

3,4-Methylenedioxymethamphetamine (“Ecstasy”) induces apoptosis of cultured rat liver cells

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Abstract

“Ecstasy” (3,4-methylenedioxymethamphetamine, MDMA) has been shown to be hepatotoxic for human users, but molecular mechanisms involved in this effect remained poorly understood. MDMA-induced cell damage is related to programmed cell death in serotonergic and dopaminergic neurons. However, until now there has been no evidence of apoptosis induced by MDMA in liver cells. Here we demonstrate that exposure to MDMA caused apoptosis of freshly isolated rat hepatocytes and of a cell line of hepatic stellate cells (HSC), as shown by chromatin condensation of the nuclei and accumulation of oligonucleosomal fragments in the cytoplasm. In both cell types, apoptosis correlated with decreased levels of bcl-x_L, release of cytochrome *c* from the mitochondria and activation of caspase 3. In HSC, but not in hepatocytes, MDMA induced poly(ADP-ribose)polymerase (PARP) proteolysis. These results suggest that apoptosis of liver cells could be involved in the hepatotoxicity of MDMA.

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1. Introduction

3,4-Methylenedioxymethamphetamine (MDMA, “Ecstasy”) is a derivative of amphetamine that has become extensively used as a recreational drug. Although MDMA has been popularly considered as a safe drug, there are increasing evidences of its toxicity. MDMA is neurotoxic for serotonergic and dopaminergic neurons and produces a decrease of 5-HT transporters [1,2]. Other side-effects of MDMA are hyperthermia, rhabdomyolysis and cardiac dysrhythmias [3,4]. Severe liver injury and hepatitis in the presence or absence of systemic features have also been described in MDMA users. MDMA hepatotoxicity is idiosyncratic and appears with different intensity, from mild hepatitis that resolves spontaneously, to fulminant liver failure requiring liver transplant. The latency is also variable, raging from days to weeks after the intake [5–9].

Programmed cell death is probably involved in the neurotoxic effects of MDMA, since MDMA induces apoptosis of serotonergic and neocortical neurons [10,11] and immortal-

ized neuron cells [12] through regulation of proteins belonging to the bcl-2 family. However, the underlying mechanisms for the toxic effect of MDMA in liver cells are not fully established yet. MDMA has been shown to produce a decrease in the viability of freshly isolated hepatocytes [13] and to induce collagen production in hepatic stellate cells (HSC) [14]. To our knowledge, the possibility of MDMA exerting an apoptotic effect in liver cells as a mechanism involved in liver damage elicited by this drug, has not been studied until now.

In the present study, the ability of MDMA to induce programmed cell death of freshly isolated rat hepatocytes and a cell line of HSC has been examined. MDMA was found to exert a pro-apoptotic effect in both cell types, as demonstrated by biochemical and morphological parameters.

2. Materials and methods

2.1. Reagents

MDMA–HCl was a gift from the “Audiencia Provincial de Navarra”. Tumor necrosis factor α (TNF- α) was from Boehringer Mannheim (Mannheim, Germany); Actinomycin

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D (ActD) was purchased from Sigma (St. Louis, MO); Ac-DEVD-CHO, Hoechst 33342 and camptothecin were from Calbiochem (La Jolla, CA). Cell culture reagents were from Gibco BRL (Grand Island, NY). Poly(ADP-ribose)polymerase (PARP), bcl-x_L polyclonal rabbit and bax monoclonal mouse antibodies were from Santa Cruz Biotechnologies (Santa Cruz, CA). Cytochrome *c* monoclonal antibody was from BD PharMingen.

2.2. Cell culture and treatments

The HSC cell line CFSC-2G [15] was used for some experiments. Cells were cultured in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and nonessential amino acids for 36 h; after which the medium was replaced with a serum-free medium. Treatments were carried out 12 h later.

Freshly isolated hepatocytes were obtained by liver collagenase perfusion from male Wistar rats as described elsewhere [16]. Isolated hepatocytes were resuspended and plated in MEM supplemented with 10% FBS and nonessential amino acids. Four hours after plating the cells the medium was replaced by MEM supplemented with 1% FBS, and treatments were carried out 12 h later.

Cells were treated with the indicated concentrations of MDMA for 8 or 24 h. In some experiments hepatocytes were treated with camptothecin (10 µg/ml) or TNF-α (10 ng/ml) for 8 h. Prior to the addition of TNF-α, cells were sensitized by a 30-min pre-treatment with ActD (15 ng/ml).

2.3. Nuclear staining

Cell nuclei were visualized using the DNA-binding fluorochrome Hoechst 33342. Cells were fixed with methanol for 8 min at –20 °C. After washing with PBS, the cells were incubated at room temperature for 5 min with Hoechst 33342 (0.5 µg/ml), gently washed three times with PBS, embedded in glycerol/PBS (1:1) and examined under UV light on a fluorescence microscope Labophot-2 (Nikon).

2.4. Determination of oligonucleosomal (histone-associated) DNA fragments

The presence of soluble histone–DNA complexes, was measured by the Cell Death Detection Assay (Boehringer Mannheim). For this assay, HSC were seeded on 24-well plates at a density of 80,000 cells/well and hepatocytes in 35-mm culture dishes at a density of 3×10^6 cells/dish. In some cell cultures Ac-DEVD-CHO (0.1 mM) was added 3 h before treating hepatocytes and HSC with 1.0 and 5.0 mM MDMA, respectively. Cell death ELISA assays were performed according to the manufacturer's instructions. Specific enrichment of mono- and oligonucleosomes released into the cytoplasm (enrichment factor, EF) was calculated as

the ratio between the absorbance values of the samples obtained from treated and control cells.

2.5. Western blot

After treatment with MDMA, cells were washed with PBS and whole-cell lysates were obtained. Equal amounts of protein were size-fractionated by 10% (PARP) or 12% (bcl-x and bax) sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to nitrocellulose membranes. The membranes were incubated with anti-PARP, anti-bcl-x polyclonal rabbit or anti-bax monoclonal mouse antibodies, diluted 1:2000; and for 1 h with either anti-rabbit immunoglobulin horseradish peroxidase conjugate (Promega, Madison, WI) or anti-mouse immunoglobulin horseradish peroxidase conjugate (Amersham Pharmacia Biotech), diluted 1:4000. Bound antibodies were detected by enhanced chemiluminescence autoradiography with ECL-Plus (Amersham Pharmacia Biotech). Equivalent loading was confirmed by Coomassie staining of an identical gel.

Cytochrome *c* was analyzed in cytosolic and mitochondrial fractions obtained by differential centrifugation in 250 mM sucrose buffer as described previously [17]. Five micrograms of protein were subjected to 15% SDS-PAGE. Membranes were exposed to mouse anti-cytochrome *c* monoclonal antibody at a 1:1000 dilution, followed by incubation with anti-mouse immunoglobulin horseradish peroxidase conjugate at a 1:2000 dilution.

2.6. Measurement of caspase 3 activity

Caspase 3 activity was measured using the Caspase-3/ CPP32 Colorimetric Assay Kit (BioVision, Palo Alto, CA, USA). Cells (3×10^6 hepatocytes or 2×10^6 HSC) were scraped in culture medium, pelleted, resuspended in lysis buffer and caspase 3 activity was measured following manufacturer's instructions.

2.7. Statistical analysis

The data were analyzed using the Kruskal–Wallis test to determine differences between all independent groups. When significant differences were obtained ($P < 0.05$), differences between two groups were tested using the Mann–Withney *U* test.

3. Results

3.1. MDMA induces morphological changes and chromatin condensation in hepatocytes and HSC

The morphological effects of MDMA on hepatocytes and HSC were first evaluated by light microscopy. Hepatocytes exposed to 1.0 mM MDMA showed an

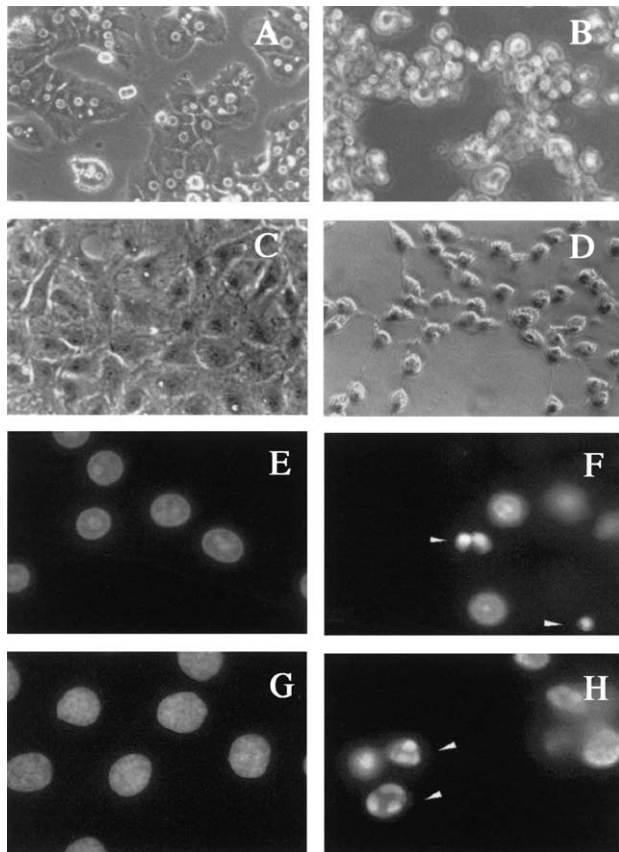


Fig. 1. Morphology of hepatocytes and HSC after treatment with MDMA. (A–D) Morphology by light microscopy of control and MDMA-treated hepatocytes and HSC. Original magnification $\times 200$. (E–H) Nuclear staining of hepatocytes and HSC using Hoechst 33342. Arrowheads point to chromatin condensation and fragmented nuclei in MDMA-treated cells. Original magnification $\times 1000$. (A, E) Control hepatocytes. (B, F) Hepatocytes incubated for 8 h with 1.0 mM MDMA. (C, G) Control HSC. (D, H) HSC incubated for 24 h with 5.0 mM MDMA.

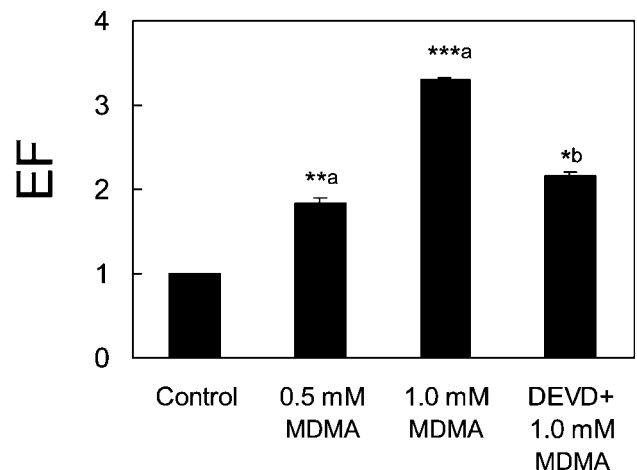
altered morphology 8 h after treatment. As shown in Fig. 1B, MDMA-treated hepatocytes presented loss of membrane differentiations and were highly refringent, as compared to untreated cells. HSC required a longer treatment (24 h) and a higher dose of MDMA (5.0 mM) to show morphological modifications. HSC acquired a netlike disposition and cells became round-shaped and refringent in response to MDMA treatment (Fig. 1D). The concentrations of MDMA used were similar to those described in toxicity studies carried out either in neurons or hepatocytes [11,13].

In order to visualize the nuclei of the cells, preparations of hepatocytes and HSC were subjected to the same treatments as above and stained with Hoechst 33342. In both cell types, MDMA treatment resulted in chromatin condensation and nuclear fragmentation (Fig. 1F and H), alterations commonly associated with programmed cell death [18].

3.2. Accumulation of oligonucleosomal fragments in the cytoplasm of hepatocytes and HSC in response to MDMA treatment

The cytoplasmic levels of DNA (histone-associated) oligonucleosomal fragments in cells treated for 24 h with different concentrations of MDMA were determined. The presence of these fragments in the cytoplasm reflects the extent of DNA fragmentation and nuclear disruption that are characteristic of apoptosis [19].

A Hepatocytes



B HSC

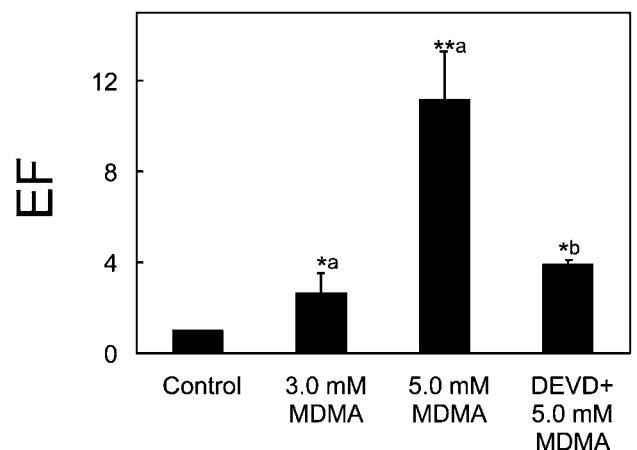


Fig. 2. Determination of oligonucleosomal fragments in cytoplasmic extracts from hepatocytes and HSC treated with MDMA. Hepatocytes and HSC were treated for 24 h with the indicated concentrations of MDMA. Oligonucleosomal fragments content was expressed as EF, as described in Materials and methods. In some cell cultures Ac-DEVD-CHO was added before treating hepatocytes and HSC with 1.0 and 5.0 mM MDMA, respectively. Each bar represents the mean $\pm$ S.D. of quadruplicate determinations from at least two independent experiments. (A) EF of hepatocytes treated with MDMA (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; a, vs. control; b, vs. 1.0 mM MDMA). (B) EF of HSC treated with MDMA (* $P < 0.05$, ** $P < 0.01$; a, vs. control; b, vs. 5.0 mM MDMA).

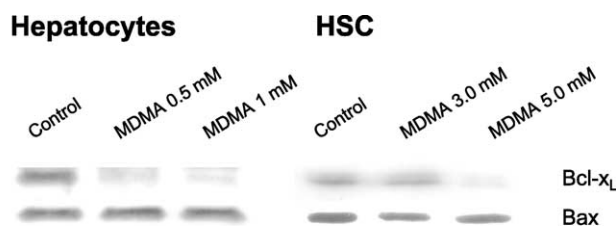


Fig. 3. Western Blot analysis of bcl-x_L and bax in MDMA-treated hepatocytes and HSC. Hepatocytes and HSC were treated for 8 h with the indicated concentrations of MDMA.

As shown in Fig. 2, values of two- to threefold increase in the cytoplasmic content of oligonucleosomal fragments were obtained in hepatocytes treated with 0.5 and 1.0 mM MDMA, and three- to tenfold increase in HSC treated with 3.0 and 5.0 mM MDMA. In some experiments hepatocytes were exposed to higher concentrations of MDMA (3.0 and 5.0 mM), but despite the fact that chromatin condensation and nuclear fragmentation were observed, no significant accumulation of oligonucleosomal fragments was detected (data not shown). A post-apoptotic necrosis resulting in the degradation of the histone–DNA complexes could explain this finding.

The cytoplasmic accumulation of oligonucleosomal fragments was diminished in both cell types by pre-treatment with the inhibitor of the caspase 3 family Ac-DEVD-CHO (Fig. 2), suggesting the involvement of caspases in the cleavage of DNA associated with MDMA-induced apoptosis.

3.3. Bcl-x_L and bax protein levels in hepatocytes and HSC treated with MDMA

Bcl-2 family members are proteins that modulate apoptosis mainly by controlling the release of pro-apoptotic

factors from the mitochondrial intermembrane to the cytoplasm [20]. These proteins are regulated by different molecular mechanisms, including changes in protein levels through transcriptional and post-transcriptional alterations. Since MDMA has been shown to modify mRNA levels of bcl-x_L and bax in neurons [11], we studied if these proteins were as well altered in liver cells exposed to the drug.

Protein levels of bcl-x_L and bax were analyzed by Western blot after treating the cells for 8 h with MDMA at the previously tested concentrations (0.5 and 1.0 mM for hepatocytes, and 3.0 and 5.0 mM for HSC). The anti-apoptotic protein bcl-x_L was decreased by MDMA treatment both in hepatocytes and HSC. However, protein levels of bax, a pro-apoptotic member of the bcl-2 protein family, remained unchanged in both cell types after treatment with MDMA (Fig. 3).

3.4. Cytochrome *c* release, caspase 3 activity and PARP proteolysis in hepatocytes and HSC treated with MDMA

Cytochrome *c* release from the mitochondria has been described as a central event in the stress-induced apoptotic pathway [21]. Cytochrome *c* release leads to the activation of downstream effector caspases, which cleave a number of cellular proteins facilitating DNA fragmentation and cell death [22]. We evaluated by Western blot the cytochrome *c* levels in cytoplasmic and mitochondrial extracts obtained from hepatocytes and HSC treated for 8 h with MDMA, at the concentrations previously indicated. MDMA treatment of both cell types resulted in increased cytosolic levels of cytochrome *c* that correlated with decreased levels of the protein in the mitochondria (Fig. 4). Since cytochrome *c* release has been related to caspase 3 activation [23], we also analyzed the activity of this enzyme in hepatocytes and HSC exposed to MDMA as above, to evaluate its involvement in MDMA-induced apoptosis. In both cell types, MDMA

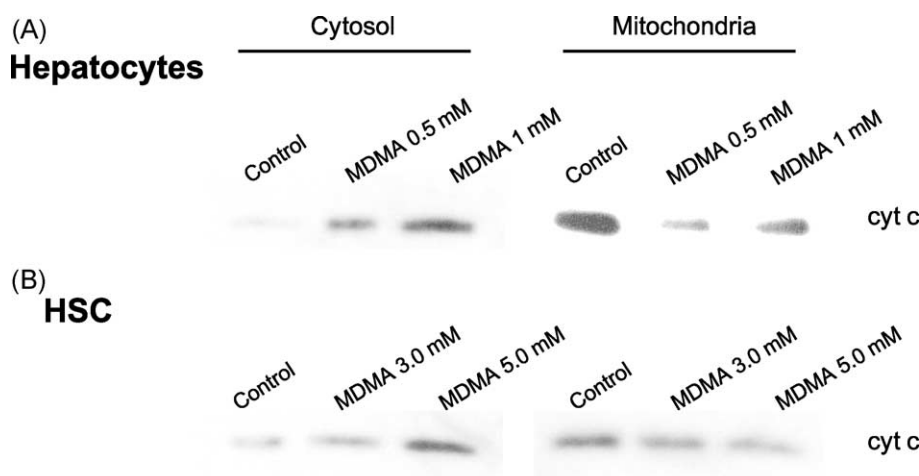
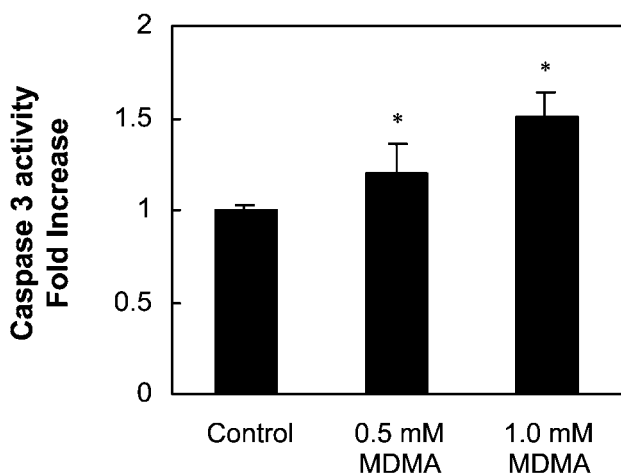


Fig. 4. Cytochrome *c* release from the mitochondria in MDMA-treated hepatocytes and HSC. Cells were treated for 8 h with the indicated concentrations of MDMA and cytosolic and mitochondrial extracts obtained. Western blot analysis using a monoclonal cytochrome *c* antibody was performed in extracts from hepatocytes (A) or HSC (B).

caused a moderate but significant increase in caspase 3 activity, which was assessed using a commercial colorimetric kit (Fig. 5).

PARP degradation by caspases is considered a biochemical marker for the execution phase of the apoptotic response [24]. PARP cleavage was evaluated in extracts from MDMA-treated cells by the appearance of the 85-kDa proteolytic fragment and the disappearance of the 116-kDa intact PARP, both of them recognized by the same antibody. As Fig. 6B shows, HSC presented an increased degradation of PARP in response to treatment with 3.0 and 5.0 mM MDMA for 8 h. On the contrary, MDMA treatment of

A Hepatocytes



B HSC

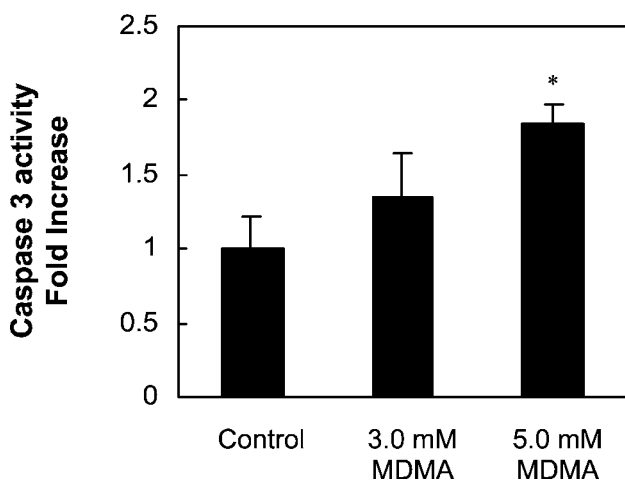
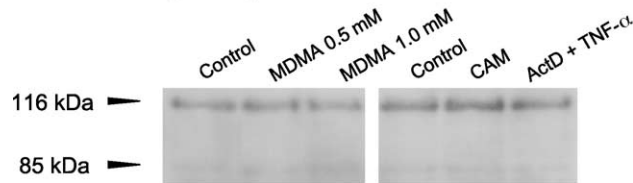


Fig. 5. Caspase 3 activity in hepatocytes and HSC treated with MDMA. Caspase 3 activity was assessed as described in Materials and methods, using 150 μ g of protein extracts from hepatocytes (A) or HSC (B), treated with the indicated concentrations of MDMA for 8 h. Activity is referred to control, nontreated cells. Each bar represents the mean $\pm$ S.D. of quadruplicate determinations (* $P < 0.05$ vs. control).

A Hepatocytes



B HSC

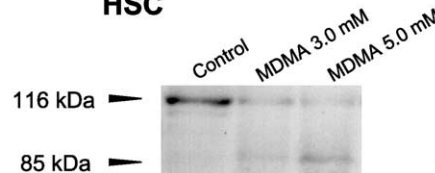


Fig. 6. Western Blot analysis of PARP in MDMA-treated hepatocytes and HSC. Hepatocytes and HSC were exposed for 8 h to treatment with the indicated concentrations of MDMA. Hepatocytes were also treated with camptothecin and ActD/TNF- α , as described in Materials and methods. Arrows indicate the 116-kDa band (intact PARP) and the 85-kDa band (PARP proteolytic fragment) from hepatocytes (A) or HSC (B), treated with the indicated apoptotic inducers.

primary hepatocytes for 8 h did not result in an increased degradation of PARP, as compared to samples obtained from untreated cells. Two widely used apoptotic inducers, such as camptothecin and ActD/TNF- α [25], also failed to enhance PARP proteolysis in this cell type (Fig. 6A).

4. Discussion

Apoptosis is a highly regulated process involved in an array of physiological and pathological responses, including cell damage associated with toxic substances. In the liver, apoptosis has been shown to be responsible for damage induced by endotoxic shock [26], copper accumulation in Wilson's disease [27] and hypoxia/reoxygenation injury [28]. Since MDMA had been described as a pro-apoptotic agent for neurons [10–12], we sought to investigate whether apoptosis of liver cells could contribute to the hepatotoxic effect of this drug, focussing on two of the cell types present in the liver, hepatocytes and HSC. MDMA was found to exert a pro-apoptotic effect in both cell types, as determined by morphological and biochemical alterations commonly associated with programmed cell death, such as chromatin condensation of the nuclei and accumulation of oligonucleosomal fragments in the cytoplasm. The involvement of caspases in the fragmentation of DNA induced by MDMA was demonstrated by pre-treatment of both cell types with the caspase 3 family inhibitor Ac-DEVD-CHO, which significantly prevented the cytoplasmic accumulation of oligonucleosomal fragments in both cell types. This inhibitory effect could be due to the participation of a caspase-activated DNase [29] in the apoptotic action of MDMA.

Previous data have shown that D-amphetamine, a drug structurally related to MDMA, can increase apoptosis in preneoplastic and neoplastic nodules in an animal model for hepatocellular carcinoma [30]. However, as the authors indicate, in these lesions liver cells are already undergoing high constitutive apoptosis as a result of treatment with a chemical agent, and are more sensitive to other substances. Our data show that MDMA is an apoptotic inducer for normal liver cells in the absence of other agents. This fact could be related to the increasing reports of severe liver damage caused by this particular derivative of amphetamine.

Proteins belonging to the bcl-2 family seem to play a key role in the apoptotic response triggered by derivatives of amphetamines. MDMA induces changes in the expression of the splice variants of the bcl-x gene in neurons [11], D-amphetamine increases basal apoptosis of neoplastic liver lesions through dysregulation of bcl-2 family genes [30], and an increased expression of bcl-2 can prevent apoptosis of immortalized neuron cells induced by methamphetamine [12]. Therefore, we sought to investigate whether alterations in the expression of members of the bcl-2 family could also be involved in the apoptotic effect of MDMA in normal liver cells. We found a similar pattern of alterations in the proteins obtained either from hepatocytes or HSCs treated with MDMA. The anti-apoptotic member of the bcl-2 family, bcl-x_L, was diminished in MDMA-treated cells, while no changes were elicited by MDMA in the levels of the pro-apoptotic protein bax. Similar results have been described in neurons exposed to MDMA [11]. The fact that MDMA diminished bcl-x_L protein levels pointed out to mitochondria as a target for its pro-apoptotic effect, since bcl-2 family proteins have been shown to modulate the permeabilization of mitochondrial membranes and the subsequent liberation of pro-apoptotic factors such as cytochrome *c* [20]. MDMA treatment caused the release of cytochrome *c* in both cell types, as well as caspase 3 activation, thus suggesting the sequence of events already described for other apoptotic agents: alteration of mitochondrial membranes through changes in bcl-2-like proteins, release of cytochrome *c* and activation of effector caspases. The previously reported role of MDMA as an inducer of cellular oxidative stress [31], could explain these findings because apoptosis caused by redox disruption has been shown to happen in many instances through alterations in mitochondria [32]. PARP is a common substrate of caspases, mainly those belonging to caspase 3 family. In HSC, apoptosis induced by MDMA was accompanied by PARP proteolysis. On the contrary, in primary rat hepatocytes, neither MDMA nor other apoptotic agents such as camptothecin and ActD/TNF- α induced the proteolysis of PARP. In accordance with these findings, some authors find that caspase activation induced by several apoptotic stimuli does not result in proteolysis of PARP in hepatocytes [33,34].

We have previously described that exposure of HSC to MDMA produces a pro-fibrogenic effect, increasing the

expression of $\alpha 1(I)$ procollagen mRNA [14]. In the present study, higher concentrations of the drug that reduced viability of HSC by inducing programmed cell death were used. Therefore, we can deduce from our previous and present data that there is a double response of HSC to MDMA exposure: induction of either fibrogenesis or programmed cell death, depending on the dose of the drug. These responses could reflect differences between chronic hepatotoxicity or fibrosis and acute liver failure caused by MDMA. However, further investigation is required to determine the correlation between our results, either in hepatocytes or HSC, and the *in vivo* hepatotoxic action of MDMA. The doses of MDMA used in our cell culture experiments (0.5 to 5.0 mM) are significantly higher than peak plasma levels found in animal models for the neurotoxic effect of MDMA (15 μ M) [35], but, to our knowledge, plasma levels of MDMA have not been determined in animal models for MDMA-induced liver damage, and therefore cannot be properly compared. Interestingly, in the aforementioned work MDMA levels in the brain have been found to be higher than plasma levels, reaching values of 0.1–0.25 mM [35]. A similar effect could happen in other target organs for MDMA like the liver. The study of the mechanisms of hepatotoxicity induced by MDMA, both in cellular and animal models, is particularly interesting due to the key role played by the liver as the organ responsible for detoxification.

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