Leydig Cell Apoptosis in Response to Ethane Dimethanesulphonate After Both *In Vivo* and *In Vitro* Treatment

ANDREW J. MORRIS, MATTHEW F. TAYLOR, AND IAN D. MORRIS

From the Division of Pharmacology, Physiology and Toxicology, School of Biological Sciences, University of Manchester, Manchester, UK.

ABSTRACT: The biological effects of ethane dimethanesulphonate (EDS) are unique since cytotoxicity in the adult rat is almost exclusively confined to the Leydig cells. For this reason, EDS has been used extensively to investigate the physiological role of the Leydig cell and its products. Experiments were conducted to determine whether the Leydig cell will undergo apoptosis in response to EDS or methylprednisolone (MP), a glucocorticoid known to cause apoptosis in a number of other cell types. Percoll-purified Leydig cells were incubated for 24 hours with EDS (750 μg/ml), at which time the cells attached to the culture plate became rounded up while control cells were flattened and polyhedral. Following incubation with EDS or MP (10 μM), cells that became detached from the plate were characteristically apoptotic when stained with the fluorescent DNA dye, acridine orange. These cells had shrunk and the nuclear chromatin had become condensed, which is an early characteristic of apoptosis in other cells; eventually, apoptotic bodies formed, reflecting a later apoptotic stage. Electrophoresis of DNA extracted from the treated Leydig cells exhibited the characteristic ladder of the apoptotic process. Increasing the concentration of EDS or MP resulted in a dose-dependent increase in the incidence of apoptosis that reached a maximum of 25% (EDS) or 12% (MP) of detached cells. Administration of EDS *in vivo* caused a 20-fold increase in the number of apoptotic cells observed in interstitial cell preparations. In conclusion, the data indicates that programmed cell death, apoptosis, can occur in the Leydig cell and that this is the likely mechanism by which EDS kills the cells *in vivo* and *in vitro*.

Key words: Testis, cell culture, methylprednisolone, glucocorticoid, rat.

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The methane sulphonic ester of ethylene glycol, ethane 1,2-dimethanesulphonate (EDS), is a unique testicular toxicant and has been shown, when injected into adult rats, to cause temporary infertility (Jackson, 1966), reduced serum and intertesticular testosterone, and elevated pituitary secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Jackson and Morris, 1977; Morris and McCluckie, 1979; Tena-Sempere et al, 1993). The Leydig cell population is destroyed and subsequently regenerates, apparently from mesenchymal fibroblast-like precursors (Jackson et al, 1986a, b; Kerr et al, 1986; Morris et al, 1986;).

The *in vivo* treatment of rats with EDS leads to alterations in the cytoplasm and nuclei of the interstitial Leydig cells. The principal changes observed were vesiculation in the smooth endoplasmic reticulum, focal hypertrophy of the Golgi apparatus, and clumping of the nuclear chromatin (Kerr et al, 1986; Morris et al, 1986). Subsequently, the Leydig cells exhibit further degenerate alter-

ations in their cytoplasm and nuclei leading to fragmentation followed by their disappearance through the phagocytic activity of macrophages (Kerr et al, 1985, 1986; Jackson et al, 1986b; Morris et al, 1986). These morphological changes are reminiscent of those described for apoptosis in other cell systems (Wyllie, 1980; Earnshaw, 1995; Kroemer et al, 1995), which suggests that Leydig cells can engage in programmed cell death rather than die by necrosis in response to certain stimuli. In support of this, rats treated *in vivo* with EDS showed increased testicular levels of internucleosomal DNA cleavage 24 hours after drug administration, a time at which only Leydig cell degeneration is thought to be occurring (Henriksen et al, 1995). Furthermore, crude interstitial cell preparations from rats in which the Leydig cell population is regressing after hypophysectomy exhibit increased DNA fragmentation (Tapanainen et al, 1993), suggesting that Leydig cell apoptosis may be a more general phenomena than previously suspected.

The hallmarks of classical apoptosis such as cell shrinkage, plasma membrane blebbing, chromatin condensation, and DNA fragmentation have been well defined (Wyllie 1980; Earnshaw 1995; Kroemer et al, 1995). These processes can be contrasted to those found in necrosis such as increases in cell volume, swelling of intra-

cellular organelles, and the eventual lysis of the cell. *In vivo*, these apoptotic cells would be rapidly phagocytosed, their intact membranes preventing an inflammatory response.

To investigate the role of apoptosis in the response of Leydig cells to EDS, we undertook the present study to determine the *in vivo* and *in vitro* effects of EDS on some of the morphological and biochemical parameters of apoptosis. We also investigated the potential for programmed cell death being a more general phenomena in the Leydig cell by examining the effects of methylprednisolone (MP), a drug that can induce apoptosis in a number of cell types by acting through the glucocorticoid receptor (Wyllie et al, 1980; Kiefer et al, 1995), which is present within the Leydig cell (Stalker et al, 1989).

Materials and Methods

Reagents

All chemicals were of reagent grade. Penicillin, streptomycin, and 10× M199 media (with Hanks' balanced salts) were obtained from Gibco Ltd. (Paisley, Scotland). Percoll was purchased from Pharmacia Lkb. (Upsalla, Sweden). Collagenase type I, DNase I, nitro-blue tetrazolium, NAD$^+$, 5-androstane-3-ol-17-one, methylprednisolone, RNase A, and proteinase K were all obtained from Sigma Chemical Co. (St. Louis, Missouri). Ethane dimethanesulphonate was synthesized in our laboratory from ethylene glycol and methanesulphonyl chloride according to the method described by Jackson and Jackson (1984).

Animals

Adult male Sprague-Dawley rats (250–300 g), purchased from Charles Rivers UK Ltd. (Margate, Kent, UK), were housed four per cage in a light-controlled room (12 hours light : 12 hours dark; lights on at 0700 hours). The animals were handled daily for at least 1 week prior to the beginning of experimentation, with food and water supplied *ad libitum*. In some experiments, rats were injected i.p. with either ethane dimethanesulphonate (100 mg/kg body weight) in a vehicle of dimethyl sulphoxide : water (1:3 volume ratio) or with an equivalent volume of vehicle only (2 ml/kg body weight); in other experiments, rats were injected i.p. with MP (100 mg/kg body weight) in a vehicle of corn oil or with vehicle alone. The animals were killed 24 hours later by cervical dislocation, and the testes were removed. Leydig cells were prepared as described below.

Leydig Cell Preparation

Leydig cells were prepared by a combination of collagenase digestion and Percoll gradient separation as described elsewhere (Lin et al, 1989; Moore and Morris, 1993). Briefly, rats were killed by stunning, followed by cervical dislocation. The testes were removed, decapsulated, and digested with collagenase type I (0.25 mg/ml) in M199 media (1× M199, pH 7.4, containing 4.2 mM sodium bicarbonate, 20 mM HEPES, 1 mg/ml BSA, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1,000 Kunitz

units DNase I) for 20 minutes at 34° C in a shaking water bath (200 cycles/minute). A crude interstitial cell preparation was obtained by filtering the digest first through coarse nylon gauze and then through 60 μm nylon mesh. The resultant cell suspension was centrifuged at 120 × *g* for 20 minutes, washed twice in M199, and resuspended in 1 ml.

The cell suspension was loaded on top of a discontinuous Percoll gradient. The gradients were comprised of six density steps of 1.0900, 1.0625, 1.0450, 1.0400, 1.0350, and 1.0300 g/ml. The gradient was centrifuged at 800 × *g* for 30 minutes, and cell fractions were collected from the bottom of the tube. The interface of densities 1.0900 and 1.0625 was collected and denoted as the Leydig cell fraction, this fraction was diluted and centrifuged at 120 × *g* for 20 minutes, resuspended, and washed twice in M199. Leydig cell purity was assessed using 3β-hydroxysteroid dehydrogenase staining method (Weibe, 1976; Ziegler et al, 1983) with the formation of blue-purple formazan granules in the cytoplasm indicating the presence of Leydig cells. Greater than 70% of the cells consistently stained positive for Leydig cells.

Leydig Cell Culture

Leydig cells prepared as described above were diluted to 5 × 10^5 cells/ml in M199 media and plated at 0.5 ml/well on Falcon 3847 Primeria plates (Becton Dickinson, Cowley, UK). After 4 hours, the media was aspirated to remove contaminating and damaged cells and fresh media (0.5 ml) was added containing either vehicle alone dimethylsulfoxide (DMSO), EDS (either 500 or 750 μg/ml), or MP (10 or 20 μM). The doses of EDS were chosen from previous experiments that determined the *in vitro* cytotoxicity and inhibition of steroidogenesis by EDS in immature rat Leydig cells (Verhoeven et al, 1989). Plates were incubated at 34°C in a humidified 5% CO$_2$/95% air atmosphere for either 24 or 48 hours. Detached cells were pelleted at 250 × *g* for 5 minutes (cell viability assessed using Trypan blue exclusion) and fixed in 2% paraformaldehyde. The morphology of the detached cells was determined by staining the cells with acridine orange and examining the fluorescence on a BioRad MRC 600 confocal microscope (BioRad Ltd., Hemel Hempstead, UK).

DNA Isolation and Analysis

DNA was extracted and analyzed by agarose gel electrophoresis as described by Wilson et al (1995). Briefly, approximately 1 × 10^6 unattached cells were collected 24 hours posttreatment and were microfuged at low speed for 5 minutes at 4°C. The supernatant was discarded and the pellet resuspended in 20 μl of lysis buffer (50 mM Tris-HCl, pH 8.0, containing 10 mM ethylenediamine–tetraacetic acid (EDTA), 0.5% sodium lauryl sarkosinate, and 0.5 μg/ml proteinase K) and incubated for 1 hour at 50°C. RNase A (10 μl, 0.5 mg/ml) was added and incubated for a further 1 hour at 50° C. Low melting temperature agarose (10 μl, 1%) was added to the sample and 40 μl of each sample placed into wells of 2% agarose gel (containing 10 μg/ml ethidium bromide), which was electrophoresed at 40 V for 2 hours. DNA bands were visualized by UV fluorescence.
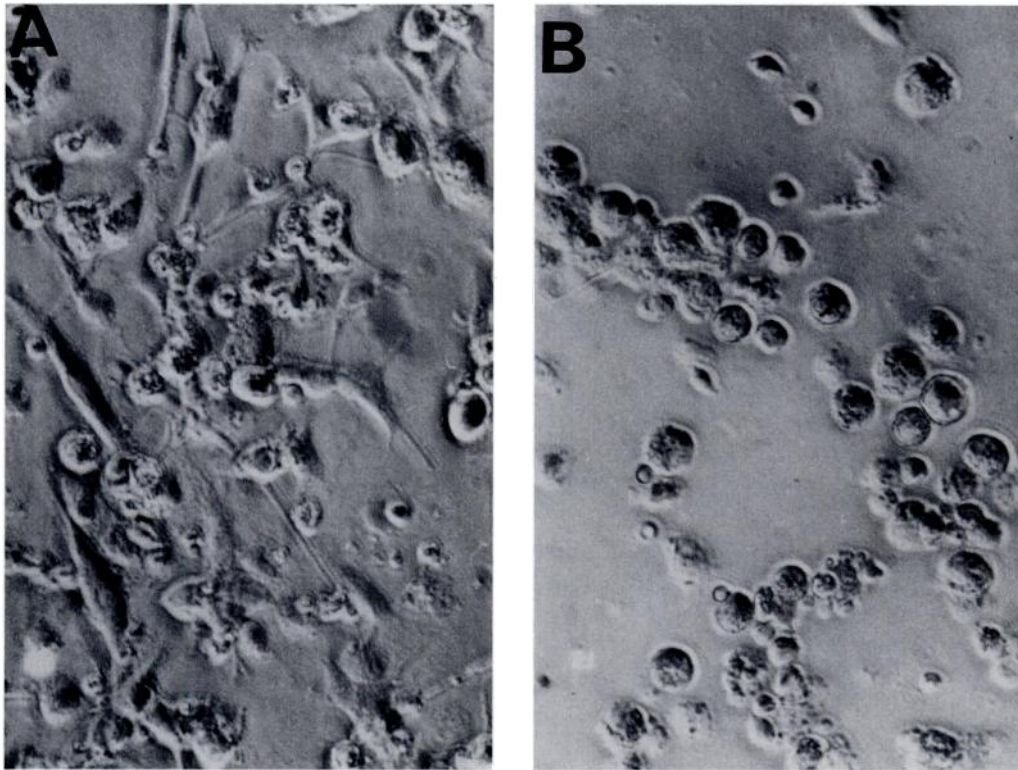
FIG. 1. Phase-contrast micrographs of Leydig cells from control (**A**) and EDS (**B**) treated populations. 400× magnification. (**A**) Leydig cells were plated at a density of 2.5 × 10⁵ cells/well and treated with vehicle (dimethylsulfoxide [DMSO], final concentration 0.05%) and incubated for 24 hours at 34°C in a humidified 5% CO_2/95% air atmosphere. (**B**) Leydig cells were treated as in **A** above except they were incubated with ethane dimethanesulphonate (EDS) (750 µg/ml) in vehicle.

Results

Morphology

Figure 1 shows a phase-contrast photomicrograph of Leydig cells attached to the plate following 24 hours' treatment with either vehicle alone (control) or with 750 µg/ml EDS. The EDS-treated cells round up, appear smaller, and lose contact with the plate. This is in contrast to control cells that are flattened and are polygonal in shape with a large irregular surface. Staining the Leydig cells from the supernatant with acridine orange followed by confocal fluorescence microscopy (Fig. 2A and 2B) shows that the EDS-treated population contains cells with intense, well-demarcated areas of nuclear fluorescence, suggesting that condensation of the chromatin has occurred, which is an indicator of apoptosis (Fig. 2B). A similar nuclear morphology was seen after MP treatment (not shown). The control cells (Fig. 2A), however, show no chromatin condensation; both control and treated Leydig cells excluded trypan blue uptake, suggesting that the cells were viable.

In Vitro Effects of EDS and MP

The DNA prepared from cells cultured for 24 hours in the presence of vehicle, (750 µg/ml EDS or 20 µM MP)

was subjected to agarose gel electrophoresis. The characteristic apoptotic DNA ladder representing the cleavage of DNA into multimers of 200 base pairs can be seen in all sample lanes (Fig. 3).

In the control incubations, there was a spontaneous increase in the number of apoptotic cells identified by acridine orange staining (Figs. 4 and 5) suggesting that some apoptosis is attributable to the culture conditions. Treatment of Leydig cell preparations with either EDS or MP caused a significant further increase in the number of apoptotic cells. In the case of EDS treatment, these effects are both time (Fig. 4) and concentration (Fig. 5A) dependent compared to control cells treated with vehicle (DMSO). Cells treated with MP (Fig. 5B) also exhibited concentration dependence, although the extent to which the Leydig cells underwent apoptosis appears to be ~50% less than that observed with EDS treatment.

In Vivo EDS and MP Treatment

Crude interstitial cell preparations prepared from rats 24 hours after i.p. injection of EDS (100 mg/kg body weight) or vehicle (2 ml/kg body weight; DMSO:water [1:3]) were isolated as described and stained with acridine orange. The percentage of apoptotic cells is shown in Figure 6. There was a small percentage of apoptotic cells in the
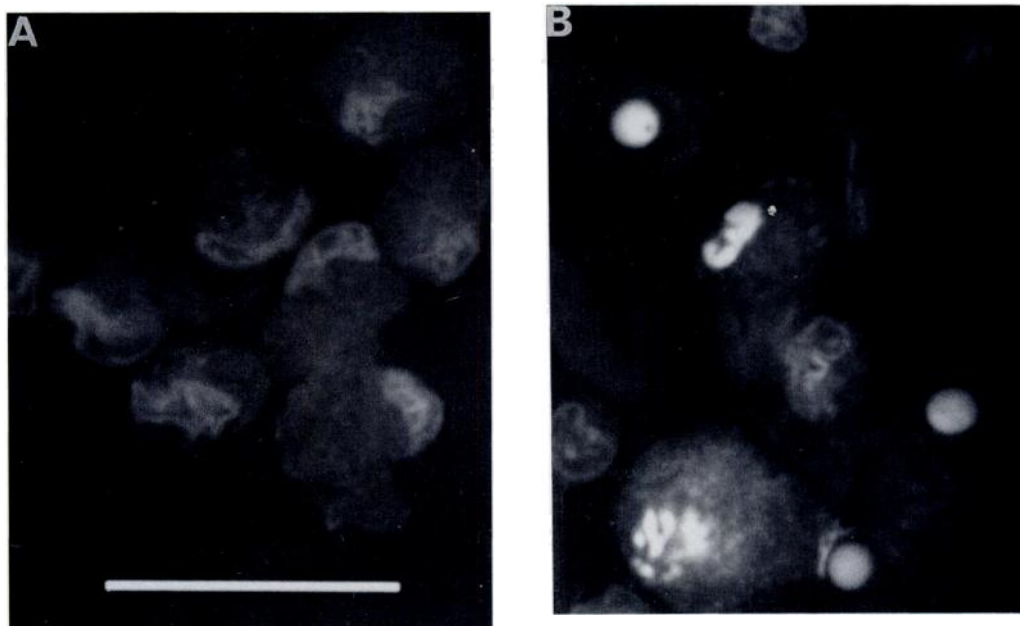
FIG. 2. Confocal micrographs of Leydig cell from control and treated populations stained with acridine orange. The scale bar represents 100 μm. (A) Purified Leydig cells were cultured in M199 media and treated with vehicle (DMSO, 0.05% final concentration) for 24 hours. The detached cells were collected, stained using acridine orange, and examined using the fluorescence confocal microscope. The cell nuclei are diffusely stained and the cells are not apoptotic. (B) Purified Leydig cells incubated as in A except that the vehicle was replaced with 750 μg/ml EDS in DMSO (0.05% final concentration). Both apoptotic (bright condensed nuclei) and nonapoptotic (diffuse-staining) cells are present.

vehicle-treated rat; whereas the EDS-treated rat shows about a 10-fold increase in apoptotic staining. In a similar experiment, 24 hours after the injection of rats with either a pharmacological dose of MP (100 mg/kg body weight) or a vehicle of corn oil (2 ml/kg body weight), no apoptotic cells were observed in either groups (data not shown).

Discussion

The morphology of apoptotic cells has been described at length elsewhere (Searle et al, 1982; Earnshaw, 1995; Kroemer et al, 1995). The appearance of the Leydig cells treated with EDS or MP is consistent with the characteristics of cells undergoing apoptosis. Microscopic studies have shown that an early apoptotic event is the aggregation of chromatin into compact, dense granular masses that often fragment as the cell forms apoptotic bodies. This morphological feature has allowed the identification of apoptotic cells by staining the condensed chromatin with DNA-specific fluorescent dyes such as acridine orange. Using this technique, we have been able to show the appearance of apoptotic Leydig cells after treatment *in vivo* or *in vitro* with EDS or *in vitro* with MP. These observations are also in agreement with *in vivo* studies using EDS (Kerr et al, 1986; Morris et al, 1986), where apoptotic changes in cell morphology were also noted. The major difference between the *in vivo* and *in vitro*

systems appears to be in the terminal stages of apoptosis, where *in vivo* the dying cells are phagocytosed by neighboring macrophages (Kerr et al, 1986; Morris et al, 1986) *in vitro* the cell undergoes secondary necrosis in the culture medium. Leydig cells are notoriously difficult to maintain *in vitro* and after 24 hours, steroidogenesis is often markedly impaired although not absent (Klinefelter and Ewing, 1989; Klinefelter and Kelce, 1996). After *in vitro* incubation of Leydig cells, a small percentage of control cells are apoptotic, which is probably symptomatic of the difficulties in achieving appropriate culture conditions. Apoptosis in cultured Leydig cells may arise as a consequence of the disruption of the normal cellular environment (Marti et al, 1994; Boudreau et al, 1995) coupled with the withdrawal of cytokine and growth factor stimulation (Tamm and Kikuchi, 1990; Rodriguez-Tarduchy et al, 1992; Harrington et al, 1994) that have been reported to activate the cell death program in other cells. Further, time and dose-dependent increases in apoptosis can be induced by the addition of either EDS or a glucocorticoid to the culture media.

One of the best characterized biochemical markers for apoptosis is the activation of an endonuclease that cleaves genomic DNA at the internucleosomal-linker regions leading to the characteristic DNA ladder pattern following gel electrophoresis (Cohen and Duke, 1984; Arends et al, 1990). Early studies showed that treatment of immature thymocytes with physiological concentrations of glucocorticoids, *in vitro*, induces the fragmentation of the DNA
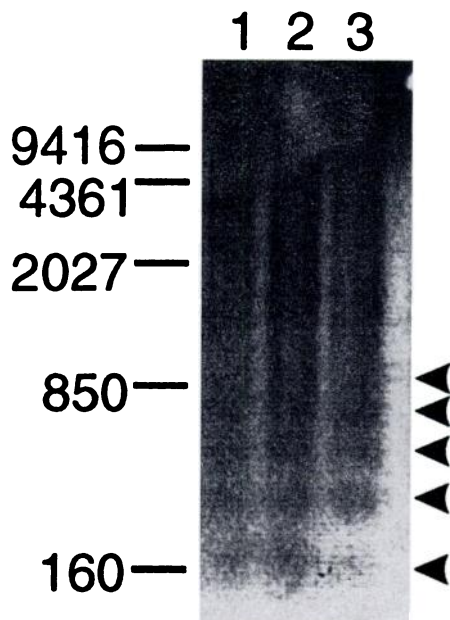
FIG. 3. Agarose gel electrophoresis of DNA extracted from Leydig cells treated with EDS methylprednisolone (MP) or vehicle for 24 hours at 34°C. **(Lane 1)** DNA extracted from vehicle treated cells. **(Lane 2)** DNA extracted from Leydig cells incubated with EDS (750 μg/ml). **(Lane 3)** DNA from cells incubated with MP (20 μM). The DNA was vizualised under UV light after staining with ethidium bromide. DNA markers (Kb) are shown on the left, and the arrowheads on the right indicate the positions of the DNA fragments.

FIG. 5. Concentration dependence of apoptosis with EDS **(A)** or MP **(B)** after a 24-hour incubation. **(A)** Leydig cells were incubated for 24 hours in the presence of vehicle (0.05% DMSO final concentration) or EDS (either 500 μg/ml or 750 μg/ml in vehicle) as indicated. The detached cells were collected, and condensed nuclear chromatin was identified by staining with acridine orange. CON indicates the level of apoptosis occurring at time 0. The bars represent the mean ± SEM of six independent experiments. * indicates $P < 0.05$ and ** $P < 0.005$ as compared to the control values at the same time point. **(B)** Leydig cells were treated as in **A** except that 10 or 20 μM MP was used.

into discrete units that are multiples of 180 base pairs (Wyllie et al, 1980). Leydig cells following treatment with either EDS or MP also exhibited a classical DNA ladder indicative of apoptotic cell death rather than necrotic cell death, which would have produced a DNA smear. However, such data is only qualitative and does not provide any information on the percentage of cells undergoing apoptosis.

The 24-hour *in vivo* treatment of adult male rats with EDS followed by the isolation of the crude interstitial cell population and the staining of these cells with acridine orange shows that >20% of the cells, presumably Leydig
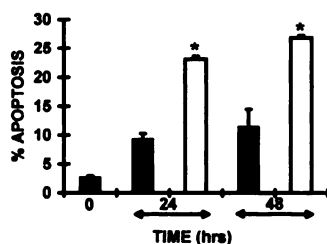
cells, were apoptotic. Histological examinations have estimated that ~75% of the Leydig cell population has been eliminated 24 hours after EDS administration (Kerr et al, 1987), and completely disappears by 3 days (Molenaar et al, 1985; Kerr et al, 1986; Morris et al, 1986). These experiments support our *in vitro* data that cell death after EDS is due to engagement of apoptosis. However, when the effects of *in vitro* and *in vivo* treatments are compared, the *in vitro* activity is not as potent, which may be explained by the need to biochemically activate the EDS molecule (Kelce, 1994). Apoptotic interstitial cells were

FIG. 4. The percentage apoptotic Leydig cells was determined following either a 24-hour or 48-hour incubation with vehicle (DMSO, final concentration 0.05%, closed bars) or 750 μg/ml EDS (open bars) in vehicle. The detached cells were collected and the apoptotic condensed nuclear chromatin identified by staining with acridine orange. The bars represent the mean ± SEM of five independent experiments. * indicates $P < 0.005$ as compared to the control value at the same time point.
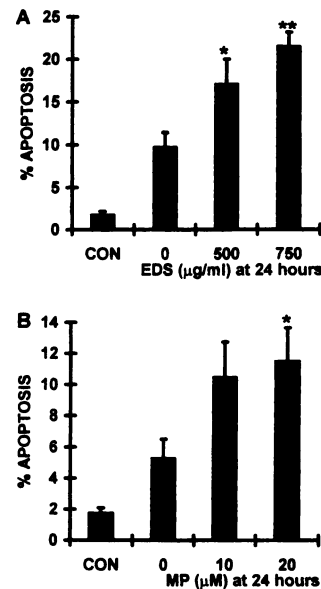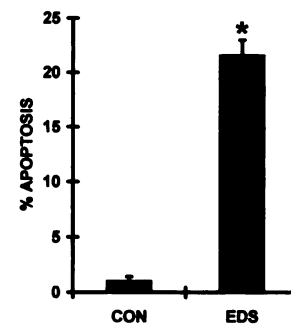

FIG. 6. Percentage of apoptotic cells within an interstitial cell preparation from rats treated with either vehicle (DMSO:water, 1:3, 2 ml/kg body weight) or EDS (100 mg/kg body weight in vehicle). The bars represent the mean ± SEM with * indicating $P < 0.005$; $n = 4$.

extremely rare in the cell population from the vehicle-treated rat, which would suggest that Leydig cells *in vivo* do not normally undergo apoptosis in contrast to the situation *in vitro*. MP, on the other hand, promoted the Leydig cell preparation to undergo apoptosis *in vitro* at a dose of 10 μM; whereas *in vivo,* a pharmacological dose of 100 mg/kg body weight appeared to have no effect. We are not able to indicate the *in vivo* significance of this result; however, glucocorticoids will decrease testosterone secretion after *in vivo* administration, although this is thought to occur by an inhibition of steroidogenic enzymes (Orr and Mann, 1992; Agular and Vind, 1995). Glucocorticoids have been shown to activate the cell death program in thymocytes (Wyllie, 1980), and EDS treatment of rats causes involution of the thymus (Leeming et al, 1991) as well as apoptosis of thymocytes *in vitro* (Morris et al, unpublished data).

Although the exact mechanism by which EDS causes apoptosis in Leydig cells is at present unknown, experiments suggest that the covalent binding of EDS to glutathione and its cellular depletion is essential for cytotoxicity (Kelce and Zirkin, 1993; Kelce, 1994). Depletion of cellular glutathione has been suggested to be the trigger for apoptosis in response to cytotoxic treatments in other cells (Beaver and Waring, 1995; Sugimoto et al, 1996). Another interesting observation was that the cytotoxic effects of EDS on adult rat Leydig cells were independent of new protein synthesis (Kelce and Zirkin, 1993). These experiments suggest that EDS induces the apoptotic response in Leydig cells by the alkylation of DNA and proteins without requiring new RNA or protein synthesis. This would appear to contradict the generally accepted view that suggests that new RNA and protein synthesis is required for programmed cell death. However, several reports have argued that active gene transcription and/or translation are not always required for apoptosis (Polunovsky et al, 1994; Gabai et al, 1995; Powell et al, 1995).

In summary, we have shown that Leydig cells can undergo apoptosis in response to the cytotoxin EDS both *in vitro* and *in vivo*. However, whether the apoptotic phenomena is purely a toxicological response of the Leydig cell or whether this process has some physiological significance remains to be elucidated.

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