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Role of reactive oxygen species, glutathione and NF-κB in apoptosis induced by 3,4-methylenedioxymethamphetamine (''Ecstasy'') on hepatic stellate cells

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Abstract

''Ecstasy'' (3,4-methylenedioxymethamphetamine, MDMA), is a derivative of amphetamine with hepatotoxic effects that has been shown to induce apoptosis of cultured liver cells. In the present work, we studied the role played by oxidative stress in the apoptotic response caused by MDMA on a cell line of hepatic stellate cells (HSC). MDMA-treatment provoked oxidative stress determined as reactive oxygen species (ROS) accumulation and decrease of intracellular reduced glutathione levels. Pre-treatment with the antioxidant pyrrolidine dithiocarbamate blocked ROS production but did not prevent MDMA-induced apoptosis of HSC. The pro-oxidant menadione induced in HSC ROS production and apoptosis that were prevented by pyrrolidine dithiocarbamate, showing HSC to be susceptible to oxidative stress-induced apoptosis. Addition of exogenous GSH or its precursor NAC potentiated the apoptotic action of MDMA but blocked apoptosis induced by menadione. Pre-treatment of HSC with the cytochrome P450 inhibitor quinine diminished the extent of apoptosis caused by MDMA, suggesting the involvement of a metabolic derivative of MDMA on its apoptotic effect. Nuclear factor NF-κB was activated by MDMA in a oxidative stress independent fashion and played a protective role in the apoptotic response, since inhibition of NF-κB by treatment with parthenolide or by viral infection with a dominant-negative form of NIK (Ad5dnNIK) resulted in an increase of MDMA-induced cell death. In summary, MDMA-induced apoptosis of HSC is accompanied, but not caused by oxidative stress; a metabolic derivative of the drug is responsible for the apoptotic effect of MDMA, which is partially blocked by NF-κB activation.
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Keywords: MDMA; Apoptosis; Hepatic stellate cells; Glutathione; Reactive oxygen species; NF-κB

1. Introduction

Apoptosis is an endogenous cell death program that can be triggered by different stimuli and is characterized by morphological features such as reduction in cell volume, membrane blebbing, chromatin condensation and nuclear DNA fragmentation [1,2]. A family of specific cysteine proteases, caspases, plays a key role in the apoptotic response [3]. There are reports suggesting that oxidative stress is involved in the induction of programmed cell death in some systems. Addition of oxidants like hydrogen peroxide or menadione can lead to cell death by apoptosis [4,5], and an increased production of reactive oxygen species (ROS) seems to be a critical step for apoptosis associated with ionizing radiation or chemotherapeutic drugs [6–8]. Moreover, antioxidants such as *N*-acetylcysteine (NAC) and reduced glutathione (GSH) can block apoptosis induced by agents different from oxidants [9,10]. Mitochondria could be involved in some of these events, since they are both a source of ROS and a target for ROS-induced toxicity, and mitochondria disfunction has been proposed as one of the pathways leading to caspase activation and cell death [11].

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Abbreviations: MDMA, 3,4-methylenedioxymethamphetamine; GSH, glutathione; HSC, hepatic stellate cells; PDTC, pyrrolidine dithiocarbamate; NAC, *N*-acetylcysteine; MEM, minimum essential medium; FBS, fetal bovine serum; BSO, buthionine sulfoximine; CM-H_2DCFDA, 5-6-chloromethyl-2′,7′-dichlorohydrofluorescein diacetate; ROS, reactive oxygen species; NIK, NF-κB-inducing kinase; DHMA, 3,4-dihydroxymethamphetamine.

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3,4-Methylenedioxymethamphetamine (MDMA) is a synthetic derivative of amphetamine that can be toxic for brain [12,13] and liver cells [14]. Cell damage caused by MDMA in several *in vitro* and *in vivo* models seems to be mediated by the induction of oxidative stress. Exposure to MDMA leads to a depletion in GSH levels that correlates with increased lipid peroxidation and cell damage [14–16], and the enzyme superoxide dismutase or NAC prevent some toxic effects of MDMA [17,18]. Other molecular mediators that can be responsible for MDMA-induced cell damage are nitric oxide [19] and metabolites of the drug [20]. We have shown that MDMA induces apoptosis of primary rat hepatocytes and of a cell line of rat hepatic stellate cells (HSC) [21]. In both cell types, apoptosis was accompanied by morphological features like nuclear condensation and fragmentation, and by a sequence of events characteristic of apoptosis mediated by alterations in mitochondria: disregulation of Bcl-x_L protein, cytochrome *c* release to the cytoplasm and caspase-3 activation [22].

HSC are the cell type responsible for collagen production in the liver. MDMA presents a dual and dose-dependent effect on HSC, inducing collagen production at low doses and cell death by apoptosis at higher concentrations [21,23]. In previous studies, we found that the profibrogenic effect of MDMA is mediated by oxidative stress, since it correlates with ROS production and GSH depletion, and is abolished by pre-treatment with antioxidants [23]. In the present work, we studied the role played by oxidative stress in the apoptotic death of HSC elicited by MDMA.

2. Materials and methods

2.1. Reagents

MDMA–HCl was a gift from the ''Audiencia Provincial de Navarra.'' Cell culture reagents were from Gibco BRL. Menadione, pyrrolidine dithiocarbamate (PDTC), NAC, GSH, buthionine sulfoximine (BSO), and quinine were from Sigma. 5,6-Chloromethyl-2′,7′-dichlorohydrofluorescein diacetate (CM-H_2DCFDA) was from Molecular Probes. Parthenolide was from Alexis Biochemicals.

2.2. Cell culture and treatments

The HSC cell line CFSC-2G [24] was used in all the experiments. This cell line has the phenotype of a transitional cell between HSC and myofibroblasts. Cells were cultured in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and non-essential amino acids for 36 hr, after which the medium was replaced for a serum-free medium. After 12 hr cells were treated with either 5 mM of MDMA or with menadione 100 μM for the indicated times. In some experiments 30 min pre-treatments with either PDTC (100 μM), GSH (1 mM), NAC (5 mM), BSO (100 μM), quinine (1 or 10 μM) or parthenolide (20 or 100 μM) were carried out.

2.3. Measurement of intracellular GSH levels

The intracellular levels of GSH (reduced form) were determined by the method of Hissim and Hilf [25]. HSC were cultured and treated as described above. After treatment, HSC were scraped and resuspended in MEM (1×10^6 cell/mL). An aliquot of the deproteinized cell suspension (50 μL) was mixed with 2.1 mL of 200 mM sodium phosphate buffer, pH 8.0, containing 5 mM EDTA. Then 100 μL of a solution of *o*-phthaldialdehyde (1 mg/mL in methanol) was added, and 15 min later the intensity of fluorescence was determined (excitation at 350 nm, emission at 420 nm).

2.4. Measurement of ROS production

Production of ROS, mainly peroxides, was measured using the fluorescent probe CM-H_2DCFDA. For these experiments HSC were grown in MEM without phenol red. For time-course studies HSC were plated to subconfluence in 12-well plates, treated for different times with 5 mM MDMA, and then incubated for 20 min with 5 μM CM-H_2DCFDA at room temperature. Fluorescence (excitation at 485 nm, emission at 530 nm) was analyzed in a Cytofluor 2350, Millipore.

2.5. 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. After treatment with 5 mM MDMA for 24 hr, cell death ELISA assays were performed according to the manufacturers 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.

2.6. 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 12% SDS–PAGE and electrotransfered to nitrocellulose membranes. The membranes were incubated with anti-IκB-α monoclonal mouse antibodies (Santa Cruz Biotechnology) diluted 1:2000, and for 1 hr with anti-mouse immunoglobulin horseradish peroxidase conjugated (Amersham Pharmacia Biotech), diluted 1:4000. Bound antibodies were detected by enhanced chemiluminiscence autoradiography with ECL-Plus (Amersham Pharmacia Biotech). Equivalent

loading was confirmed by Coomasie staining of an identical gel.

2.7. Nuclear protein extraction and electrophoretic mobility shift assays (EMSA)

HSC cultured as described above, were treated for 2 hr with 5 mM MDMA or 100 μM menadione. Nuclear proteins from control and treated cell cultures were obtained following the method described by Schreiber *et al.* [26]. The consensus sequence for NF-κB with the binding motif GGG GAC TTT CCC was obtained from Santa Cruz Biotechnology (sc-2505) and labeled with [γ^{32}P]ATP using T4 polynucleotide kinase. The reaction mixtures containing 5 μg of nuclear extracts, were incubated with the probe for 30 min at room temperature. DNA–protein complexes were separated from unbound probe by electrophoresis on a 6% polyacrylamide gel. Complexes formed were identified by autoradiography of the dried gels. For antibody interference studies, incubation mixtures contained the corresponding specific antibody (Santa Cruz Biotechnology) and electrophoresis was carried out on a 4% polyacrylamide gel.

2.8. Viral infection of HSC

The adenoviral vector delivering a dominant-negative form of NF-κB-inducing kinase (NIK) (Ad5dnNIK) was a gift from Dr. Brenner. Ad5LacZ, which contains the *E. coli* β-galactosidase (β-Gal) gene was used as a control virus throughout the study. Sub-confluent HSC were infected with Ad5dnNIK or Ad5LacZ in serum-free medium at a multiplicity of infection (MOI) of 3000 (determined as optimal MOI by galactosidase staining for AdLacZ infected cells with different MOIs) for 3 hr, and then supplemented with 2% FBS for 12 hr. The medium was changed to fresh MEM supplemented with 2% FBS, and 24 hr later experiments were carried out.

2.9. 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 oxidative stress in HSC at pro-apoptotic doses

To study the ability of MDMA to induce oxidative stress at pro-apoptotic concentrations, we analyzed intracellular levels of ROS and reduced GSH in HSC treated with 5 mM MDMA. In previous studies this concentration of MDMA, had been shown to exert a proapoptotic effect on HSC [21]. Time-course experiments showed that HSC exposed to MDMA presented an increased production of ROS, reaching values of 2-fold increase at 30 min (Fig. 1A).

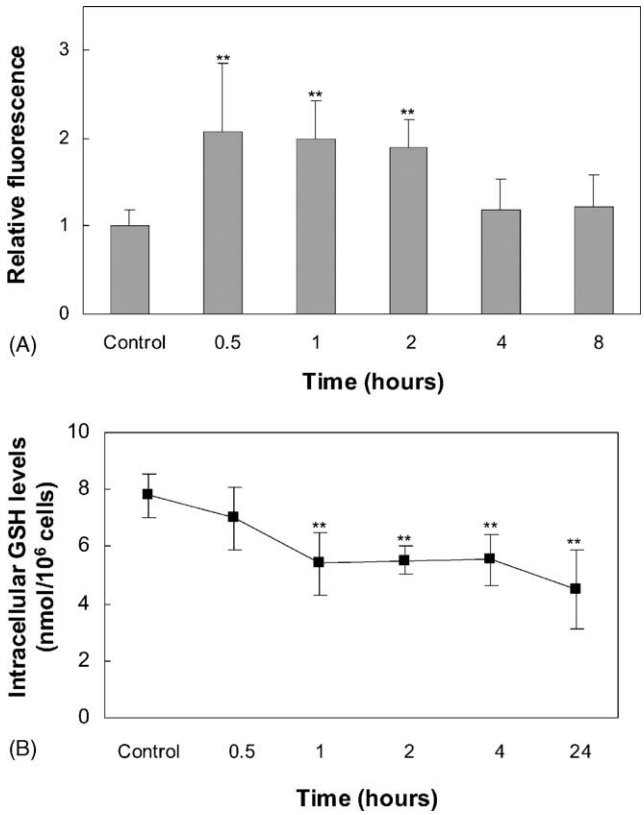

Fig. 1. Time-course analysis of hydrogen peroxide and intracellular GSH (reduced form) levels on HSC treated with MDMA. (A) HSC were exposed for 30 min, 1, 2, 4 and 8 hr to 5 mM MDMA, and hydrogen peroxide levels determined by fluorimetry, using CM-H_2DCFDA as a probe. Each bar represents the mean ± SD of fluorescence fold change compared to controls of at least quadruplicate experiments ($^{**}P < 0.01$, vs. control). (B) HSC were treated for 30 min, 1, 2, 4 and 24 hr with 5 mM MDMA, and GSH (reduced form) levels measured as described in Section 2. Values are the mean ± SD of at least quadruplicate experiments ($^{**}P < 0.01$, vs. control).

Intracellular GSH levels were also affected by MDMA, being 40% lower than untreated controls 1 hr after adding the drug, and remaining depleted for 24 hr (Fig. 1B). This effect does not seem to be a consequence of apoptotic cell death, since cell viability is not compromised by MDMA at early time points [21].

3.2. Effect of menadione on ROS production and apoptosis of HSC

Menadione (2-methyl-1,4-naphthoquinone) is a compound frequently used as an intracellular generator of hydrogen peroxide [27], that has been shown to induce apoptosis of some cell types through generation of oxidative stress [28,29]. To establish if HSC were susceptible to oxidative stress-induced apoptosis, we carried out experiments using

menadione as a pro-oxidant and PDTC as an antioxidant. This compound elicits a generic antioxidant activity, being able to act as a metal chelator or as a scavanger of different ROS through its sulfhydryl group [30].

Time-course analysis of ROS production after adding menadione to the cell cultures, showed a 3- to 4-fold increase after 1 hr treatment (Fig. 2A). As expected, pre-treatment with PDTC effectively blocked the increase on hydrogen peroxide levels caused either by MDMA or menadione in HSC (Fig. 2B).

The extent of apoptotic cell death was determined in these same conditions. Apoptosis was measured as the fold increase in histone-associated oligonucleosomal fragments that accumulate in the cytoplasm (see Section 2). As shown in Fig. 2C, pre-treatment with PDTC did not affect the apoptotic action of MDMA, suggesting that ROS do not mediate programmed cell death induced by MDMA on HSC. However, menadione induced apoptosis of HSC, and this effect was prevented by pre-treatment with PDTC (Fig. 2C), showing that HSC are susceptible to apoptosis induced by oxidative stress.

3.3. Effect of GSH, NAC and BSO on apoptosis of HSC induced by MDMA or menadione

To evaluate the role played by GSH in apoptosis induced by MDMA, we altered the GSH status by pre-treatment with several compounds. Cells were pre-treated either with GSH or the GSH precursor NAC to prevent the decrease of GSH levels caused by MDMA, and also with BSO, a compound that leads to GSH depletion through inhibition of the rate-limiting enzyme of GSH synthesis. Analysis of intracellular GSH levels showed that addition of GSH and NAC prior to treatment with MDMA prevented GSH depletion, obtaining values of GSH similar to untreated cells. Menadione-treated HSC also presented decreased GSH levels, as expected for its pro-oxidant activity, and both NAC and GSH were able to return GSH levels to values slightly higher that control when added prior to menadione. BSO effectively depleted GSH levels by itself and an additive effect was observed when added together with either MDMA or menadione (Table 1).

Once we had established the conditions for preventing or increasing GSH depletion, apoptosis was measured after treating HSC as above. Pre-treatments presented opposite effects on apoptotic cell death depending on the agent. Both GSH and NAC increased MDMA-induced apoptosis of HSC, reaching in the case of NAC values of oligonucleosomal fragment accumulation 2-fold higher than those found in cells treated only with MDMA. However, in menadione-treated cells pre-treatment with either GSH or NAC blocked the apoptotic action of the pro-oxidant. BSO added prior to MDMA reduced its apoptotic effect, while it did not affect apoptosis induced by menadione (Fig. 3). Both compounds prevented the increase of ROS levels caused by menadione or MDMA (data not shown).

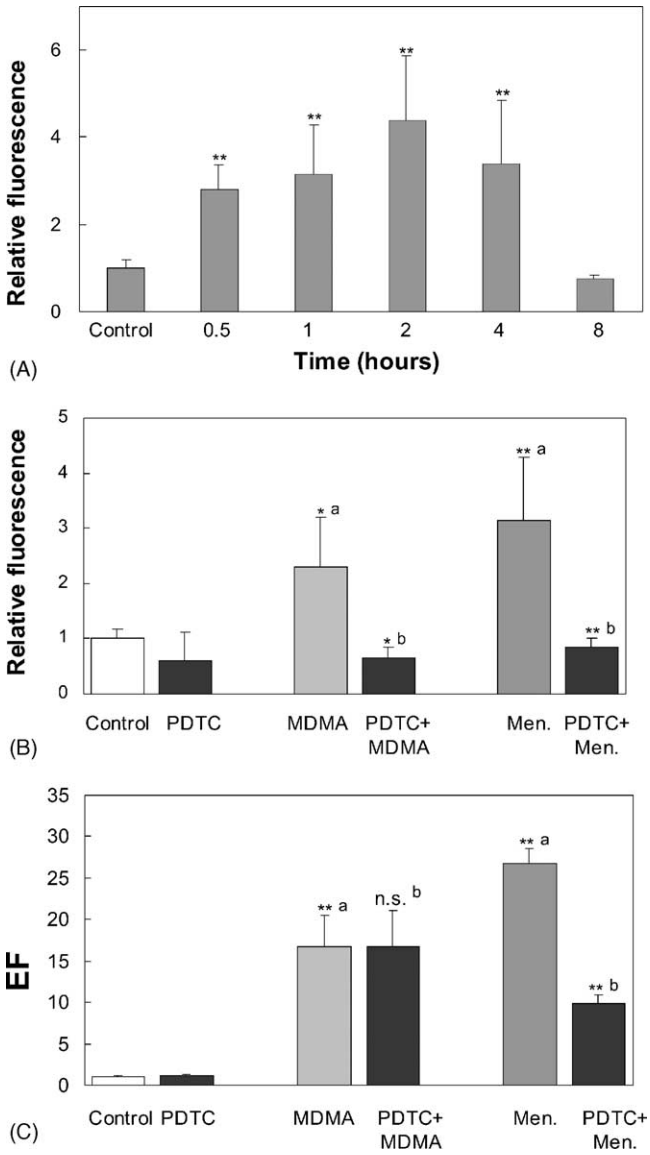

Fig. 2. Effect of menadione on HSC. (A) Time-course analysis of hydrogen peroxide levels of HSC treated with menadione 100 μM. HSC were exposed for 30 min, 1, 2, 4 and 8 hr to 100 μM menadione, and hydrogen peroxide levels determined by fluorimetry, using CM-H_2DCFDA as a probe. Each bar represents the mean ± SD of fluorescence fold change compared to controls of at least quadruplicate experiments. (B) Effect of PDTC on hydrogen peroxide production in MDMA or menadione-treated HSC. Hydrogen peroxide levels were determined in HSC treated for 1 hr with MDMA 5 mM or menadione 100 μM in the presence or absence of PDTC, using CM-H_2DCFDA as a probe. (C) Effect of PDTC on apoptosis of HSC induced by MDMA or menadione. Oligonucleosomal fragments content was expressed as EF, as described in Section 2. HSC were treated for 24 hr with MDMA 5 mM or menadione 100 μM in the presence or absence of PDTC. Each bar represents the mean ± SD of quadruplicate determinations from two independent experiments ($^{**}P < 0.01$, $^{*}P < 0.05$, n.s.: no significative; a: vs. control; b: vs. MDMA or menadione-treated cells).

3.4. Quinine prevents apoptosis induced by MDMA

Some of the toxic effects described for MDMA have been shown to be caused by metabolic transformation of the drug. Semiquinone or quinone species that react easily with nucleophiles like GSH are generated from

Table 1
Intracellular concentration of GSH (nmol/10^6 cells)

Treatment		Pre-treatment		
		GSH (1 mM)	NAC (5 mM)	BSO (100 M)
Control	7.8 ± 0.71	7.5 ± 1.23	10.64 ± 2.41^{*,a}	4.08 ± 1.14^{**,a}
MDMA (5 mM)	4.51 ± 1.37^{**,a}	7.46 ± 0.76^{**,b}	6.6 ± 2.1^{*,b}	2.59 ± 0.62^{**,b}
MEN (100 M)	1.13 ± 0.46^{**,a}	10.6 ± 1.57^{**,b}	14.21 ± 3.56^{**,b}	#

Intracellular GSH was measured as described in Section 2. Data are means ± SD of at least triplicate measurements from two to seven different experiments. (#): not detectable values; a: vs. control; b: vs. MDMA or menadione.
$^{*}P < 0.05$; $^{**}P < 0.01$.

MDMA by members of the cytochrome P450 family [31,32]. To establish whether a derivative of the drug was responsible for its apoptotic activity, an inhibitor of cytochrome P450, quinine, was added prior to MDMA treatment, and the extent of apoptotic cell death measured as before. Inhibition of cytochrome P450 by quinine prevented apoptosis of HSC induced by MDMA in a dose–response fashion (Fig. 4).

3.5. MDMA activates NF-κB

NF-κB is a nuclear factor that has been described to be activated by oxidative stress in some systems [33–35] and that is involved in the apoptotic response. We evaluated whether MDMA had an effect on the activity of nuclear factor NF-κB and the role played by this factor on the apoptotic action of MDMA. Time-course analysis of NF-κB binding activity by electrophoretic mobility shift assay, revealed an increased binding activity of the factor to its consensus sequence in MDMA-treated HSC. Unlike MDMA, menadione did not increase the binding activity of NF-κB (Fig. 5A). By super-shift analysis the protein complex that bound to the labeled probe was identified as the transcriptionally active heterodimer p50/p65. Western blot analysis of the inhibitory subunit of the factor, IκB, showed an increased degradation induced by MDMA (Fig. 5B). Activation of NF-κB by MDMA was not affected by pre-treatments that altered the GSH status, such as GSH, NAC or BSO (Fig. 5C).

The role played by NF-κB on the apoptotic response elicited by MDMA was studied using two different experimental approaches to inhibit the factor. The chemical compound parthenolide is a sesquiterpene lactone with anti-inflammatory properties that exerts its action through inhibition of NF-κB [36]. HSC were pre-treated with two concentrations of parthenolide before addition of MDMA and after 24 hr apoptosis was determined as accumulation

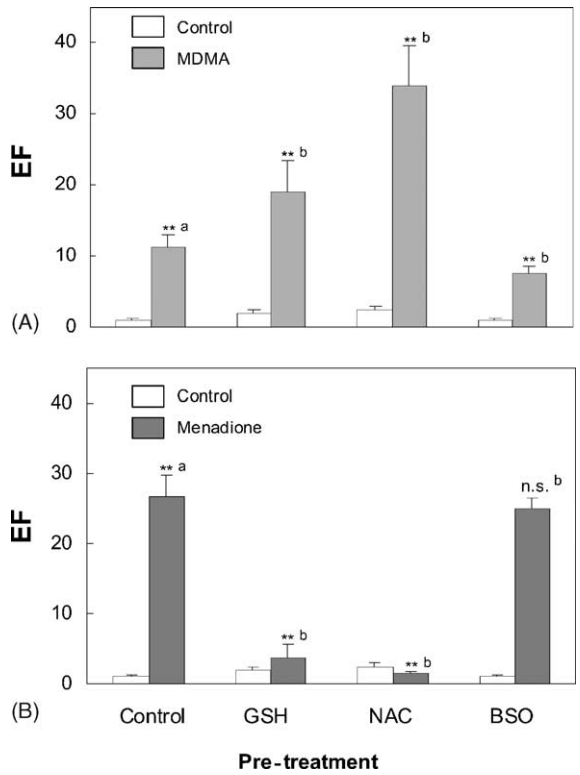

Fig. 3. Effect of GSH, NAC and BSO on the accumulation of cytoplasmic oligonucleosomal fragments in HSC treated with MDMA or menadione. HSC were treated for 24 hr with 5 mM MDMA (A) or 100 μM menadione (B) in the presence or absence of GSH, NAC and BSO. Oligonucleosomal fragments content was expressed as EF, as described in Section 2. Each bar represents the mean ± SD of quadruplicate determinations from at least two independent experiments ($^{**}P < 0.01$, n.s.: no significative; a: vs. control; b: vs. MDMA or menadione-treated cells).

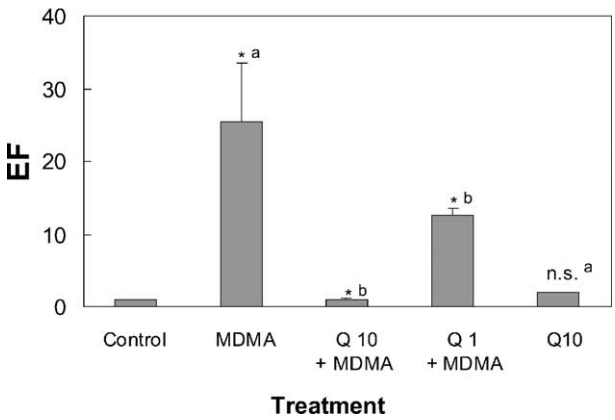

Fig. 4. Effect of quinine on the accumulation of oligonucleosomal fragments in cytoplasmic extracts from HSC treated with MDMA. HSC were treated for 24 hr with 5 mM MDMA in the presence or absence of the cytochrome P450 inhibitor quinine, at concentrations of 1 or 10 μM. Oligonucleosomal fragments content was expressed as EF, as described in Section 2. Each bar represents the mean ± SD of quadruplicate determinations ($^{*}P < 0.05$, n.s.: no significative; a: vs. control; b: vs. MDMA-treated cells).

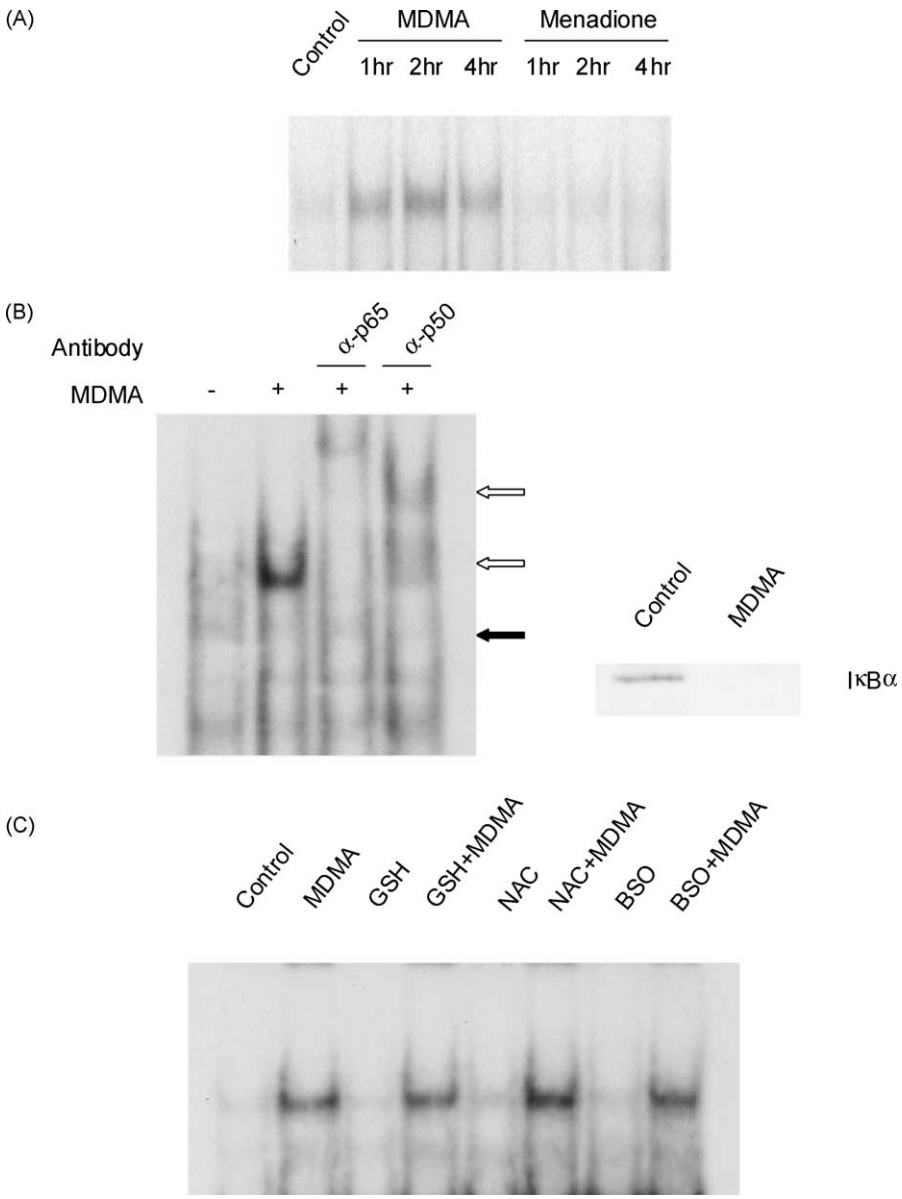

Fig. 5. NF-κB binding activity of MDMA-treated cells. (A) Time-course analysis of NF-κB binding activity determined by EMSA in extracts of HSC treated with MDMA or menadione. HSC were treated for 1, 2 or 4 hr with 5 mM MDMA or 100 μM menadione and nuclear protein extracts obtained as described in Section 2. (B) Identification of NF-κB by super-shift and Western blot analysis of IκB-α degradation in HSC treated with MDMA for 2 hr. C, NF-κB binding activity in extracts of HSC pre-treated for 30 min with GSH, NAC or BSO, before treatment with MDMA (2 hr).

of oligonucleosomal fragments. Pre-treatment with parthenolide increased the extent of apoptotic death induced by MDMA. Moreover, parthenolide by itself had an apoptotic effect on HSC that was dose-dependent (Fig. 6A). Other experiments were aimed at inhibiting NF-κB through the blockage of its signaling cascade. NF-κB activation commonly takes place as a result of protein phosphorylation by specific kinases called IKK (IκB kinases). IKKs are also a substrate for NIK (NF-κB-inducing kinase), which can be activated through its interaction with components of the death receptor such as TRAF. A dnNIK delivered by adenoviral (Ad5dnNIK) vector has been shown to inhibit IKK phosphorylation and NF-κB activation [37]. HSC were infected with the adenovirus Ad5dnNIK prior to treatment with MDMA and apoptosis evaluated 24 hr later. Ad5dnNIK-infected cells presented an increased level of cytosolic oligonucleosomal fragments compared to control cells, and were more susceptible to apoptosis induced by MDMA (Fig. 6B).

4. Discussion

Intracellular oxidative stress due to accumulation of ROS mediates several physiological and pathological responses, including drug-toxicity. The effects of oxidative stress are different depending, among other factors, on the extent of ROS accumulation. An excessive accumulation

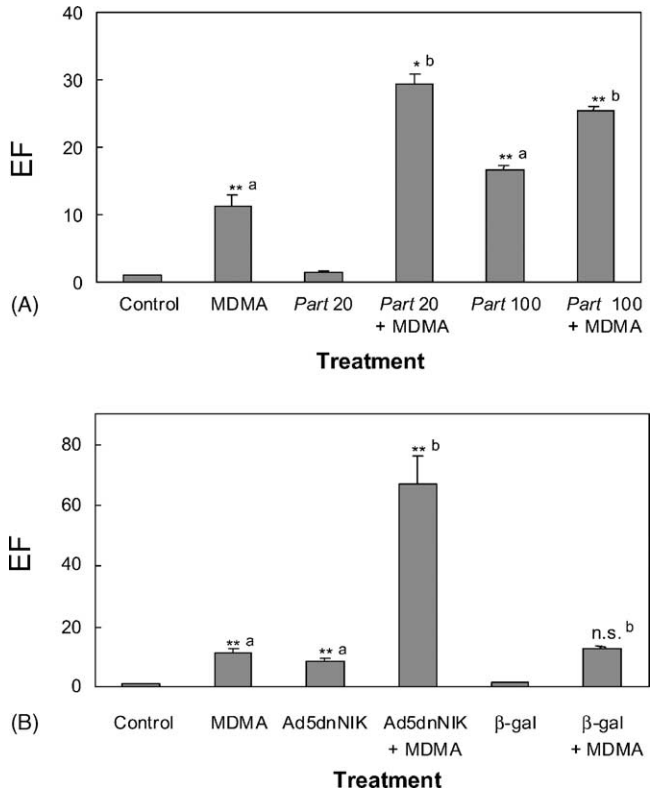

Fig. 6. Effect of NF-κB inhibition on the accumulation of oligonucleosomal fragments in HSC treated with MDMA. (A) Effect of parthenolide. HSC were treated for 24 hr with 5 mM MDMA in the presence or absence of parthenolide 20 and 100 μM. (B) Effect of the dnNIK delivered by adenoviral vector Ad5dnNIK. HSC were infected for 3 hr with Ad5dnNIK or Ad5LacZ (infection control). The oligonucleosomal fragments content was expressed as EF, as described in Section 2. Each bar represents the mean ± SD of quadruplicate determinations from at least two independent experiments ($^{**}P < 0.01$, n.s.: no significative; a: vs. control; b: vs. MDMA-treated cells).

of ROS leads to alterations and damage of all type of macromolecules and eventually to necrotic cell death. However, a moderate increase of ROS can elicit effects such as activation of signaling pathways and/or transcription factors that could be involved in physiological responses. In HSC, hydrogen peroxide production has been shown to up-regulate collagen expression at the transcriptional level in response to cytokines or acetaldehyde metabolism [38,39] as well as to exposure to MDMA [23], adding to the many reports pointing out to a relationship between oxidative stress and fibrogenesis. However, to our knowledge, the role played by oxidative stress in programmed cell death of HSC remains to be fully elucidated. Our results show that apoptosis of HSC can be triggered by oxidative stress, since treatment with the pro-oxidant menadione had an apoptotic effect that was inhibited by three sulfhydryl-containing antioxidants: PDTC, GSH (reduced form, GSH) and NAC. The fact that MDMA induced oxidative stress at apoptotic doses in HSC, and that this cell type was sensitive to pro-oxidants with apoptotic potential, suggested a relationship between both events. However, this possibility was ruled out by several results. On the one hand, PDTC had no effect on MDMA-induced apoptosis. On the other, pre-treatment with GSH and the GSH precursor NAC produced a dramatic increase of the apoptotic action of MDMA. In some cell types there is a threshold response to hydrogen peroxide with respect to apoptosis, with a protective effect at low concentrations and a pro-apoptotic effect at higher ones, that could explain the potentiation of apoptosis observed in HSC treated with both MDMA and GSH or NAC. However, PDTC also blocked ROS production induced by MDMA without affecting cell viability, pointing out to an specific effect of GSH-related molecules.

Glutathione plays a key role in controlling the redox state of the cell through several mechanisms, including scavanging of hydrogen peroxide and other ROS, and keeping the enzyme GSH peroxidase in a reduced state [40]. Although GSH and its precursor NAC can potentiate apoptosis in some transformed cell lines [41,42], most of the reports indicate a role for GSH as a protective agent against apoptosis. In hepatocytes GSH depletion has been shown to induce apoptosis by itself [43] or to sensitize the cells towards TNF-α-induced apoptosis [44], and the effect of some anti-apoptotic agents has been demonstrated to be mediated by stabilizing the GSH pool [45]. MDMA-induced apoptosis of HSC correlates with a decrease in GSH, but the prevention of this effect by pre-treatment with exogenous GSH or NAC potentiated the apoptotic action of the drug. This could be explained by other actions of the tripeptide different from its role as an antioxidant. Glutathione can form adducts with 3,4-dihydroxymethamphetamine (DHMA), the main product of MDMA metabolization, which is synthesized by members of the cytochrome P450 family, mainly *CYP*2D6 [31,46,47]. We found that pre-treatment of HSC with the *CYP*2D6 inhibitor quinine abolished the apoptotic effect of MDMA, suggesting an apoptotic role of a by-product of MDMA metabolization. The pro-apoptotic effect of exogenous GSH or NAC and the protective action of BSO point out to the adduct as a mediator of apoptotic cell death induced by MDMA.

NF-κB is a factor activated in response to cellular stress that is involved in the regulation of apoptosis. Depending on the cell type and the apoptotic agent, NF-κB has been reported to mediate or prevent apoptosis. In TNF-α or Fas-ligand-induced apoptosis most of the studies, including recent reports on HSC [48,49], show that NF-κB plays a protective role, since its inhibition enhances the cell death rate or sensitizes cells towards the apoptotic effect of these factors [50,51]. However, in models of apoptosis induced by oxidative stress, NF-κB activation can be pro-apoptotic, and addition of antioxidants block both the activation of the factor and apoptotic cell death [33–35]. We found that NF-κB binding activity was enhanced in HSC by treatment with MDMA, and our results suggest that this activation has a protective action against apoptosis induced by the drug, since inhibition of the factor either by pre-treatment

with parthenolide or infection with Ad5dnNIK resulted in an enhancement of apoptotic cell death. NF-κB activation by MDMA was not affected by pre-treatment with either GSH or NAC, and therefore does not seem to be caused by oxidative stress elicited by the drug. This result also indicates that the potentiation of MDMA-induced apoptosis due to pre-treatment with GSH or NAC is not a consequence of an inhibition of the anti-apoptotic action of NF-κB by antioxidants, and should be explained by other mechanisms, like the above-mentioned adduct formation. Moreover, the fact menadione failed to increase the binding activity of NF-κB in HSC, questions the ability of oxidative stress to induce NF-κB activity in this cell type.

In summary, MDMA induces programmed cell death on HSC and this effect is accompanied by oxidative stress, but not mediated by it. MDMA-treatment of HSC results both in the triggering of the apoptotic cascade and in the activation of at least one anti-apoptotic factor, NF-κB, that fails to totally block apoptosis but partially protects the cells from apoptotic death.

Acknowledgments

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