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ORGAN TOXICITY AND MECHANISMS

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Hepatotoxicity of 3,4-methylenedioxyamphetamine and α-methyldopamine in isolated rat hepatocytes: formation of glutathione conjugates

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Abstract The amphetamine designer drugs 3,4-methylenedioxymethamphetamine (MDMA or "ecstasy") and its *N*-demethylated analogue 3,4-methylenedioxyamphetamine (MDA or "love") have been extensively used as recreational drugs of abuse. MDA itself is a main MDMA metabolite. MDMA abuse in humans has been associated with numerous reports of hepatocellular damage. Although MDMA undergoes extensive hepatic metabolism, the role of metabolites in MDMA-induced hepatotoxicity remains unclear. Thus, the aim of the present study was to evaluate the effects of MDA and α-methyldopamine (α-MeDA), a major metabolite of MDA, in freshly isolated rat hepatocyte suspensions. The cells were incubated with MDA or α-MeDA at final concentrations of 0.1, 0.2, 0.4, 0.8, or 1.6 mM for 3 h. The toxic effects induced following incubation of hepatocyte suspensions with these metabolites were evaluated by measuring cell viability, the extent of lipid peroxidation, levels of glutathione (GSH) and glutathione disulfide (GSSG), the formation of GSH conjugates, and the activities of GSSG reductase (GR), GSH peroxidase (GPX), and GSH *S*-transferase (GST). MDA induced a concentration- and time-dependent GSH depletion, but had a negligible effect on lipid peroxidation, cell viability, or on the activities of GR, GPX, and GST. In contrast, α-MeDA (1.6 mM, 3 h) induced a marked depletion of GSH accompanied by a loss on cell viability, and decreases in GR, GPX and GST activities, although no significant effect on lipid peroxidation was found. For both metabolites, GSH depletion was not accompanied by increases in GSSG levels; rather, 2-(glutathion-*S*-yl)-α-MeDA and 5-(glutathion-*S*-yl)-α-MeDA were identified by HPLC-DAD/EC within cells incubated with MDA or α-MeDA. The results provide evidence that one of the early consequences of MDMA metabolism is a disruption of thiol homeostasis, which may result in loss of protein function and the initiation of a cascade of events leading to cellular damage.

Keywords 3,4-Methylenedioxyamphetamine · α-Methyldopamine · Rat hepatocytes · Glutathione conjugates

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Introduction

The amphetamine designer drugs 3,4-methylenedioxymethamphetamine (MDMA or "ecstasy") and its *N*-demethylated analogue 3,4-methylenedioxyamphetamine (MDA or "love") have been used as recreational drugs of abuse, mainly among young people, due to their stimulant and hallucinogenic properties. However, MDMA and MDA produce a variety of systemic and organ-specific effects, including serotonergic neurotoxicity (Battaglia et al. 1987; Ricaurte et al. 2000), convulsions, hyperthermia, disseminated intravascular coagulation, rhabdomyolysis, and acute renal failure

(Simpson and Rumack 1981; Henry et al. 1992; Hegadoren et al. 1999; Walubo and Seger 1999). In the past decade, clinical evidence has increasingly shown that the liver is also a target of MDMA toxicity. Jaundice, hepatomegaly, centrilobular necrosis, hepatitis and fibrosis represent some of the adverse effects caused by MDMA in the liver (Henry et al. 1992; Dykhuizen et al. 1995; Khakoo et al. 1995; Ellis et al. 1996; Milroy et al. 1996; Fineschi et al. 1999; Varela-Rey et al. 1999). Various factors probably play a role in MDMA-induced liver toxicity, including the metabolism of MDMA, the increased efflux of neurotransmitters, the oxidation of biogenic amines, and hyperthermia. In fact, our group has shown recently that the toxic effects induced by MDMA in isolated mouse hepatocytes are exacerbated under hyperthermic conditions (Carvalho et al. 2001). However, in spite of the extensive hepatic metabolism of MDMA, the role of metabolites in MDMA-induced hepatotoxicity is still unclear. In view of this, the present work was performed to evaluate the effects of two main MDMA metabolites, MDA and α-methyldopamine (α-MeDA), in freshly isolated rat hepatocytes. MDA is not only a synthetic drug of abuse but it is also an important metabolite of MDMA (Cho et al. 1990). MDMA is metabolized to *N*-methyl-α-MeDA and α-MeDA (Lim and Foltz 1988; Kumagai et al. 1991), and MDA to α-MeDA (Marquardt et al. 1978). *N*-Methyl-α-MeDA and α-MeDA are catechols that can undergo oxidation to the corresponding *ortho*-quinones. Quinones are highly redox-active molecules, which can enter redox cycles through their semiquinone radicals, leading to the formation of reactive oxygen species (ROS), including the superoxide anion, hydrogen peroxide, and the hydroxyl radical. Alternatively, since the reactive *ortho*-quinone intermediates are Michael acceptors, they can promote cellular damage through an alkylation process with biomolecules such as crucial cellular proteins and/or DNA (Bolton et al. 2000). In the presence of glutathione (GSH), the *ortho*-quinone of α-MeDA undergoes conjugation with GSH to form (glutathion-*S*-yl)-α-MeDA adducts (Hiramatsu et al. 1990). The GSH conjugates remain redox active and may be readily oxidized to the *ortho*-quinone thioethers, which can be followed by reductive addition of a second molecule of GSH to yield 2,5-*bis*(glutathion-*S*-yl)-α-MeDA (Miller et al. 1997). It must be stressed that these redox-active metabolites have been recently implicated in the mechanisms underlying MDMA-induced neurotoxicity (Miller et al. 1995, 1997; Bai et al. 1999, 2001) and nephrotoxicity (Carvalho et al. 2002b). To further elucidate the potential role of metabolites in MDMA-elicited hepatotoxicity, we evaluated the effects of MDA and α-MeDA on cell viability, the extent of lipid peroxidation, levels of glutathione and glutathione disulfide (GSSG), the formation of GSH conjugates, and the activities of GSSG reductase (GR), GSH peroxidase (GPX), and GSH *S*-transferase (GST) in isolated rat hepatocytes. Finally, high-performance liquid chromatography methodology with simultaneous combined electrochemical and diode-array analysis (HPLC-DAD/EC) was utilized to identify the GSH conjugates of these metabolites formed within the cells.

Materials and methods

Chemicals

All reagents used in this study were of analytical grade. Collagenase (type I), bovine serum albumin (fraction V), *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), ethylene glycol-*bis*(β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), reduced GSH, GSSG, GR (EC 1.6.4.2), 2-vinylpyridine, β-nicotinamide adenine dinucleotide phosphate reduced form (β-NADPH), β-nicotinamide adenine dinucleotide reduced form (β-NADH), 5,5'-dithio-*bis*(2-nitrobenzoic acid) (DTNB), pyruvic acid, 2-thiobarbituric acid (TBA), 1-octanesulfonic acid, γ-glutamyl transpeptidase (γ-GT; EC 2.3.2.2) and all the reagents for enzymatic determinations were obtained from Sigma Chemical Company (St. Louis, MO, USA). Chloroacetic acid, citric acid, methanol (gradient grade), perchloric acid, and all other chemicals were obtained from Merck (Darmstadt, Germany). 3,4-Methylenedioxyamphetamine (HCl salt) and α-methyldopamine (HBr salt) were synthesized in the Organic Chemistry Department, Faculty of Pharmacy, Porto University, Portugal. 5-(Glutathion-*S*-yl)-α-MeDA was synthesized in the Center for Molecular and Cellular Toxicology, College of Pharmacy, University of Texas, Austin, USA.

Animals

Male Wistar rats (200–250 g) were obtained from Charles-River Laboratories (Barcelona, Spain). Animals were acclimatized in polyethylene cages, lined with wood shavings, with wire-mesh tops, at an ambient temperature of 20 ± 2°C, humidity between 40 and 60% and 12 h/12 h light/dark cycle. The animals had free access to standard rat chow and drinking water, and were kept in the animal house for at least 1 week prior to use. Surgical procedures for the isolation of hepatocytes were performed under anesthesia and were carried out always between 9:00 a.m. and 10:00 a.m.

Hepatocyte isolation and incubation

Hepatocytes were isolated by collagenase perfusion as previously described (Moldéus et al. 1978). Cell viability at the initiation of the experiments was between 85% and 97%.

Incubations were performed in a shaking water bath (90 oscillations/min) at 37°C, using 10^6 cells/ml in Krebs-Henseleit buffer supplemented with 25 mM HEPES (pH 7.4), and gassed with an air stream of humidified carbogen. Isolated hepatocytes were pre-incubated for 30 min at 37°C and then incubated with MDA chloride or α-MeDA bromide at final concentrations of 0, 0.1, 0.2, 0.4, 0.8, or 1.6 mM. Since α-MeDA is a light-sensitive unstable compound, all incubations were performed under light-protection conditions. Aliquots of the cell suspensions were taken at time zero and at hourly intervals for 3 h for the evaluation of cell viability, lipid peroxidation, GSH and GSSG levels. Selenium-dependent GPX, GR and GST activities were also quantified in cell suspension aliquots taken at time zero and after 3 h of incubation (samples were kept frozen at −80°C until assay, usually within 1 week). At the end of the experiments, aliquots of cell suspensions (10^6 cells) were collected and immediately centrifuged at 300 rpm for 2 min. The pellet was used for detection of GSH conjugates.

Biochemical analysis

Cell viability was determined after isolation by the Trypan blue exclusion test. During the course of the experiments cell viability was determined by the lactate dehydrogenase (LDH) leakage method, randomly confirmed by the Trypan blue exclusion test.

The extent of lipid peroxidation was measured by the thiobarbituric acid reactive substances (TBARS) assay at 535 nm as described before (Carvalho et al. 1997).

The GSH and GSSG contents of cell suspensions were determined by the DTNB–GSSG reductase recycling assay as described by Anderson (1985), with some modifications. Briefly, 200 µl of cell suspension aliquots were precipitated with 200 µl perchloric acid (5% final acid concentration) and centrifuged for 10 min at 13,000 rpm in a refrigerated centrifuge (4°C). After this time, 100 µl acidic supernatant was neutralized with 100 µl 0.76 M $KHCO_3$ and the sample centrifuged for 1 min at 13,000 rpm. Fresh reagent, containing 0.69 mM NADPH and 4 mM DTNB in 72 mM phosphate buffer, was prepared daily. For measurement of total glutathione, 100 µl/well of samples, standards or blank were added in duplicate to 96-well microtiter plates, followed by 65 µl/well of the freshly prepared reagent. Plates were then incubated in a thermomax plate reader (PowerWaveX; Bio-Tek, Winooski, VT, USA) at 30°C for 10 min prior to the addition of 40 µl GR per well (10 IU/ml in phosphate buffer). The stoichmetric formation of 5-thio-2-nitrobenzoic acid (TNB) was followed for 2 min at 415 nm and compared with a standard curve. For the determination of GSSG, aliquots (200 µl) of acidic supernatant were treated with 10 µl 2-vinylpyridine and mixed continuously for 1 h for derivatization of GSH. GSSG was then measured as described above for total glutathione. The GSH levels were calculated by subtracting GSSG content from the total glutathione content ($GSH = GSH_t - 2 \times GSSG$).

For measurement of the activities of selenium-dependent GPX, GR and GST, aliquots of cell suspensions were sonicated for 12 s at medium intensity and then centrifuged at 13,000 rpm for 5 min. GPX, GR, and GST activities were then determined in the supernatant as previously described (Carvalho et al. 2002a).

HPLC-DAD/EC analysis of GSH conjugates

The separation and characterization of GSH conjugates was performed by high-performance liquid chromatography (HPLC), equipped with an electrochemical (EC) and a photodiode array detector (DAD). HPLC analysis was performed using an autosampler injector (Gilson model 231-401), and a Spherisorb RP-18 (5 µm) column (Waters, Milford, MA, USA). A Coulochem II electrochemical detector (ESA, Chelmsford, MA, USA) equipped with a guard cell (ESA 5020) and an analytical cell (ESA 5010) was used. The electrochemical potential settings of the Coulochem II multidetector were: guard cell +0.45 V, detector 1 −0.1 V, and detector 2 +0.35 V. A current of 1–5 µA full-scale was used. A photodiode array detector (Waters model 2690) was placed before the analytical cell and the detection was performed at 292 nm, corresponding to the maximum absorption wavelength of the mono GSH-conjugates. A Compaq computer fitted with Millennium software (Waters) processed the chromatographic and spectral data.

The mobile phase (50 mM citric acid, 0.46 mM 1-octanesulfonic acid and 15% methanol, adjusted to pH 3.0 with 10 M NaOH) was filtered through a 0.45-µm membrane (Millipore, Madrid, Spain) and degassed under a helium stream. An isocratic elution was performed at 1.0 ml/min at room temperature.

Sample aliquots were treated with methanol for precipitation of protein and centrifuged for 10 min at 13,000 rpm. The supernatant was evaporated under a stream of nitrogen and then reconstituted to 150 µl with water. A volume of 100 µl was then injected into the HPLC system. The presence of the conjugates was confirmed by co-chromatography with 2-(glutathion-*S*-yl)-α-MeDA and 5-(glutathion-*S*-yl)-α-MeDA standards.

To strengthen sample peak identification of GSH conjugates, cell samples were sonicated (12 s at medium intensity) and then treated with γ-GT (8 IU in Krebs-Henseleit buffer) for 1 min before precipitation of proteins with methanol. UV spectra of peaks present in samples were obtained to corroborate identification of GSH conjugates.

Synthesis of 2-(glutathion-*S*-yl)-α-MeDA conjugate

A mixture of α-MeDA (2 mM), GSH (10 mM), and tyrosinase (5000 U) in 5 ml potassium phosphate buffer (50 mM, pH 7.4) was incubated for 1 min at 37°C. The reaction was then terminated by addition of 1 ml 15% perchloric acid. The reaction mixture was centrifuged, and 100 µl supernatant was injected into the HPLC-DAD/EC system. The mobile phase consisted of 10 mM ammonium acetate and 10% methanol (adjusted to pH 3.0), at a flow rate of 1.0 ml/min. The HPLC chromatogram revealed the presence of two major peaks corresponding to 2-(glutathion-*S*-yl)-α-MeDA and 5-(glutathion-*S*-yl)-α-MeDA conjugates, with retention times of 8 min and 16 min, respectively. The peak corresponding to 5-(glutathion-*S*-yl)-α-MeDA was confirmed after co-elution with 5-(glutathion-*S*-yl)-α-MeDA standard. Fractions containing 2-(glutathion-*S*-yl)-α-MeDA were collected and the structure confirmed by mass spectrometry (adapted from Miller et al. 1995).

Statistical analysis

Results are presented as means ± SEM (from five to eight experiments of different preparations of hepatocytes). Statistical comparisons between groups were performed by one-way analysis of variance (ANOVA) followed by Scheffé's test. Changes in the studied parameters with time of incubation were evaluated by one-way repeated measures ANOVA (Huynh-Feldt adjustment). Significance was accepted at $P < 0.05$.

Results

The toxic effects induced by MDA or α-MeDA on hepatocyte suspensions were evaluated by measuring cell viability, the extent of lipid peroxidation, levels of GSH, GSSG and total glutathione, and antioxidant enzyme activities. Figure 1 illustrates the viability of hepatocytes incubated with MDA or α-MeDA (both at 0.1–1.6 mM). At all tested concentrations, MDA had a negligible effect on cell viability, as measured by LDH leakage (Fig. 1A). In contrast, α-MeDA at the highest concentration tested (1.6 mM) induced a significant decrease in cell viability (cell death up to 43 ± 5%) after 3 h of incubation (Fig. 1B).

No lipid peroxidation was observed when cells were incubated with MDA or α-MeDA (0.1–1.6 mM, 3-h incubation) as measured by TBARS assay (data not shown). Both MDA and α-MeDA (0.1–1.6 mM) induced a concentration- and time-dependent GSH depletion (Fig. 2A,B, respectively). GSH depletion was clearly more marked for the catechol metabolite at the highest concentration studied. In fact, 1.6 mM MDA caused nearly 42% depletion of GSH after 2 h of incubation, whereas 1.6 mM α-MeDA caused an ~85% depletion of GSH, compared with controls. Differences were not evident at the lower tested concentrations. An important finding was that glutathione depletion induced by MDA

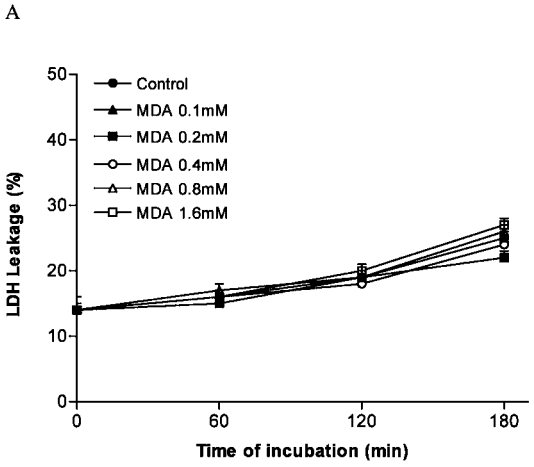

Fig. 1A,B Effect of 3,4-methylenedioxyamphetamine (MDA; **A**) and its major metabolite α-methyldopamine (α-MeDA; **B**) on cell viability of hepatocyte suspensions as measured by percentage of lactate dehydrogenase (LDH) leakage. Data represent means ±SEM, $n = 5–8$; $^{*}P < 0.05$, compared with control

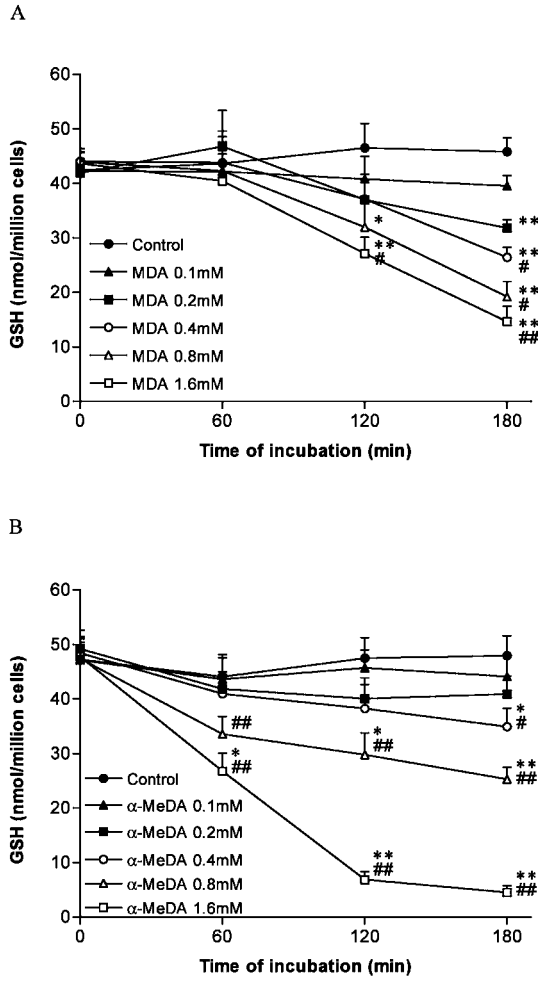

Fig. 2A,B Effect of MDA (**A**) and α-MeDA (**B**) on glutathione (GSH) levels in hepatocyte suspensions. Data represent means ±SEM, $n = 5–8$; $^{*}P < 0.05$, $^{**}P < 0.01$, compared with control; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, compared with time zero

or α-MeDA was not due to glutathione oxidation (Fig. 3A,B, respectively). In fact, no significant differences in GSSG levels were found when cells were treated with MDA (0.1–1.6 mM) or α-MeDA (0.1–0.8 mM). It is noteworthy that after the third hour of incubation, GSSG levels were significantly decreased by 1.6 mM α-MeDA, when compared with those of controls. Total glutathione content of cells incubated with 1.6 mM MDA was significantly reduced to 42% of control levels after 3 h of incubation, whereas with 1.6 mM α-MeDA total glutathione levels of cells were significantly reduced to 63% and 72% of control levels after 2 and 3 h of incubation, respectively (data not shown).

One of the aims of the present work was to identify putative GSH conjugates formed within cells incubated with MDA or α-MeDA; thus, an HPLC methodology was implemented to separate and identify these conjugates. In all HPLC-EC analyses of samples obtained from incubations with MDA, a predominant electroactive peak (with retention time of 12.5 min) was detected and identified as α-MeDA after coelution with authentic α-MeDA standard. Within this samples, the HPLC-DAD/EC system also demonstrated the presence of two mono GSH-conjugates in cells treated with 1.6 mM MDA for 3 h. The incubation of hepatocytes with 0.1–1.6 mM α-MeDA for 3 h resulted in a more expressive presence of the two mono GSH-conjugates (Fig. 4). Retention times for the 2-(glutathion-*S*-yl)-α-MeDA and 5-(glutathion-*S*-yl)-α-MeDA conjugates were 10.0 min (Fig. 4A) and 20.5 min (Fig. 4B), respectively, and were confirmed by co-chromatography with authentic standards. The appearance of two peaks in each of the standards and samples is due to the presence of diastereomers. In previous work, using different chromatographic conditions, we were able to completely separate these two peaks and confirm their identity by mass spectrometry (unpublished data). Furthermore, it was observed that the peaks corresponding to the GSH conjugates completely disappeared when samples were treated with γ-GT, an enzyme known to cleave the γ-glutamyl bond of GSH

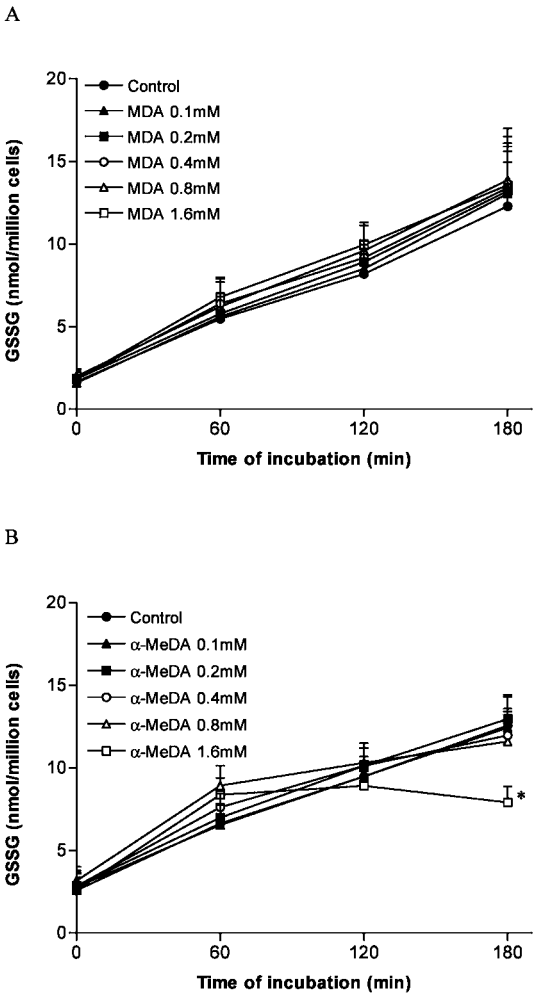

Fig. 3A,B Effect of MDA (**A**) and α-MeDA (**B**) on glutathione disulfide (GSSG) levels in hepatocyte suspensions. Data represent means ± SEM, $n = 5$–8; $^*P < 0.05$, compared with control

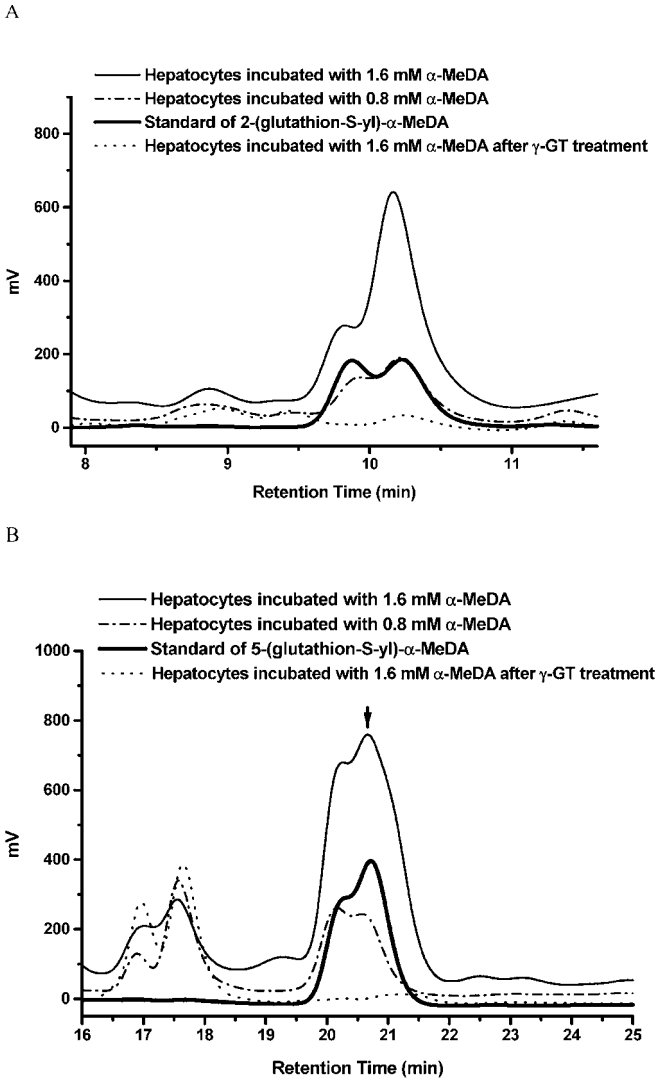

Fig. 4A,B Chromatograms from electrochemical detection HPLC showing the peaks corresponding to the 2-(glutathion-*S*-yl)-α-methyldopamine (**A**) and 5-(glutathion-*S*-yl)-α-methyldopamine (**B**) conjugates formed within cells incubated with 0.8 mM or 1.6 mM α-methyldopamine (α-MeDA) for 3 h, and their respective standards. Peaks obtained from cells incubated with 1.6 mM α-MeDA after treatment with γ-glutamyl transpeptidase (γ-GT) are also presented. Experimental details are stated in "Materials and methods"

(Fig. 4A,B). Although the purpose of the present work was not the quantification of these GSH conjugates, it was observed that 2-(glutathion-*S*-yl)-α-MeDA and 5-(glutathion-*S*-yl)-α-MeDA formation in cells incubated with α-MeDA correlated with decreases in the concentration of α-MeDA in the incubation medium (as shown in Fig. 4A,B by hepatocytes incubated with α-MeDA at a final concentration of 0.8 mM and 1.6 mM). A comparison of the UV spectra of the 5-(glutathion-*S*-yl)-α-MeDA conjugate present in the α-MeDa treated cells with that of the respective standard is shown in Fig. 5. The same UV pattern was also observed for 2-(glutathion-*S*-yl)-α-MeDA conjugate (data not shown). UV spectra of the GSH-conjugate peaks detected in samples exhibited three λ_{max}, at 236 nm, 256 nm and 292 nm, which are characteristic of *S*-glutathionyl conjugates of catecholamines (Spencer et al. 1998).

Finally, the enzymatic activities of GR, GST, and GPX were also evaluated. No significant differences in GR, GPX and GST activities were observed after incubation of hepatocyte suspensions with 0.1–1.6 mM MDA (data not shown). In contrast, the activity of each of these enzymes was significantly lower in samples incubated with 1.6 mM α-MeDA (3 h) than in control samples (Fig. 6). GPX was the most susceptible to inhibition, decreasing to 32% of initial activity, whereas GR and GST activities decreased to only 62% and 55% of initial activity, respectively.

Discussion

MDA is a hepatic-derived metabolite of MDMA (Lim and Foltz 1988). Metabolic activation to a reactive

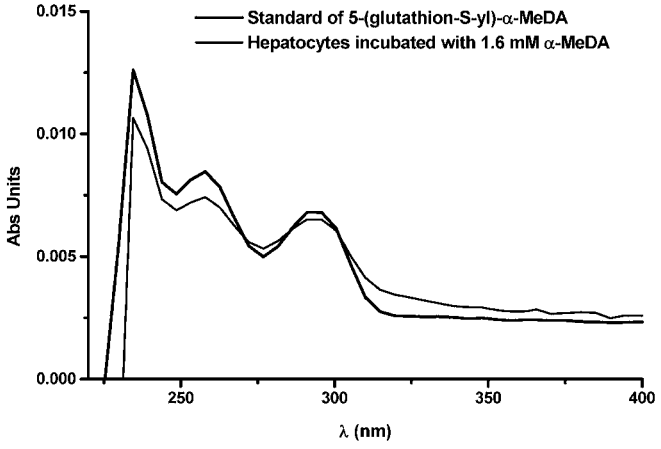

Fig. 5 Comparative UV spectra of the 5-(glutathion-*S*-yl)-α-MeDA conjugate present in hepatocyte suspensions incubated with α-methyldopamine (α-MeDA) and the respective standard

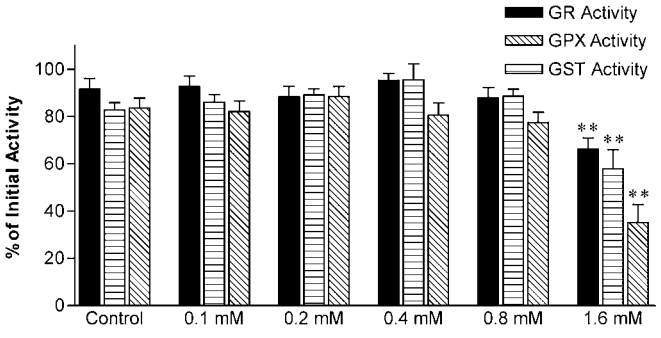

Fig. 6 Percentage of GSSG reductase (GR), GSH peroxidase (GPX) and GSH *S*-transferase (GST) activities in hepatocyte suspensions after 3 h of incubation with α-MeDA, relative to initial activities. Data represent means ±SEM, $n = 7–8$; $^{**}P < 0.01$, compared with control

metabolite capable of conjugating with GSH has been shown for MDMA and MDA (Hiramatsu et al. 1990). MDMA is *N*-demethylated to MDA, and MDA is demethylenated to α-MeDA by cytochromes P450 2D, 2B and 3A1 (Bai et al. 1999; Kreth et al. 2000). α-MeDA undergoes spontaneous oxidation to the corresponding *ortho*-quinone, which is a highly reactive molecule that can redox cycle with its semiquinone radical, leading to the generation of ROS or reactive nitrogen species (see Fig. 7) (Bolton et al. 2000).

Alternatively, the *ortho*-quinone formed by oxidation of α-MeDA is readily conjugated with GSH to form 2-(glutathion-*S*-yl)-α-MeDA and/or 5-(glutathion-*S*-yl)-α-MeDA, which themselves may be readily oxidized to *ortho*-quinones followed by the addition of a second molecule of GSH to form 2,5-*bis*(glutathion-*S*-yl)-α-MeDA (Miller et al. 1997). These thioether conjugates of α-MeDA not only preserve but also enhance the biological activity of the parent compounds. In fact, electrochemical studies indicate that the thioether conjugates of α-MeDA exhibit lower half-wave oxidation potentials than α-MeDA, and thus are potentially more reactive (Bai et al. 1999).

In the present study, a time- and concentration-dependent depletion of GSH was found to occur in rat hepatocytes following exposure to MDA or α-MeDA. The GSH depletion was clearly more marked for the catechol metabolite at the highest concentration studied. The fact that GSH depletion obtained with the lowest concentrations of MDA and α-MeDA was similar indicates that, probably, the effect of MDA is due to its conversion into α-MeDA. In fact, in the present study the metabolism of MDA was observed to occur in hepatocytes since the catechol metabolite α-MeDA and the conjugates of α-MeDA with GSH were detected within cells incubated with MDA. The resulting depletion of GSH has already been reported after MDMA exposure of isolated rat (Beitia et al. 1999) and mouse (Carvalho et al. 2001) hepatocytes. As an endogenous antioxidant, GSH is known to play a crucial protective role against cellular injury, which is due to its oxidant neutralizing, lipid peroxidase and/or tocopheryl-radical regenerating activities (Di Mascio et al. 1991). Thus, GSH depletion may render the cells more exposed to the effects of reactive compounds, ROS and reactive nitrogen species being formed within the cells, leading to deleterious effects in hepatocytes.

Despite changes in GSH homeostasis, incubation of freshly isolated rat hepatocytes with MDA did not result in any modification of cell viability or lipid redox status, suggesting that the lower intracellular GSH concentrations are sufficient to protect hepatocytes against oxidative damage. However, after the marked GSH depletion observed in hepatocyte suspensions incubated with the highest concentration of α-MeDA, concentrations of GSH are insufficient to prevent cell death (Fig. 1). These results are in agreement with previous reports (cited in Reed 1990), which showed that cytotoxicity as measured by lipid peroxidation, cell necrosis, and loss of intracellular enzymes in vivo and in vitro occurs only if the intracellular concentrations of GSH fall below 10–15% of the initial value. It must also be stressed that anorexia, hypoxia, and hyperthermia are well-known physiological conditions induced by MDMA in vivo that may strongly contribute to cell death when GSH is no longer available for protection.

2-(Glutathion-*S*-yl)-α-MeDA and 5-(glutathion-*S*-yl)-α-MeDA, were identified and characterized in freshly isolated rat hepatocytes after incubation with MDA or α-MeDA (Fig. 4). The intracellular formation of GSH conjugates helps to explain the observed decrease in GSH levels without the corresponding enhancement in GSSG levels.

The ability of polyphenolic thioether conjugates to undergo redox cycling and produce ROS provides a rationale for the potential role of these metabolites in MDA and MDMA hepatotoxicity. *N*-Methyl-α-MeDA, a metabolite of MDMA, and α-MeDA, a metabolite of MDA and MDMA, have been implicated in the neurotoxicity of the respective parent amphetamines following their biotransformation into the corresponding thioether conjugates (Miller et al. 1996, 1997; Bai et al.

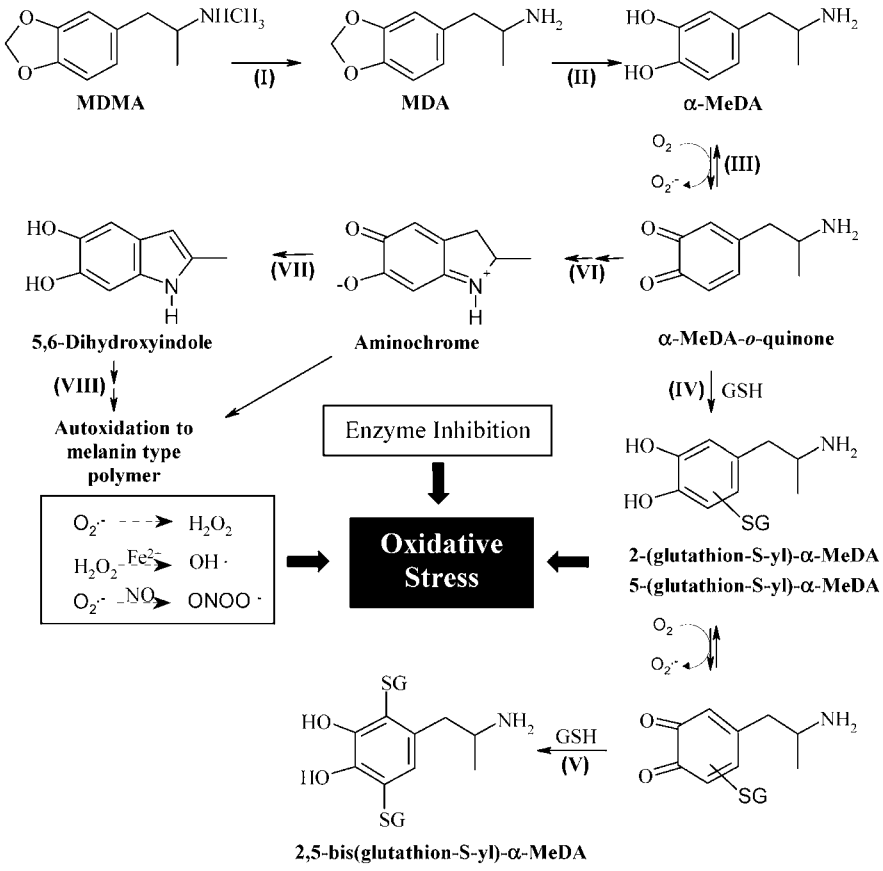

Fig. 7 Schematic presentation of possible metabolic pathway of 3,4-methylenedioxymethamphetamine (MDMA) and 3,4-methylenedioxyamphetamine (MDA) in liver. Microsomal *N*-demethylation of MDMA to MDA (*I*) and demethylenation of MDA to α-methyldopamine (α-MeDA) (*II*) followed by *ortho*-quinone formation (*III*) and glutathione conjugation (*IV*, *V*). On cyclization, the *ortho*-quinone of α-MeDA may give rise to the formation of an aminochrome (*VI*) and related compounds, such as 5,6-dihydroxyindoles (*VII*), which can undergo further oxidation and polymerization to form brown or black insoluble pigments of melanin type (*VIII*)

1999). In fact, when injected directly into brain, MDMA and MDA are not neurotoxic. In contrast, direct injection of 5-(glutathion-*S*-yl)-α-MeDA and 2,5-*bis*(glutathion-*S*-yl)-α-MeDA reproduces the toxicity observed following peripheral administration of MDA (Miller et al. 1996, 1997; Bai et al. 1999).

Consistent with known pathways of catecholamine oxidation, incubation of rat hepatocytes with α-MeDA resulted in the formation of orange-colored aminochromes. Furthermore, as the oxidation progressed, dark brown/black turbidity appeared in the medium, a consequence of melanin-type polymer formation (Bindoli et al. 1992; Zhang and Dryhurst 1994). It was already reported that oxidation of dopamine by tyrosinase gives rise to a black, insoluble polymer only when the concentrations of sulfhydryls such as GSH and cysteine are low relative to those of dopamine (Carstam et al. 1991; Zhang and Dryhurst 1994). Consistent with these reports, the formation of black pigments in the incubation medium of cells incubated with α-MeDA only occurs as a late-stage event, probably resulting from the depleted GSH concentrations (and of the antioxidant defense system) being unable to react with the *ortho*-quinones being formed within cells (with the result of further oxidation of quinones to melanin-type polymers). In fact, α-MeDA-*ortho*-quinone can be further oxidized in a process that involves an irreversible 1,4-intramolecular cyclization reaction, resulting in the formation of aminochromes (colored pigments) and related compounds, such as 5,6-dihydroxyindoles, which eventually leads to the appearance of brown or black insoluble polymers of the melanin type (see Fig. 7) (Bindoli et al. 1989, 1992). Importantly, the catecholamine autoxidation process can be accelerated under physiological conditions by oxidative enzymes, such as xanthine oxidase, peroxidase, lipoxygenase, several copper-containing catechol oxidases, or in presence of metal ions such as Cu^{2+}, Mn^{2+}, Fe^{3+}, and several Cu^{2+} and Fe^{3+} chelates (Bindoli et al. 1992).

Melanins represent a large group of chemically active and potentially toxic substances (Hegedus 2000). In the presence of Fe^{3+}, synthetic melanin can catalyze a Fenton-type reaction, which generates the hydroxyl radical and initiates lipid peroxidation (Ben-Shachar et al. 1991). Thus, future work is therefore clearly required to investigate the toxicity exerted by these polymers in hepatocytes.

Ortho-quinones and aminochromes can interact with various nucleophiles in the cell; of particular importance are the reactions with thiol groups, which are present in cysteine, GSH and proteins. The ability of 1.6 mM α-MeDA to inhibit GR, GPX, and GST activities may thus be related to either the oxidation or alkylation of sulfhydryl groups present within the enzymes' active site. *Ortho*-quinones and aminochromes can inhibit enzymes that possess either a GSH-binding site and/or cysteine residues that are critical for enzyme function (Ommen et al. 1991; Bindoli et al. 1992). Several quinones are

known to cause irreversible inhibition of GST (Ommen et al. 1991; Monks and Lau 1992). The GSTs catalyze the nucleophilic attack of GSH on electrophilic substrates, thereby protecting various cell components from potentially damaging reactive electrophiles (Mannervik and Danielson 1988; Armstrong 1991; van Bladeren 2000). Most GST isoenzymes possess a nucleophilic amino acid, which is rapidily modified by quinones, a reaction that inactivates the enzyme (Ploemen et al. 1991), and the 5-(*S*-glutathionyl) conjugates of dopamine and α-methyldopa inhibit human GSTs (Ploemen et al. 1994). Interestingly, GSH conjugates of several quinones also react rapidly with GST (thereby inactivating the enzyme). Therefore, the presence of a GSH moiety may serve to target the quinone to the enzyme (Ommen et al. 1991). The activity of GR, which catalyzes the NADPH-dependent reduction of GSSG to GSH, is crucial to maintain a high cellular GSH/GSSG ratio. Inhibition of GR and GST by quinones, as well as GR, GPX and GST by aminochromes, has been reported (Remião et al. 1999, 2002).

Taken together, it seems likely that *ortho*-quinones, aminochromes and ROS generated during catecholamine oxidation reactions may all participate in α-MeDA-induced enzyme inhibition. Inhibition of these enzymes can increase the oxidative stress resulting from α-MeDA oxidation.

The toxicity of MDA and α-MeDA in freshly isolated rat hepatocytes has not been previously reported. The present results indicate that oxidative stress may play a part in the first step of MDMA-induced liver damage. The loss of GSH and the modification of enzyme activities by oxidized α-MeDA clearly shows that one of the early consequences of MDMA and MDA metabolism is a disruption of thiol homeostasis, which may result in loss of protein function and initiation of a cascade of events leading to cellular damage. GSH in conjunction with GPX/GR is responsible for the elimination of cellular hydrogen peroxide (H_2O_2) and organic peroxides. Thus, depletion of GSH and/or decreased activity of these enzymes may compromise this pathway, thereby allowing H_2O_2 to accumulate to toxic levels. It must be stressed that hepatic mitochondria are especially vulnerable to this effect since they lack the H_2O_2 metabolizing enzyme catalase (DeLeve and Kaplowitz 1991). Alternatively, the loss of GSH may lead to alterations in normal cellular metabolism and lead to the increased accumulation of ROS.

In conclusion, present findings show that MDA and α-MeDA induce toxicity in freshly isolated rat hepatocytes. The toxic effects are characterized by a loss of GSH homeostasis due to conjugation of GSH with α-MeDA, decreases in the antioxidant enzyme activities, and cell death. Thus, it can be postulated that the metabolism of MDMA and MDA, resulting in the formation of the highly reactive compounds *N*-Me-α-MeDA and α-MeDA, and α-MeDA, respectively, is one of the main causes of their hepatotoxic effects.

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