Comparative effects of 3,4-methylenedioxymethamphetamine and 4-methylthioamphetamine on rat liver mitochondrial function

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A B S T R A C T

Ecstasy, which is used as a recreational drug, is a common street name for 3,4-methylenedioxymethamphetamine (MDMA). Another drug of abuse chemically related, though less common than MDMA, is the amphetamine derivative 4-methylthioamphetamine (MTA). MDMA and MTA induce different systemic and organ-specific effects, including neurotoxicity, hyperthermia, nephrotoxicity, cardiotoxicity and hepatotoxicity. Therefore, it is clear that MDMA and MTA are responsible for inducing organ toxicity. The mechanisms underlying MDMA and MTA-induced hepatotoxicity are multifactorial, and therefore not completely understood. Recent findings indicate interference with cellular bioenergetics as an important toxicological feature of ecstasy. However, less is known about the involvement of mitochondria in MTA-induced hepatotoxicity. Thus, we compared the direct influence of MDMA and MTA on rat liver mitochondrial function [mitochondrial permeability transition (MPT), mitochondrial oxidative stress, and mitochondrial bioenergetics]. It was shown that MTA (from 0.025 up to 0.1 mM) was more potent than MDMA (from 0.2 up to 0.5 mM) in decreasing the sensitivity of rat liver mitochondria to MPT. However, higher concentrations of MTA (from 0.5 up to 2 mM) were highly toxic to mitochondria. MTA simultaneously increased H2O2 production in a monoamine oxidase (MAO)-dependent way, and uncoupled and inhibited mitochondrial respiration. In contrast, MDMA had only limited or no effects on these mitochondrial parameters. According to these results, it is possible to postulate that, depending on the concentration, MTA can potentially be more efficient in its effects on liver mitochondria than MDMA and, also, that its harmful effects may contribute to its hepatotoxicity.

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1. Introduction

Ecstasy is a common street name for 3, 4-methylenedioxymethamphetamine (MDMA), a synthetic amphetamine derivative widely used as recreational drug (Cohen, 1995). Another drug of abuse chemically related, though less common than MDMA, is the amphetamine derivative 4-methylthioamphetamine (MTA), also known as “Flatliner” (De Boer et al., 1999; Poortman and Lock, 1999; Elliott, 2001; Kirkbribe et al., 2001; Winstock et al., 2002).

MDMA produces different systemic and organ-specific toxic effects, including serotonergic neurotoxicity, hyperthermia, cardiac arrhythmias, hypoponatremia, convulsions, hepatotoxicity, and fulminant liver and renal failure (Burgess et al., 2000; Hall and Henry, 2006; Capela et al., 2009). A great deal of research, focusing on the neurotoxic effects of MDMA to clarify the mechanism(s) underlying neurotoxicity, has reported that oxidative stress, excitotoxicity, and mitochondrial dysfunction play a major role in mediating MDMA-induced neurotoxicity (Brown and Yamamoto, 2003; Quinton and Yamamoto, 2006; Capela et al., 2007a,b; Alves et al., 2007, 2009). Notice has also been taken on the hepatotoxic effects of MDMA (Henry et al., 1992; Ellis et al., 1996; Andreu et al., 1998; Beita et al., 2000). Evidence for the involvement of oxidative stress and mitochondrial dysfunction in mediating MDMA-induced hepatotoxicity comes from in vivo studies performed with rats (Beita et al., 2000; Moon et al., 2008; Pontes et al., 2008b) and from in vitro studies performed with isolated rat hepatocytes (Deita et al., 2000; Carvalho et al., 2004; Pontes et al., 2008b; Nakagawa et al., 2009) and hepatic stellate cells (Varela-Rey et al., 1999; Montiel-Duarte et al., 2002, 2004). Direct evidence that MDMA-induced hepatotoxicity is mediated through its effects at the mitochon-
drial level comes from the observations that MDMA caused: (1) ATP depletion in both short-term cultured rat hepatocytes (Beitia et al., 1999; Pontes et al., 2008b) and freshly isolated hepatocytes (Pontes et al., 2008c; Nakagawa et al., 2009), as well as a marked increase at the free calcium levels ([Ca^{2+}]_i) in short-term cultured rat hepatocytes (Beitia et al., 1999); (2) oxidative inactivation of rat liver mitochondrial proteins involved in energy supply in MDMA-exposed animals (Moon et al., 2008); (3) apoptotic cell death in hepatic stellate cells (Montiel-Duarte et al., 2002, 2004).

MTA is a new amphetamine derivative which has been associated with severe intoxications causing several deaths (De Boer et al., 1999; Elliot, 2000, 2001; De Letter et al., 2001; Declaestecker et al., 2001). However, information about organ-specific toxic effects of MTA is still scarce. In the reported MTA-related intoxications in humans, symptoms for the sympathomimetic effects of amphetamine-like compounds were observed, such as tachycardia, tremors, stomach cramps, headache and sweating (De Boer et al., 1999; Elliot, 2000; De Letter et al., 2001). In vitro studies showed that MTA induces neurotoxicity in rat hypothalamic culture cells (Hurtado-Guzmán et al., 2002), and in vivo studies showed that MTA induces hyperthermia in mice, another potentially lethal consequence of amphetamine intoxications (Carmo et al., 2003). The only evidence that MTA induced hepatotoxicity, and that this is mediated through its effect on mitochondria, comes from the observation that high MTA concentrations (1 mM) significantly reduced the ATP content in primary hepatocytes isolated from different animal species, namely human, monkey, dog, rabbit, rat and mouse (Carmo et al., 2004). So, studies of direct effects of MDMA and MTA on mitochondria seem to be important to a better understanding of their mechanisms of hepatotoxicity.

Mitochondria are intracellular organelles mainly devoted to energy production but they also play a crucial role on programmed cell death by increasing the production of reactive oxygen species (ROS) and by being prone to Ca^{2+}-induced mitochondrial permeability transition (MPT) (Belizário et al., 2007; O’Rourke et al., 2007; Yamada and Harashima, 2008). It is also increasingly recognised that mitochondria are targets for the toxicological actions induced by xenobiotics and, also, that changes on mitochondrial function have contributed to explain the side effects of several toxic compounds (Dykens and Will, 2007; Scatena et al., 2007; Neustadt and Pieczenik, 2008; Wallace, 2008).

The goal of this study was to compare the direct influence of MDMA and MTA on rat liver mitochondrial function [mitochondrial permeability transition (MPT), mitochondrial oxidative stress, and mitochondrial bioenergetics]. The concentrations of MDMA and MTA tested in this study are within the concentration range used by other investigators in in vitro studies performed with isolated rat liver mitochondria (Rusyniak et al., 2004; Nakagawa et al., 2009), isolated hepatocytes of different species (Montiel-Duarte et al., 2002; Carmo et al., 2004; Carvalho et al., 2001, 2004; Pontes et al., 2008b;c; Nakagawa et al., 2009), and hepatic stellate cells (Varela-Rey et al., 1999; Montiel-Duarte et al., 2002, 2004).

2. Materials and methods

2.1. Animals

Male Wistar rats (250–350 g), housed at 22 ± 2 °C under artificial light for 12-h light/dark cycle and with access to water and food ad libitum, were used throughout the experiments. The experiments reported here were carried out in accordance with the National Requirements for Vertebrate Animal Research and with the European Convention for the Protection of Animals used for Experimental and other Scientific Purposes.

2.2. Isolation of rat liver mitochondria

Liver mitochondria were isolated from Wistar rats, as previously described (Custódio et al., 1998). Briefly, liver was quickly removed from decapitated rats, minced and homogenized in an ice-cold homogenization medium containing 250 mM sucrose, 10 mM Hepes, pH 7.4, 1 mM EGTA, and 0.1% defatted bovine serum albumin (BSA). The homogenate was centrifuged for 10 min at 800 × g and mitochondria were recovered from the supernatant by centrifugation at 10,000 × g for 10 min. The mitochondrial pellet was washed in the homogenization medium and adjusted to pH 7.2, in the absence of EGTA and BSA. All operations were performed at 0–4 °C. Mitochondrial protein was measured by the Biuret method (Gornall et al., 1949). The purity of the mitochondrial fractions, at about 90%, was checked by electron microscopy.

2.3. Measurement of oxygen consumption

Mitochondrial oxygen consumption was monitored polarographically at 30 °C with a Clark-type oxygen electrode (Yellow Springs Instrument, Model YSI 5311) connected to a suitable recorder, in a chamber with constant stirring (Custódio et al., 1994). Liver mitochondria (1 mg) were incubated, for 3 min, in 1 ml of standard respiratory medium (130 mM sucrose, 50 mM KCl, 5 mM MgCl₂, 0.1 mM EGTA, 5 mM K_H₂PO₄, 5 mM Hepes), pH 7.4 (in the absence or presence of different concentrations of MDMA or MTA) and energised with 5 mM glutamate/2.5 mM malate, or 5 mM succinate. When succinate was used as respiratory substrate, the medium was supplemented with 2 μM rotenone. The addition of substrate to the mitochondrial suspensions induced a slight increase of the oxygen consumption rate (state 2 respiration). After 2 min of energisation, the addition of 150 nmol of ADP/ing of liver mitochondrial protein significantly increased the oxygen consumption rate (state 3 respiration). After phosphorylation of all the ADP added, the oxygen consumption rate (state 4 respiration) normally decreased to values identical to those of state 2 respiration rate. Uncoupled respiration was initiated by the addition of 1 μM p-trifluoromethoxyphenylhydrazone (FCCP) to mitochondria respiring in state 4 respiration. The respiratory control ratio (RCR), which is calculated by the ratio between state 3 respiration and state 4 respiration, is an indicator of mitochondrial membrane integrity. ADP/O ratio, which is expressed by the ratio between the amount of ADP added and the oxygen consumed for its phosphorylation, is an index of oxidative phosphorylation efficiency. Respiratory rates were calculated considering a saturation oxygen concentration of 240 μM at 30 °C and the values are expressed in percentage (%) of control.

2.4. Measurements of mitochondrial transmembrane potential (ΔΨ)

The mitochondrial transmembrane potential (ΔΨ) was estimated by an ion-selective electrode of tetrathenylphosphonium (TPP⁺) according to previously established methods (Kano et al., 1979). The ΔΨ was estimated from the following equation: ΔΨ = 59 log(v/V) − 59 log(1/v^0.5−0.9), where v, v', and ΔE stand for inner mitochondrial volume, incubation medium volume, and deflection of the electrode potential from the baseline, respectively. A mitochondrial matrix volume of 1.1 μl/mg protein was assumed. No correction was made for the “passive” binding of TPP⁺ to the mitochondria membranes because the purpose of the experiments was to show relative changes in the transmembrane potential rather than absolute values. As a consequence, we can anticipate some over-estimation for the ΔΨ values. MDMA and MTA did not affect TPP⁺ binding to mitochondria membranes or the electrode response. To monitor ΔΨ associated to mitochondrial respiration, liver mitochondria (1 mg/ml) were incubated in the standard respiratory reaction medium, supplemented with 3 μM TPP⁺ (and 2 μM rotenone if succinate is the respiratory substrate), at 30 °C, in the absence or presence of different concentrations of MDMA and MTA, for 3 min, prior to the addition of 5 mM glutamate/2.5 mM malate or 5 mM succinate to start the reaction.

To monitor ΔΨ associated to MPT, liver mitochondria (0.5 mg/ml) were incubated, for 3 min, in 2 ml of MPT reaction medium (200 mM sucrose, 10 mM Tris–Mops (pH 7.4), 1 mM KH₂PO₄, 10 μM EGTA) supplemented with 2 μM rotenone and 1 μg oligomycin and 3 μM TPP⁺, in the absence or presence of MDMA or MTA. The assays were started by the addition of 5 mM succinate followed by the addition of Ca^{2+} (150 nmol/mg protein) or Ca^{2+} (50 nmol/mg protein) plus t-BuOOH (50 μM) as described elsewhere (Cardoso et al., 2004). Control assays with Ca^{2+}, in the presence or absence of CyA (0.75 nmol/mg protein), added 3 min before or after mitochondria energisation, and control assays in the presence of t-BuOOH alone, were also performed.

2.5. Determination of mitochondrial thiol groups

A variation of Ellman’s method was used to determine the mitochondrial content in protein thiol groups (Riddles et al., 1983). At the end of the ΔΨ experiments, 1 ml of mitochondrial suspension (1 mg protein/2 ml) was added to 0.5 ml of 0.5% (w/v) trichloroacetic acid and the protein sedimented by centrifugation at 10,000 rpm for 10 min (Sigma 3-16K). The supernatant was discarded and the pellet was suspended in 1 ml of phosphate buffer and titrated (20 mM Na_H₂PO₄, 110 mM NaCl and 10 mM EDTA, pH 7.4). To 0.5 ml of this suspension 4.5 ml of phosphate buffer and a 50 μl aliquot of 10 mM 5,5'-dithiobis(2-nitro-benzoic acid) (Ellman’s reagent or DTNB) dissolved in methanol were added and mixed well. The absorbance was measured at 412 nm within 15 min.
2.6. Hydrogen peroxide generation

Hydrogen peroxide ([H$_2$O$_2$]) generation was measured fluorometrically using a modification of the method previously described (Barja, 2002). Briefly, mitochondria (0.2 mg) were incubated at 30 °C in 0.75 ml of a medium containing 5 mM KH$_2$PO$_4$, pH 7.4, 0.1 mM EGTA, 3 mM MgCl$_2$, 145 mM KCl, 30 mM Hepes, supplemented with 0.1 mM homovalinic acid, and 6 U/ml horseradish peroxidase. The reactions were started by adding 5 mM glutamate/2.5 mM malate, or 5 mM succinate. When succinate was used as a respiratory substrate, the medium was supplemented with 2 μM rotenone. The reactions were stopped at 15 min with 0.25 ml of cold 2 M glycine–NaOH (pH 12) containing 25 mM EDTA. The mitochondrial suspensions were centrifuged at 850 × g for 10 min. The fluorescence of supernatants was monitored using a Spectramax GEMINI EM fluorocytometer (Molecular Devices), at 312 nm excitation and 420 nm emission wavelengths. The hydrogen peroxide generation was calculated using a standard curve of H$_2$O$_2$. The standards and samples were incubated under the same conditions.

2.7. Statistical analysis

All the experiments were performed using three independent experiments with different mitochondrial preparations. The values are expressed as mean ± S.E. Means were compared using one-way ANOVA test, followed by the post hoc Tukey test. Statistical significance was set at $p < 0.05$.

2.8. Chemicals

All chemicals, except amphetamines, were purchased from Sigma Chemical Company (St. Louis, MO, USA). 3,4-Methylenedioxyamphetamine hydrochloride (HCl salt) was prepared and purified from high-purity tablets provided by the Portuguese Criminal Police Department. 4-Methylnoramphetamine (HCl salt) was synthesized at REQUIMTE, Toxicology Department, Faculty of Pharmacy, University of Porto. The obtained salts were pure and fully characterized by NMR and mass spectrometry methodologies. The compounds under study were dissolved in ultrapure water.

3. Results

3.1. Comparative effects of MDMA and MTA on mitochondrial permeability transition (MPT)

The effects of MDMA and MTA were evaluated measuring ΔΨ with the TPP$^+$-electrode. Fig. 1 shows the comparative effects of MDMA (from 0.2 up to 0.5 mM), and MTA (from 0.025 up to 0.1 mM) on MPT induced by: (1) inorganic phosphate (Pi) (1 mM) plus a high Ca$^{2+}$ concentration (150 nmol/mg protein) (Fig. 1A and B) and (2) inorganic phosphate (Pi) (1 mM) plus the pro-oxidant tert-BuOOH (50 (M) plus a lower Ca$^{2+}$ concentration (50 nmol/mg protein, which did not induce MPT by itself) (Fig. 1C).

In the absence of drugs (Fig. 1A and B), addition of Ca$^{2+}$ (150 nmol/mg protein) to succinate-energised mitochondria caused a transient depolarization followed by fast repolarization, at about 1 min, after Ca$^{2+}$ accumulation. This repolarization is sustained for a short lag time before irreversible ΔΨ dissipation caused by MPT. Cyclosporin A (0.75 nmol/mg protein CyA), a known MPT inhibitor (Broekemeier et al., 1989), totally blocked this effect, confirming ΔΨ dissipation induced by MPT. Treatment of mitochondria with MDMA (0.2, 0.3, 0.4 and 0.5 mM) (Fig. 1A) or MTA (0.025, 0.050 and 0.1 mM) (Fig. 1B), 3 min before the addition of succinate and Ca$^{2+}$, enlarged the lag time required for Ca$^{2+}$-induced irreversible depolarization. This means that, in the presence of these drugs, after Ca$^{2+}$ accumulation, mitochondria developed sustained ΔΨ, indicating a protective effect of the drugs on Ca$^{2+}$-induced MPT at the tested concentrations, with a higher efficiency of MTA as compared with MDMA. Just as in Fig. 1A and B, the protective effect of MTA (0.1 mM) on tert-BuOOH plus Ca$^{2+}$-induced MPT was higher than that of MDMA (0.5 mM) (Fig. 1C).

As the opening of MPT is regulated, among others, by the alteration of protein thiol redox status (−SH/−SS−), MDMA and MTA were also compared for their effects on the mitochondrial protein thiol groups. Fig. 2 shows that tert-BuOOH (50 nmol/mg protein) per se induced a significant decrease in the amount of thiol groups as compared with Ca$^{2+}$ alone (50 nmol/mg protein). This effect of tert-BuOOH was exacerbated in the presence of Ca$^{2+}$ (50 nmol/mg protein). Treatment of mitochondria with MTA (0.1 mM) or MDMA (0.5 mM), before the addition of Ca$^{2+}$ + tert-BuOOH, significantly decreased the content of thiol groups induced by Ca$^{2+}$ + tert-BuOOH. At the concentrations tested, no significant difference was observed between the two drugs.
3.2. Comparative effects of MDMA and MTA on mitochondrial oxidative stress

MDMA and MTA (from 0.05 up to 0.5 mM) were compared for their effects on mitochondrial oxidative stress by evaluating H$_2$O$_2$ generated by mitochondria (Figs. 3 and 4).

In the absence of exogenous respiratory substrates, both MDMA and MTA stimulated mitochondrial H$_2$O$_2$ generation in a concentration dependent manner (Fig. 3). Again the effect of MTA was more potent than MDMA (at 0.5 mM, the stimulatory effect was at about 645% and 260% for MTA and MDMA, respectively). This effect was significantly reduced by catalase to levels below those
observed in the absence of MDMA or MTA (more than 80% inhibition), considering this as an H2O2 assay control. Similar results were obtained for the effects of MDMA and MTA on H2O2 production when mitochondria were energised by the addition of the exogenous respiratory substrates glutamate/malate (Fig. 3B) or succinate (Fig. 3C). The maximal stimulatory effect of MTA (0.5 mM) on mitochondrial H2O2 generation was also analysed when the respiratory chain activity was inhibited by its classic inhibitors, namely 2 μM rotenone (complex I inhibitor), 0.5 μg/ml antimycin A (complex III inhibitor), 1 mM cyanide, and 2.5 mM sodium azide (complex IV inhibitors) (Fig. 4A). Rotenone and sodium azide produced no significant effect, but antimycin A increased the level of H2O2 production either in the absence or presence of MTA. In contrast, it was observed that cyanide inhibited the MTA effect (Fig. 4A). However, this is not a significant effect since we observed that cyanide interferes with the assay used to measure H2O2 (results not shown).

As both drugs stimulated mitochondrial H2O2 generation in the absence of exogenous respiratory substrates, the stimulatory effect of MTA (0.5 mM) on mitochondrial H2O2 generation was also analysed in the presence of the classic inhibitors of monoamine oxidases (MAO); selegiline (MAO-B inhibitor), pargyline (MAO-A and MAO-B inhibitor), and clorgyline (MAO-A inhibitor). This effect was significantly decreased (at about 70%) by selegiline (10 μM) and pargyline (10 μM), but some stimulation was detected with 1 μM clorgyline (Fig. 4B).

3.3. Comparative effects of MDMA and MTA on rat liver mitochondrial bioenergetics

MDMA and MTA (from 0.5 up to 2 mM) were compared for their effects on mitochondrial bioenergetics by evaluating several respiratory chain mitochondrial parameters [state 3, state 4, FCCP-stimulated respiration (FCCP-uncoupled respiration), respiratory control ratio (RCR), ADP/O ratio, mitochondrial transmembrane potential (Δψ), and phosphorylation rate] using both glutamate/malate and succinate as respiratory substrates.

The effects of MTA on glutamate/malate-supported respiratory rates are depicted in Fig. 5A and B. MTA concentrations of up to 0.5 mM did not significantly affect state 3, state 4 and FCCP-uncoupled respirations (Fig. 5A). However, for concentrations higher than 0.5 mM, it significantly inhibited state 3 and FCCP-uncoupled respirations, and stimulated state 4 respiration (Fig. 5A). At 2 mM, MTA inhibited state 3 respiration by about 50%, FCCP-uncoupled respiration by about 90% and stimulated state 4 respiration at about 240% (Fig. 5A). At the same concentration, MTA significantly inhibited RCR (from about 5.5 to about 2.5) but it did not significantly affect ADP/O ratio (Fig. 5B).

The effects of MTA on succinate-supported respiratory rates, in state 3 and state 4 respiration, and respiratory indices (RCR and ADP/O ratio) of rat liver mitochondria were similar to those described above for glutamate/malate. However, in contrast to the significant inhibitory effect with glutamate/malate, MTA did not significantly affect succinate-supported FCCP-uncoupled respiration (results not shown).

The effects of MTA on mitochondrial transmembrane potential (Δψ) and phosphorylation time supported by glutamate/malate are shown in Fig. 6. MTA concentrations higher than 0.5 mM dissipated Δψ and decreased the phosphorylation rate. Treatment of liver mitochondria with MDMA concentrations of up to 2 mM did not significantly affect state 3 and state 4 respiration rates, using glutamate/malate or succinate as the respiratory substrates. With MDMA, the respiratory indices (RCR and ADP/O ratio), the Δψ, and the ADP phosphorylation rates were also not affected (results not shown).

4. Discussion

Considering the effects that have been demonstrated for MDMA and MTA, commonly used as recreational drugs, we delineated our efforts to compare their actions on rat liver mitochondrial function, namely, mitochondrial permeability transition (MPT), mitochondrial oxidative stress, and mitochondrial bioenergetics.

The concentrations of MDMA and MTA used in our experiments (from 0.05 up to 2 mM) are significantly higher than the peak plasma levels found in fatalities implicating “ecstasy” ingestion: from 0.17 μM to 80 μM MDMA (De Letter et al., 2004), and from 0.41 μM to 14.5 μM MTA (Elliott, 2000, 2001). However, we must take into consideration that both drugs accumulate in the intracellular compartments, and it must be assumed that blood levels grossly underestimate intracellular concentrations. In fact, it has been reported that there are many factors affecting MDMA plasma concentration and, as such, plasma MDMA level, per se, is not a reliable predictor of acute MDMA-induced medical complications (Irwin et al., 2006).

Our results showed that MTA (from 0.025 up to 0.1 mM) was more efficient than MDMA (from 0.2 up to 0.5 mM) in protecting mitochondria against MPT induction by high concentrations of Ca2+ (Fig. 1A) and by the pro-oxidant t-BuOOH plus low concentrations of Ca2+ (Fig. 1B). A critical factor for induction of MPT is the oxidation of protein thiol groups of the MPT complex, creating diethyl cross-links (Costantini et al., 1996, 1998; Halestrap et al., 1997; McStay et al., 2002). Therefore, the most plausible hypothesis to explain the partial MPT protection induced by these drugs is concerned with changes on the redox-state of protein thiol groups of the MPT complex avoiding diethyl cross-links. This assumption is supported by the observation that 0.1 mM MTA was more efficient than 0.5 mM MDMA in protecting from the oxidation of protein thiol groups dependent on MPT induction (Fig. 2).

We also demonstrated that, in the concentration range of 0.05 of up to 0.5 mM, MTA is a more efficient pro-oxidant than MDMA, as revealed by its capacity to increase mitochondrial H2O2 formation (Fig. 3). It is currently believed that the majority of the H2O2 generated by mitochondria results primarily from dismutation of the superoxide anion (O2-*) produced at the complexes I and III levels of the respiratory chain (Murphy, 2009). However, mitochondrial H2O2 produced by MTA is not directly related to the electron transport chain. This was demonstrated by the fact that: (1) low concentrations of MTA (from 0.05 up to 0.5 mM) did not affect mitochondrial bioenergetics using either glutamate/malate or succinate as respiratory substrates (Figs. 5 and 6), an observation which corresponds well with data in literature showing that 100 μM MTA did not affect the ATP cellular content of hepatocytes obtained...
from several species (Carmo et al., 2004) and (2) mitochondrial 
H$_2$O$_2$ generation by MTA was not affected either by the presence 
of exogenous mitochondrial respiratory substrates (Fig. 3A–C) or 
by the treatment of mitochondria with the specific inhibitors of 
the mitochondrial respiratory chain, namely rotenone, antimycin 
A, and sodium azide (Fig. 4A).

Another important mitochondrial source of H$_2$O$_2$ is monoamine oxidase (MAO), a flavoprotein localized on the outer mitochon-
drial membrane (Orrenius et al., 2007; Di Lisa et al., 2009a,b). We 
observed that the stimulatory effect of MTA on H$_2$O$_2$ production 
was depressed by pargyline (a MAO-A and MAO-B inhibitor) and 
by seleagine (a MAO-B inhibitor), but not by clorgyline (a MAO-
A inhibitor) (Fig. 4B), indicating that MTA-induced mitochondrial 
H$_2$O$_2$ generation is mediated through MAO-B activity. However,
MTA-induced mitochondrial H$_2$O$_2$ generation is not a result of its 
obstructive deamination catalysed by mitochondrial MAO-B because 
the alpha carbon does not allow the metabolism of amphetamine 
substrates to MAOs. In addition to their actions as MAO-B inhibitors, seleagine 
and other propargylamines have antioxidant effects by acting 
like Cu/Zn superoxide dismutase, Mn superoxide dismutase and 
glutathione peroxidase (Tatton et al., 2003). Therefore, the hypoth-
esis that these compounds depressed MTA-induced mitochondrial 
H$_2$O$_2$ generation by acting as antioxidants cannot be excluded. The 
possibility that these compounds depressed MTA-induced mitochondrial 
H$_2$O$_2$ generation is mediated through MAO-B activity. However, 
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protective action in the respiratory chain complex I is more sensi-
tive to MTA than complex II. This effect was confirmed by using 
leaky mitochondria (frozen and thawed three times) respiring 
NADH (results not shown). However, it is difficult to explain 
why glutamate/malate dependent mitochondrial respiratory rate 
on state 3 is significantly less affected (50%) as compared with 
glutamate/malate dependent FCCP-uncoupled respiration (90%) 
(Fig. 3A). It seems that the inhibitory action induced by MTA, at the 
level of complex I of the respiratory chain, is more efficient when total 
$\Delta \Psi$ dissipation occurs.

In contrast to MTA, MDMA did not significantly affect rat liver 
mitochondrial bioenergetics either using glutamate/malate or succ-
inate as the respiratory substrates (results not shown). These 
data are in line with the results published by others (Rusyniak 
et al., 2004). Evidence for uncoupling of mitochondrial oxidative 
phosphorylation, revealed by an increase in state 4 respiration, a 
decrease in state 3 respiration, and the corresponding decreases 
in the RCR, $\Delta \Psi$ and phosphorylation efficiency, without affecting 
the ADP/O ratio (Figs. 5 and 6). MTA concentrations higher than 
0.5 mM also significantly inhibited glutamate/malate-supported 
FCCP-uncoupled respiration, without having a significant effect 
on succinate-supported FCCP-uncoupled respiration. This suggests 
that mitochondrial respiratory chain complex I is more sensi-
tive to MTA than complex II. This effect was confirmed by using 
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In conclusion, our data provided evidence that both MDMA 
(from 0.2 up to 0.5 mM) and MTA (from 0.025 up to 0.1 mM) pro-
tected rat mitochondria against MPT, but MTA was more efficient 
than MDMA. However, MTA (from 0.5 up to 2 mM) simultaneously 
increased mitochondrial H$_2$O$_2$ production in an M AO-dependent 
way. On the other hand, higher concentrations of MTA (0.5 mM) 
uncoupled and inhibited mitochondrial respiration, indicating 
a high level of toxicity. Under the same concentration range, MDMA 
effects on mitochondrial H$_2$O$_2$ production and respiration were not 
very significant. Therefore, it is possible to postulate that, depend-
ing on the concentration, MTA can potentially be more efficient 
in its effects on liver mitochondria than MDMA and also, that its 
harmful effects may contribute to its hepatotoxicity.

Conflicts of interest

None.

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Nakagawa et al., 2009) and in hepatic stellate cells exposed to 
MDMA (Varela-Rey et al., 1999; Montiel-Duarte et al., 2004).

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