various factors may contribute to MDMA induced liver toxicity, including the metabolism of MDMA (De la Torre et al., 2004b), the increased efflux of neurotransmitters (De la Torre and Farre, 2004a), the oxidation of biogenic amines (Capela et al., 2009), and hyperthermia (Green et al., 2005).

Hyperthermia corresponds to a well known life-threatening effect of MDMA *in vivo* (Hall and Henry, 2006) that is highly dependent on ambient temperature (Von Huben et al., 2007). It is well known that the administration of MDMA to laboratory animals causes hyperthermia that is a pro-oxidant aggressive condition, which leads to irreversible hepatocellular injury *in vitro* (Carvalho et al., 1997; Skibba et al., 1991). The increase in extracellular dopamine after MDMA leads to the formation of quinone metabolites; the reactive intermediates produced during the oxidation of dopamine into reactive *ortho*-quinones and/or aminochromes can be conjugated with intracellular glutathione (GSH) to form the corresponding glutathionyl adducts (Riezzo et al., 2010). It was also shown that thioether MDMA metabolites time dependently increased reactive species generation, concentration dependently depleted intracellular GSH and increased protein bound quinones, and finally induced oxidative stress and neuronal death (Capela et al., 2007). Besides, it was shown that incubation of rat hepatocytes with amphetamine-derived designer drugs causes cell death accompanied by a significant rise in cellular reactive oxygen species formation, loss of intracellular ATP and adenine nucleotides, GSH depletion and mitochondrial membrane potential collapse (Nakagawa et al., 2009).

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Ecstasy users may be particularly predisposed to hyperthermia by the direct effects of this drug on the thermoregulatory system. In fact, many patients who have developed hepatocellular damage after ingesting ecstasy have been hyperpyrexial for several hours. However, little is known regarding the differences of mechanistic pathways in MDMA induced liver toxicity under two different thermal conditions (37 °C and 41 °C). The aim of this study was to search and compare biochemical and cellular mechanisms involved in MDMA induced liver toxicity in both 37 °C and 41 °C and figure out their significant differences.

2. Materials and methods

2.1. Chemicals

3, 4-Methylenedioxymethamphetamine (MDMA), rhodamine 123, collagenase (from *Clostridium histolyticum*), bovine serum albumin (BSA), N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (HEPES), O-phthalaldehyde, acridine orange, 2′,7′-dichlorofluorescin diacetate (DCFH-DA), Trichloroacetic acid, dimethyl sulfoxide (DMSO), 1-bromoheptane, butylated hydroxyanisole, butylated hydroxytoluene, cyclosporine, diphenyliodonium chloride, trypan blue and heparin were purchased from Sigma-Aldrich Co. (Taufkrichen, Germany). All other chemicals were of the highest commercial grade available.

2.2. Animals

Male Sprague–Dawley rats weighing 280 to 300 g were used in the study. All rats were housed in a room at a constant temperature of 25 °C on a 12/12 h light/dark cycle with food and water available *ad libitum*. All experiments were conducted according to the ethical standards and protocols approved by the Committee of Animal Experimentation of Shaheed Beheshti University of Medical Sciences, Tehran, Iran.

2.3. Isolation and incubation of hepatocytes

Hepatocytes were obtained by collagenase perfusion of the liver and viability was assessed by plasma membrane disruption determined by trypan blue (0.2 w/v) exclusion test (Pourahmad et al., 2006). Cells were suspended at a density of 10^6 cells/ml in round-bottomed flasks rotating in a water bath maintained at 37 °C in Krebs–Henseleit buffer (pH = 7.4), supplemented with 12.5 mM HEPES under an atmosphere of 10% O_2, 85% N_2, and 5% CO_2. Hepatocytes were preincubated for 30 min prior to addition of chemicals. Stock solutions of all chemicals (×100 concentrated for the water solutions or ×1000 concentrated for the methanolic solutions) were prepared fresh prior to use. To avoid either non-toxic or very toxic conditions in this study, we used EC_{50} concentrations for MDMA. The EC_{50} of a chemical in hepatocyte cytotoxicity assessment technique (with the total 3 h incubation period) is defined as the concentration, which decreases the hepatocyte viability down to 50% following 2 h of incubation (Galati et al., 2000). In order to determine this value for MDMA, dose–response curves were plotted and then EC_{50} was determined based on a regression plot of three different concentrations (data and curves not shown). To incubate all water soluble treatments with the required concentration, we added 100 µl sample of its concentrated stock solution (×100 concentrated) to one rotating flask containing 10 ml hepatocyte suspension. For the chemicals, which dissolved in methanol, we prepared methanolic stock solutions (×1000 concentrated), and to achieve the required concentration in the hepatocytes, we added 10 µl samples of the stock solution to the 10 ml cell suspension. Ten microlitres of methanol did not affect the hepatocyte viability after 3 h of incubation (data not shown). GSH depleted hepatocytes were prepared by preincubation of hepatocytes with 200 µM 1-bromoheptane for 30 min as described by Khan and O'Brien (1991).

2.4. Cell viability

The viability of isolated hepatocytes was assessed from the intactness of the plasma membrane as determined by the trypan blue (0.2% w/v) exclusion test (Pourahmad et al., 2006). Aliquots of the hepatocyte incubate were taken at different time points during the 3 h incubation period.

2.5. Determination of reactive oxygen species

To determine the rate of hepatocyte reactive oxygen species generation induced by MDMA, dichlorofluorescin diacetate (DCFH-DA) (1.6 µM) was added to the hepatocytes. It penetrates hepatocyte cells and becomes hydrolyzed to non-fluorescent dichlorofluorescin. The latter then reacts with reactive oxygen species to form the highly fluorescent dichlorofluorescein (DCF), which effluxes the cell. The fluorescence intensity of DCF was measured using a Shimadzu RF5000U fluorescence spectrophotometer. Excitation and emission wavelengths were 500 and 520 nm, respectively. The results were expressed as fluorescent intensity per 10^6 cells (Pourahmad et al., 2008).

2.6. Mitochondrial membrane potential assay

Mitochondrial uptake of the cationic fluorescent dye, rhodamine 123 (1.5 µM), has been used for estimation of mitochondrial membrane potential (Andersson et al., 1987). The amount of rhodamine 123 remaining in the incubation medium was measured fluorimetrically using a Shimadzu RF5000U fluorescence spectrophotometer set at 490 nm excitation and 520 nm emission wavelengths. The capacity of mitochondria to take up the rhodamine 123 was calculated as the difference (between control and treated cells) in rhodamine 123 fluorescence. Our data were shown as the percentage of mitochondrial membrane potential collapse (%$\Delta\Psi$m) in all treated (test) hepatocyte groups (Andersson et al., 1987).

2.7. Lysosomal membrane integrity assay

Hepatocyte lysosomal membrane stability was determined from the redistribution of the fluorescent dye, acridine orange (Pourahmad et al., 2009). Aliquots of the cell suspension (0.5 ml) that were previously stained with acridine orange (5 µM) were separated from the incubation medium by 1 min centrifugation at 1000 rpm. The cell pellet was then resuspended in 2 ml of fresh incubation medium. This washing process was carried out two times to remove the fluorescent dye from the media. Acridine orange redistribution in the cell suspension was then measured fluorimetrically using a Shimadzu RF5000U fluorescence spectrophotometer set at 495 nm excitation and 530 nm emission wavelengths.

2.8. Statistical analysis

Levene's test was used to check the homogeneity of variances. Data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD as the *post hoc* test. Results were presented as mean ± S.D. of triplicate samples. The minimal level of significance chosen was $P<0.05$.

2.9. Protocol design

In this research we used a wide range of inhibitors/antagonists including reactive oxygen species scavengers (mannitol, DMSO, and catalase), antioxidants (α-Tocopherol, butylated hydroxytoluene, butylated hydroxyanisole, and superoxide dismutase), ferric chelator (deferoxamine), MPT pore sealing agents (carnitine and cyclosporine), endocytosis inhibitors (monensin, methylamine, 3-methyladenine, and chloroquine), cytochrome P450 (CYP450) isoenzyme inhibitors