Increased Expression of Estrogen Receptor β in Pachytene Spermatocytes After Short-Term Methoxyacetic Acid Administration

OSCAR M. TIRADO,* DAVID M. SELVA,* NÚRIA TORÀN,† CARLOS A. SUÁREZ-QUIAN,‡ MICHELLE JANSEN,§ DONALD P. MCDONNELL,§ JAUME REVENTÓS,* AND FRANCINA MUNELL*

From the *Unitat de Recerca Biomèdica and* †Departament d'Anatomia Patològica, Hospital Materno-Infantil Vall d'Hebron, Barcelona, Spain; ‡Department of Cell Biology, Georgetown University Medical Center, Washington, DC; and the §Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, North Carolina.*

ABSTRACT: Degeneration of primary spermatocytes by apoptosis occurs during normal spermatogenesis, as well as in several pathological conditions, including exposure to specific testicular toxicants. The mechanisms that regulate the death and survival of primary spermatocytes, however, are still not well understood. The recent localization of estrogen receptor beta (ERβ) and P450 aromatase in pachytene spermatocytes suggests a role for estrogens in this step of spermatogenesis. Using a well-known model of pachytene spermatocyte apoptosis in adult rats consisting of the administration of methoxyacetic acid (MAA), we investigated the participation of ERβ during the initial phase of apoptosis, prior to germ cell loss. Adult rats were treated with a single intraperitoneal dose of MAA, and DNA laddering analysis confirmed apoptotic cell death in the testis. In enriched germ cell fractions and testis from MAA-treated animals, ERβ mRNA increased significantly at 3 and 6 hours, respectively. Next, stage-specific induction of ERβ mRNA was demonstrated by use of laser capture microdissection of seminiferous tubules in combination with semiquantitative reverse transcription–polymerase chain reaction. The ERβ protein also increased significantly after 6 hours and was mainly immunolocalized in the cytoplasm of pachytene spermatocytes of afflicted tubules. The cytoplasmic localization was confirmed by Western blot analysis of isolated cytoplasmic and nuclear fractions of testicular extracts. Finally, the MAA activation of ERβ was tested in vitro in HepG2 cells cotransfected with ERβ and a reporter construct that contained a consensus estrogen responsive element. Addition of MAA at similar doses used in vivo elicited a similar estrogenic activation as did estradiol at 1 nmol/L concentration. The present results raise the possibility that cytoplasmic ERβ participates in the apoptotic process of pachytene spermatocytes induced by MAA. Whether MAA interacts with ERβ in the cytoplasm of primary spermatocytes, preventing the progression of the first meiotic division, however, remains to be determined.

Key words: germ cell apoptosis, testicular toxicity, Laser Capture Microdissection.

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Adult mammalian spermatogenesis is a highly coordinated process that includes cellular proliferation, division, differentiation, and, paradoxically, programmed cell death. Spontaneous germ cell apoptosis, for example, has been morphologically and biochemically documented in spermatogonia (Allan et al, 1992), primary and secondary spermatocytes, and spermatids (Kerr, 1992; Brinkworth et al, 1995; Blanco-Rodríguez and Martínez-García, 1996). In this regard, the prophase of the first meiotic division is one of the most strictly controlled steps of spermatogenesis and is composed of several checkpoints common to its mitotic counterpart, as well as steps that are best characterized as specific to meiosis. When cellular defects exceed the capability of the meiotic repair machinery, the meiotic progression is arrested, and spermatocytes die by apoptosis (Sassone-Corsi, 1997). Given that meiotic arrest associated with spermatocyte apoptosis is not a rare finding in human testicular biopsies in patients with infertility of unknown origin, it is of great interest to identify those genes that control meiotic progression. As such, investigators recently used knockout mouse technology to try to identify potential genes that participate in the apoptotic process in pachytene spermatocytes (Reventós and Munell, 1997; Sassone-Corsi, 1997).

The relationship between sex steroids and germ cell survival/death in the testis has been the subject of exten-

sive analysis and is known to entail the absolute requirement for androgens (Zirkin et al, 1989; Henriksen et al, 1995; Woolveridge et al, 1999), although the precise action(s) or the somatic cell most vitally responsive to androgens remains a matter of debate (Súarez-Quian et al, 1999). Of interest, a more comprehensive and emerging view of the hormonal control of spermatogenesis raises the possibility that androgens may not be the exclusive steroid responsible for its regulation and that estrogens may also participate in such capacity. First, germ cell apoptosis induced by incubating segments of human seminiferous tubules without survival factors can be inhibited by low concentrations of estradiol (Pentikainen et al, 2000). Second, estradiol treatment of the hypogonadal mouse, which congenitally lacks gonadotrophins and presents a meiotic arrest at the pachytene stage, induces the progression and completion of spermatogenesis in the absence of measurable androgen concentrations (Ebling et al, 2000). Third, estradiol treatment also induces the initiation of spermatogenesis in photo-regressed male Siberian hamsters (Park et al, 2002). Fourth, it has been shown that germ cells express cytochrome P450 aromatase (Nitta et al, 1993; Carreau and Levallet, 1997; Janulis et al, 1998, Levallet et al, 1998; Carreau et al, 2002) and that mice lacking functional aromatase are infertile and exhibit a reduction in round and elongated spermatids and an increase in germ cell apoptosis (Robertson et al, 1999).

A recently identified form of a second estrogen receptor, the estrogen receptor β (ERβ; Kuiper et al, 1996), was found to be expressed in pachytene spermatocytes as well as in other germ and/or somatic cells of the testis (Saunders et al, 1998; Van Pelt et al, 1999; Pentikainen et al, 2000; O'Donnell et al, 2001). Given that the survival of pachytene spermatocytes depends on androgens and no evidence of androgen receptors has been demonstrated in these cells, it is possible that androgens would somehow enter freely into germ cells (or be delivered by androgen-binding protein)—once there, they would be aromatized to estrogens. Alternatively, estrogens produced by testicular somatic cells (Carreau et al, 2002) could also enter into germ cells. In this scenario, ERβ could mediate the effects of estradiol in the regulation of primary spermatocyte survival and/or apoptosis.

In the present investigation, we chose a well-established model of pachytene spermatocyte apoptosis consisting in a single intraperitoneal injection of methoxyacetic acid (MAA) in adult rats that results in almost complete loss of pachytene spermatocytes (Brinkworth et al, 1995), to examine whether the massive and stage-specific cellular apoptosis entailed the alteration of normal ERβ levels. The temporal expression of ERβ mRNA and protein was analyzed during the time course of the apoptotic process and compared with the results of morphological and biochemical apoptotic assays. Analysis of the spatial

expression of ERβ mRNA was achieved using laser capture microdissection (LCM) in combination with reverse transcription–polymerase chain reaction (RT-PCR) of stage-specific seminiferous tubules (Suárez-Quian et al, 2000; Tirado et al, 2003). Furthermore, the activation of ERβ by MAA was tested in a cell-based transcription assay system using a luciferase reporter construct that contains a consensus estrogen responsive element cotransfected with ERβ in HepG2 cells. The data presented provide evidence for the induction of ERβ mRNA and protein prior to pachytene spermatocyte cell death by apoptosis and support the involvement of this receptor in the regulation of the meiotic progression of primary spermatocytes.

Materials and Methods

Animals and Treatments

Forty-five adult male Sprague-Dawley rats, weighing approximately 400 g, were used for the present experiments. The animals were maintained in standard conditions. All experimental procedures were conducted in accordance with institutional standards, which fulfill the requirements established by the Spanish Government and the European Community (BOE 67, 3/18/88, Real Decreto 223/1988, and BOE 256, 10/25/90). Thirty-three rats were treated with a single intraperitoneal injection of MAA (650 mg/kg body weight; Sigma-Aldrich, Steinheim, Germany), buffered to 7.4 pH in 0.9% saline water, and killed at 3 (n = 6), 6 (n = 6), 9 (n = 6), 12 (n = 3), or 24 (n = 3) hours or 3 (n = 3), 5 (n = 3), or 7 (n = 3) days after the drug administration. Six rats were used as controls and were either treated with vehicle (saline water) or not treated. Animals were killed by CO_2 asphyxiation. One testis from each animal was fixed in 4% paraformaldehyde for 24 hours and subsequently embedded in paraffin. The other testis was minced, immediately frozen, and used for DNA and RNA extraction, except for 12 rats (3 controls and 3 killed at 3, 6, and 9 hours), in which germ cell isolation preceded the DNA and RNA isolation. Serial 5-μm-thick sections, obtained from the 39 paraffin-embedded testis, were used for morphological examination after hematoxylin-eosin (H&E) staining, for in situ DNA fragmentation analysis, and for immunohistochemistry. Six additional animals, 3 controls and 3 rats killed after 12 hours of MAA administration, were used for LCM experiments.

DNA Isolation and DNA Fragmentation Analysis on Agarose Gel

Total DNA was isolated from individual frozen testes by digestion in lysis buffer (20 mmol/L Tris-HCl [pH 7.4], 1 mmol/L EDTA, and 1% sodium dodecyl sulfate [SDS])

and proteinase K (10 μg/mL) at 56°C overnight. DNA was extracted with phenol/chloroform and precipitated by the addition of sodium acetate and ethanol. The DNA was resuspended in low-TE buffer (20 mmol/L Tris-HCl [pH 7.4] and 1 mmol/L EDTA [pH 8]), and its concentration was measured by spectrophotometry. An equal amount of DNA from each sample was loaded onto 2% agarose gel and blotted to Hybond-N nylon membrane (Amersham) by overnight capillary transfer using 10× standard saline citrate (SSC; 3 mol/L NaCl and 0.3 mol/L trisodium citrate). Blots were hybridized at 42°C, overnight, using rat total genomic DNA labeled by random priming with ^{32}P d-CTP as the probe (Macaya et al, 1994). Finally, the membranes were washed in decreasing concentrations of SSPE (3.6 mol/L NaCl, 0.2 mol/L Na$_2$ HPO$_4$7H$_2$O, and 0.02 mol/L EDTA)/0.1% SDS and autoradiographed.

Histological Examination and In Situ End Labeling of Fragmented DNA (TUNEL)

TUNEL assay was done as described elsewhere (Selva et al, 2000). In brief, dewaxed and rehydrated sections were treated with 20 μg/mL proteinase K for 15 minuutes and with 3% hydrogen peroxide for 5 minutes. After an incubation with terminal deoxynucleotidyltransferase (TdT) buffer (25 nmol/L Tris HCl, 200 mmol/L cacodylate acid, and 200 mmol/L KCl) for 15 minutes, sections were treated with 0.05 U/μL TdT (Roche Molecular Biochemicals, Mannheim, Germany) and 0.5 nM biotin-16-deoxy(d)-UTP (Roche Molecular Biochemicals) in TdT buffer at 37°C for 90 minutes and with 300 mmol/L NaCl and 30 mmol/L SSC at room temperature for 15 minutes. After washing and incubating with 2% bovine serum albumin, sections were exposed to avidin-biotin complex (Vector Laboratories Inc., Burlingame, CA), diluted 1 : 25, at 37°C for 45 minutes, and the peroxidase reaction was visualized with diaminobenzidine and hydrogen peroxide. Counting individually labeled cells in at least 3 sections per sample served as our quantitation protocol.

Germ Cell Isolation

Germ cells were isolated from the testis as described elsewhere (Weiss et al, 1997; Selva et al, 2000). Testes were excised and washed with phosphate-buffered saline (PBS) supplemented with penicillin-streptomycin (5 mg/mL) and amphotericin (5 mg/mL), decapsulated, minced, and incubated in 100 mL of the above PBS solution for 8 minutes. The medium was removed, and the remaining testicular pieces were digested in trypsin (80 mg/mL)/PBS at 33°C for 10 min. The reaction was stopped by the addition of 25 mg/mL trypsin inhibitor, and the resulting solution was treated with deoxyribonuclease (0.4 mg/mL) at room temperature for 5 minutes. The isolated tubules were minced in a petri dish for 30 minutes and sequentially filtered through a 100-μm pore size nylon filter, a

fine glass-fiber filter, and a 20-μm pore size nylon filter. The recovered solution was centrifuged at 95 × *g* for 10 min, and the pellet was resuspended in 15 mL Dulbecco's minimum essential medium (DMEM)/Nutrient Mixture (NUT) mix F-12 culture medium (Life Technologies Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS). The cell suspension was incubated in the same medium in tissue culture flasks for 5 hours, and the supernatant, free of Sertoli cells, was recovered and centrifuged at 95 × *g* for 10 minutes. The pellet was frozen until its use for RNA extraction. This protocol ensures the purity of the germ cell fraction, as it has been shown using vimentin as a marker for Sertoli cell contamination (Selva et al, 2002).

Messenger RNA Isolation and Analysis of Its Expression by Semiquantitative RT-PCR

Total and messenger RNA were extracted from unseparated testicular cells and from isolated germ cells by means of guanidium thiocyanate/phenol-chloroform extraction (Chomzinsky and Sacchi, 1987) and by use of the Quickprep mRNA purification Kit (Pharmacia Biotech). One microgram of total RNA or, alternatively, 0.25 μg of eluted mRNA was reverse transcribed using 200 U of Superscript II Rnase H- Reverse Trancriptase (Gibco-BRL, Bethesda, MD), according to supplier's instructions. For rat ERβ, a 735-bp product was amplified (upper primer: TGCTGGATGGAGGTGCTAATG, lower primer: ACACAACCACCCTGACTCCT) and for the rat ribosomal protein L19, a 486-bp fragment was obtained (upper primer: CAATGCCAACTCTCGTCAAC, lower primer: CTTGGTCTCTTCCTCCTTGG). Amplification consisted of 40 cycles for ERβ and 32 cycles for the control gene L19, both within the linear growth phase of the PCR reaction. Annealing was done at 57°C for 30 seconds. PCR products were separated on a 2% agarose gel and quantified by the Molecular Analyst/Macintosh data analysis software using a Bio-Rad Image Analysis System (Bio-Rad Laboratories Inc., Hercules, CA). The products of amplification were purified using the QIAquick PCR Purification Kit (Quiagen, Hilden, Germany), according to supplier's instructions, and sequenced using an Abi Prism 310 genetic analyzer (Perkin-Elmer Corp.).

ERβ Immunohistochemistry

For ERβ immunodetection, tissue sections were deparaffinized and treated with 10% methanol and 3% hydrogen peroxide in PBS for 5 min. The slides were then incubated with 3% normal rabbit serum and with 3 μg/mL of a goat anti-rat ERβ polyclonal antibody that recognizes the amino terminus domain of the rat protein (sc-6821; Santa Cruz Biotechnology, Santa Cruz, CA), at 4°C, for 16 hours. After PBS washing, sections were exposed to biotinylated rabbit anti-goat IgG as a secondary antibody

for 30 minutes (Vector Laboratories) and treated with avidin-biotin complex (Vectastain ABC Kit; Vector Laboratories) for 45 minutes. Bound peroxidase was visualized using 0.01% hydrogen peroxide and 0.05% diaminobenzidine in PBS.

Preparation of Cytoplasmic and Nuclear Extracts

Cytoplasm and nuclear extracts of total testis were prepared as follows: decapsulated testes were immersed in buffer A (10 mmol/L Tris-HCL [pH 7.4], 2 mmol/L EDTA, and 0.25 mol/L sucrose) that contained the protease inhibitors phenylmethylsulfonyl fluoride (0.2 mmol/L), aprotinin (5 mg/mL), and leupeptin (5 mg/mL). Testes were homogenized in a motorized homogenizer and then centrifuged at $800 \times g$ for 10 minutes. The supernatant was centrifuged at $10\,000 \times g$ for 30 minutes, to obtain the cytosolic fraction. The nuclear pellet was washed and resuspended in RIPA buffer (50 mmol/L Tris-Cl [pH 7.5] and 150 mmol/L NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) that contained the protease inhibitors described above, then lysed in the homogenizer and centrifuged at $10\,000 \times g$ for 30 minutes. The supernatant was kept as the nuclear fraction.

Protein Extraction and Western Blot Analysis

Fractions of total testis were lysed with RIPA buffer that contained protease inhibitors (as described above), and the lysates were centrifuged at $13\,000 \times g$ at 4°C for 30 minutes. The protein content of the supernatant was determined by the Bradford assay (Bio-Rad Laboratories). Equals amounts of protein (30 μg) were resolved by 10% SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After blocking, the membranes were incubated at 4°C overnight with 2 μg/mL of 2 rabbit polyclonal antibodies raised against the aminoterminal region of mouse ERβ (sc-6821; Santa Cruz Biotechnology, and PA1–311; Affinity Bioreagents Inc., Golden, CO) and then for 1 hour at room temperature with horseradish peroxidase–conjugated secondary antibody (1 : 2000). Peroxidase activity was analyzed with the ECL system (Amersham Pharmacia Biotech), according to the manufacturer's instructions. The ERβ content was determined densitometrically. To confirm that similar amounts of protein were loaded onto each lane, membranes were stained with Coomassie brilliant blue R-250.

LCM

Stage-specific seminiferous tubules were harvested from frozen, testicular sections of control and MAA-administered rats by LCM, as described elsewhere (Suárez-Quian et al, 2000; Tirado et al, 2003). Specifically, 5-μm frozen sections were cut on a standard cryostat with a fresh blade, and 2 sections each were mounted on Colorfrost Plus glass slides (Fisher Scientific, Pittsburgh, PA). The unfixed sections were immediately stored at −80°C. The frozen sections were thawed at room temperature for 30–60 seconds without drying and immersed in 70% ethanol to fix for 30 seconds. After fixation, slides were stained with hematoxylin (1 minutes) and eosin (15 seconds), dehydrated in ascending grades of ethanol (70%, 90%, and 100%), twice in each for 1 minute, and immersed in Xylene, twice, for 5 minutes each. The seminiferous tubules were staged using the scheme of Leblond and Clermont (1952) and microdissected using a Pixell II apparatus (Arcturus, Mountain View, CA). An infrared laser pulse of 90 mW and beam size of 30 μm were pulsed over the tubules of interest, the ethylene vinyl acetate thermoplastic film was melted directly onto these tubules, and the captured tissue was transferred to a thermoplastic polymer film–coated cap by the 1-step transfer method. The transfer film cap was placed directly into a microcentrifuge tube for RNA extraction.

Total RNA Extraction from Captured Seminiferous Tubules

Total RNA was obtained from microdissected tubules by using the Micro RNA isolation Kit (Stratagene, La Jolla, CA), according to the manufacturer's instructions.

Cell Culture and Transient Transfection Assay

Human hepatoma (HepG2) cells were maintained at 37°C, 5% CO_2 in MEM (Invitrogen Corp., Carlsbad, CA) containing phenol red and supplemented with 10% FBS (Hyclone Laboratories Inc., Logan, UT), 1 mmol/L sodium pyruvate, and 100 nmol/L nonessential amino acids (Invitrogen). Cells were seeded into 24-well plates at 60% confluence 24 hours before transfection. Cells were washed once with $1\times$ PBS and transfected with a lipofectin (Invitrogen)–DNA mixture that contained 3 μg total DNA per triplicate. Transfection mixtures contained 2000 ng $3\times$ ERE TATA Luc, 100 ng CMV β-galactosidase, 500 ng pRST7 hERβ, and 400 ng pBS-KSII+ (Stratagene). After a 5-hour transfection, medium was replaced with phenol red–free MEM supplemented with 10% dextran-stripped FBS (Hyclone Laboratories), 1 mmol/L sodium pyruvate, and 100 nmol/L nonessential amino acids. The cells were incubated for 16 hours before 17β-estradiol or increasing concentrations of MAA were added to the transfected cells and treated for 24 hours. A 20-mmol/L stock MAA solution was prepared by adding 98% pure MAA to the medium and adjusting the pH to 7.4 with 10 N NaOH. Subsequently, the cells were lysed and assayed for luciferase and β-galactosidase activities. Luciferase activity was divided by β-galactosidase activity, to normalize for transfection efficiency. Data are presented as the average of triplicate transfections, and the assay was repeated 3 times.
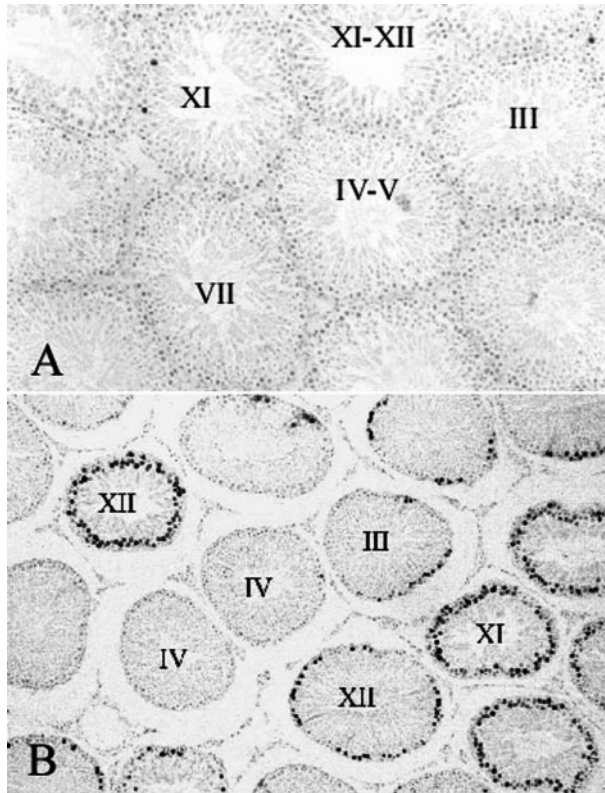
Figure 1. TUNEL assay in testicular sections of control rats **(A)** and rats killed after 24 hours of MAA administration **(B).** Note the specific TUNEL staining in some tubule sections whose stages of the cycle of the seminiferous epithelium are indicated by roman numerals.

Quantification of the TUNEL-labeled pachytene spermatocytes as a function of the seminiferous epithelium stage. Three different rats killed at each time point after the administration of MAA were used, and the TUNEL-positive pachytene spermatocytes were counted in 10 testicular cross-sections per animal. Data are expressed as the percentage of TUNEL-positive pachytene spermatocytes

Hours After MAA	I–III	VII–VIII	XI–XIII
3	0	0	0.70 ± 0.24
6	1.93 ± 0.32	0	4.03 ± 1.34
9	20.70 ± 1.75	0	24.56 ± 1.89
12	14.60 ± 2.3	0	58.50 ± 0.86
24	6.93 ± 0.77	0	71.90 ± 2.51

Statistical Analysis

For experiments shown in Figures 2, 4, 5, and 6 below, at least 3 replications were used for each group within an experiment. Analysis of variance was used to assess statistical significance between group means, and groups were considered to be statistical different at $P \leq .05$.

Results

Apoptosis in Pachytene Spermatocytes after MAA Administration

A single intraperitoneal dose of MAA leads to significant apoptosis in rat testis, as revealed by the appearance of a laddering pattern when the total genomic testicular DNA was electrophoresed in agarose gel, blotted, and hybridized with total rat DNA as a probe. This characteristic pattern, the hallmark of apoptosis, was observed in testicular samples obtained 24 hours after MAA administration, as described elsewhere (Brinkworth et al, 1995), and no laddering was detected in testes removed at 3, 5, and 7 days after MAA administration (data not shown). No DNA fragmentation was noted in control testes.

To further characterize the experimental model, TUNEL assays were done in the testis of the MAA-treated animals as well in controls. In control testes, isolated TUNEL-labeled cells were detected in some seminiferous tubules, but, in general, there was a lack of positive staining in most tubule profiles (Figure 1A). At 3 and 6 hours after MAA administration, there were no significant differences in the number of TUNEL-labeled cells, compared with control testes, but a gradual increase was noted at 9–12 hours that remained high at 24 hours. The majority of labeled cells were identified as pachytene spermatocytes (Figure 1B). Three days after the administration of MAA, primary spermatocytes were absent in most tubule stages and only a few, isolated labeled cells, identified as degenerating spermatogonia, could be discerned. Similar images were obtained in testis analyzed at 5 and 7 days after the toxicant (data not shown).

Examination of TUNEL labeling at low magnification revealed a stage-specific distribution of the reaction product in pachytene spermatocytes beginning 6 hours after the compound's administration (Table 1). Although positive TUNEL staining was present in the majority of stages, maximal staining was observed in latter stages of the cycle of the seminiferous epithelium, specifically at stages X–XIII.

ERβ mRNA Expression after MAA Administration

The levels of ERβ mRNA were examined by semiquantitative RT-PCR in both unseparated testicular cells (a total testis cell fraction) and in isolated gem cells. In the unseparated testicular cells, a significant increase in ERβ mRNA expression became noticeable at 6 hours after MAA administration and remained altered after 9 hours. In the isolated germ cells, although a progressive increase was noted starting at 3 hours (Figure 2), a significant increase in the ERβ mRNA levels was not discerned until 9 hours after MAA exposure. The sequence analysis of the products confirmed the identity of the amplified
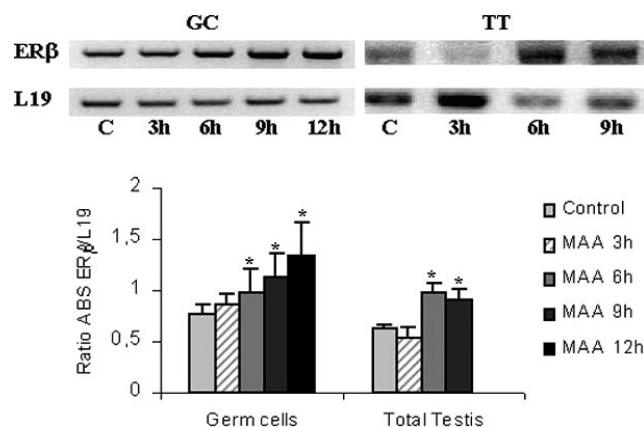
Figure 2. Changes of ERβ mRNA levels during pachytene spermatocyte apoptosis induced by MAA administration. A representative agarose gel showing the RT-PCR results obtained when the unseparated testicular cells (TT) and isolated germ cells (GC) of control (C) and MAA exposed rats at different time-points were amplified using specific primers for rat ERβ and L19. The ratios between the ERβ and L19 band intensity were calculated and represented in the lower graph. In isolated germ cells, a significant increase in ERβ mRNA versus control was noted 3–12 hours after MAA administration, whereas when testicular cells that were not separated were analyzed, a significant increase in ERβ mRNA was not detected until 6 h after MAA exposure. Asterisk represents significance at $P < .05$.

cDNA bands with the sequences used to design the primer probes.

ERβ Immunodetection in Pachytene Spermatocytes after MAA Administration

In previous immunohistochemical studies, ERβ was shown to reside in the nuclei of Sertoli cells and pachytene spermatocytes, although no differential stage-specific intensity immunostaining was observed (Saunders et al, 1998). Using an antibody against the amino-terminal region of ERβ, we also examined its distribution in control and in MAA-treated rats (Figure 3). In control animals, ERβ immunoreactivity was present in the nuclei of Sertoli cells and pachytene spermatocytes and in the cytoplasm of pachytene spermatocytes and elongated spermatids, although this latter labeling appeared as a faint but widespread immunostaining (Figure 3A). In some tubules, however, robust ERβ immunostaining could be observed in isolated pachytene spermatocytes appearing to be in a state of cell degeneration. These degenerating cells were characterized by chromatin clumping and a lack of cytoplasmic density. Beginning at 9 hours and remaining up to 24 hours after MAA administration, an intense ERβ immunostaining was noted in the cytoplasm of a significant number of pachytene spermatocytes residing in stages X–XIII of the cycle of the seminiferous epithelium (Figure 3B–E), corresponding to the stages with increased number of TUNEL-labeled cells (Figure 3F and 3G). In stages II–III, a lower (but readily detectable) number of

cells were also positive for ERβ (Figure 3C) and TUNEL (Figure 3F). In stages VII–VIII, a faint ERβ immunostaining was observed only in a few pachytene spermatocyte cells (Figure 3D), but no TUNEL labeling was ever detected in the pachytene spermatocytes inhabiting these stages (Figure 3G). Furthermore, careful inspection of the ERβ immunohistochemistry of labeled pachytene spermatocytes revealed a significant, punctuate immunostaining within the nuclei of the cells. However, because of the high intensity of the cytoplasmic reaction in pachytene spermatocytes of MAA-treated rats, the reaction was stopped earlier in these sections than in controls, and the nuclear immunostaining was often masked and easily missed unless a thorough focus of the pachytene spermatocyte nucleus was done.

Up-regulation of ERβ Protein after MAA Exposure Measured by Western Blot Analysis

To verify the results obtained by inmunohistochemistry, Western blot analysis was done in cytosolic and nuclear testicular extracts, using 2 different antibodies raised against peptidic sequences of the N-terminal region of rat ERβ. Both antibodies recognized a single 55-kDa protein species, and the specificity of the reaction was demonstrated by the disappearance of the band when one of the antibodies (Santa Cruz Biotechnology) was incubated with the corresponding peptide (Figure 4). Expression of the ERβ protein was detected in the cytoplasmic and nuclear fractions, but the most significant increase was noted in the cytoplasmic fraction during the time course of the experiment (Figure 4), in agreement with the results obtained by immunohistochemistry.

Capture of Seminiferous Tubules in a Stage-Specific Manner and mRNA Expression of ERβ

LCM was done successfully using frozen testicular sections fixed with 70% ethanol, obtained from 3 control rats and 3 rats killed 12 h after the MAA administration. The different stages of the seminiferous epithelium were optically identified in H&E-stained sections at $630\times$ magnification (Figure 5A). A total of 50 seminiferous tubules, at stages III–IV, VII–VIII, or X–XIII, respectively, were selected for microdissection. The stage-specific tubular sections were captured without interstitial contamination (Figure 5B). The mRNA expression of ERβ and L19 as a control was analyzed in the RNA extracted from these tubules by RT-PCR using the specific primers already described. A clear up-regulation of ERβ mRNA was observed in all tubules obtained from injected animals, compared with controls (Figure 5C).

MAA Effects on ERβ

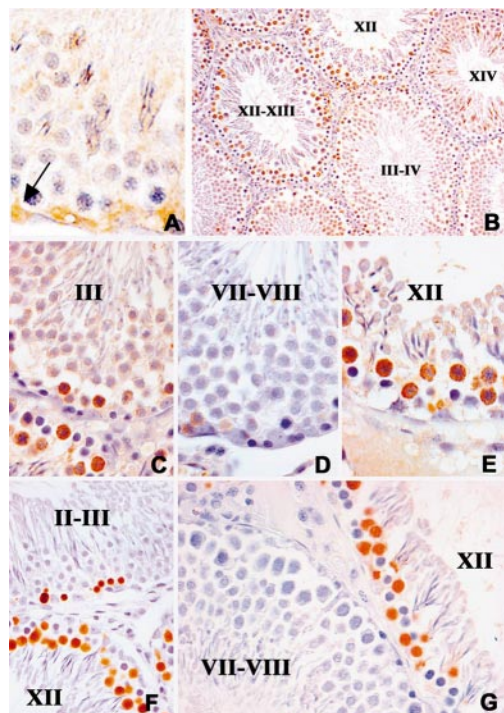Direct effects of MAA on ERβ activation were examined using HepG2 cells stably cotransfected with a specific

Figure 3. Immunohistochemical localization of ERβ in control and MAA-exposed rats and stage-specific colocalization with TUNEL labeling. In control rats, ERβ labeling was found predominantly in Leydig and Sertoli cells (arrow), although a faint staining (barely detectable at this magnification) was observed in some pachytene spermatocytes and elongated spermatids **(A).** At 24 hours after the MAA administration, a marked ERβ immunostaining was noted in the cytoplasm of a significant number of pachytene spermatocytes in a stage-dependent fashion **(B).** This enhanced cytoplasmic staining of ERβ in pachytene spermatocytes is clearly visible in panels C and E. A comparison between ERβ immunostaining **(C–E)** and TUNEL staining **(F–G)** illustrates that robust ERβ cytoplasmic staining is coincident with TUNEL staining of pachytene spermatocytes residing in late-stage tubules. The stages of the cycle of the seminiferous epithelium are indicated with roman numerals.

ERβ-responsive element and a luciferase reporter system. Results indicated that MAA at 5 mmol/L had an identical ability to activate the ERβ as estradiol at 1 nmol/L concentration (Figure 6).

Discussion

We investigated the possible role of ERβ in pachytene spermatocyte apoptosis induced by the well-known in vivo and in vitro testicular toxicant MAA (Foster et al, 1987; Bartlett et al, 1988). As previously demonstrated, the administration of MAA to adult rats caused a massive and highly specific depletion of primary spermatocytes 24 hours after treatment (Brinkworth et al, 1995; Clark et al, 1997). To further examine the short-term effect of MAA on spermatogenesis, MAA was administered via a single intraperitoneal injection instead of by gavage, a route previously demonstrated to also exercise a long-term, deleterious effect on pachytene spermatocytes (Krishnamurthy et al, 1998). In the present study, DNA fragmentation was assayed by means of TUNEL, and it was first detected in pachytene spermatocytes 9 hours after the MAA administration and reached maximum levels at 24 hours. After 3 days, the majority of primary spermatocytes were absent in most tubular stages, a finding that corroborates previous reports (Clark et al, 1997). Except for the robust labeling of pachytene spermatocytes, only a few other, isolated cells, identified as degenerating spermatogonia, could be discerned that were labeled by TUNEL. Similar images were obtained in testis analyzed at 5 and 7 days after administration of the toxicant. The apoptotic nature of this degeneration was also confirmed by the presence of the characteristic ladder pattern of DNA fragmentation in the testis of rats killed 24 after MAA treatment.

All results of the present investigation point to the fact that the short-term exposure of rats to MAA leads to the rapid increase in ERβ protein and mRNA levels in pachytene spermatocytes destined to undergo apoptosis. The authenticity of the immunostaining results was confirmed using Western blot analysis of cytosolic and nuclear testicular extracts, using 2 distinct commercial antibodies, and, again, testicular cells treated with MAA rendered a more robust signal for cytoplasmic ERβ than control cells. Taken together, these results are consistent with the interpretation that MAA action resulting in pachytene spermatocyte apoptosis entails the disruption of the normal expression levels and disposition of ERβ in these cells.

These observations led us to pose 3 questions. First, does MAA directly and/or indirectly affect ERβ levels, or are the effects mediated via other cells of the seminiferous tubules, possibly the Sertoli cell? Second, does MAA exhibit ERβ activity? Third, does aberrant ERβ exert a direct role in pachytene spermatocyte apoptosis? Regarding question 1, aside from data presented in the present article, there is no evidence either way to definitively settle this question in germ cells. Unfortunately, isolated pachytene spermatocytes do not withstand prolonged cultured conditions to rigorously test this hypothesis. Nevertheless, given our in vivo findings that both ERβ mRNA and protein are elevated in afflicted pachytene spermatocytes, a reasonable conclusion is that MAA alters ERβ levels in these cells. Whether it does so directly or via Sertoli cells, however, remains to be determined. Regarding question 2, we demonstrated that the addition of MAA at similar doses used in vivo (5 mmol/L) to HepG2 cells cotransfected with ERβ and a luciferase reporter construct containing a consensus estrogen responsive element elicited a similar estrogenic activation as estradiol at 1 nmol/L concentration. Thus, these results confirm
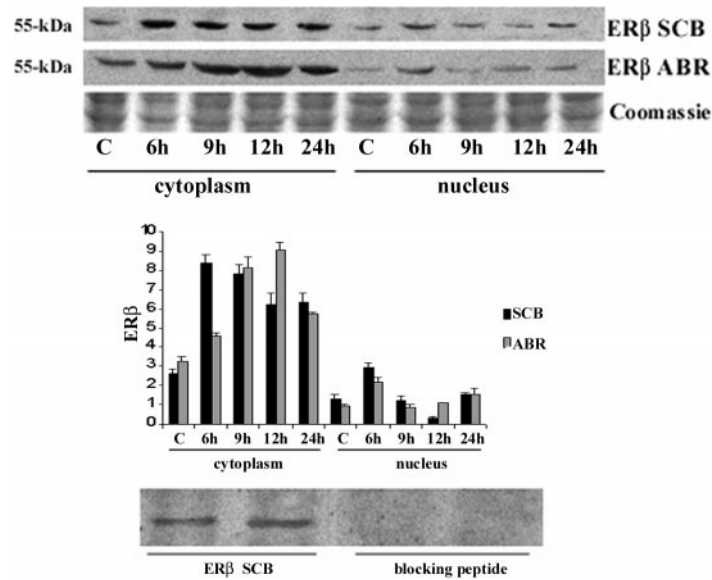
Figure 4. Changes of ERβ protein levels during pachytene spermatocytes apoptosis induced by MAA administration. Western blot analysis of proteins isolated from cytoplasmic and nuclear testicular extracts of control rats (C) and rats killsed at different time points (6, 9, 12, and 24 hours) after MAA administration were done using 2 antibodies (SCB: Santa Cruz Biotechnology, and ABR: Affinity Bioreagents Inc.) raised against the N-terminal region of the ERβ. Representative blots and the mean and standard deviation values of ERβ are illustrated. Both antibodies recognized a single 55-kDa–specific band that was blocked when the SCB antibody was incubated with the corresponding peptide (lower panel). Although the expression of the ERβ protein was present in both the cytoplasmic and nuclear extracts, the most significant increase was detected in the cytoplasm 6–24 hours after the MAA administration.
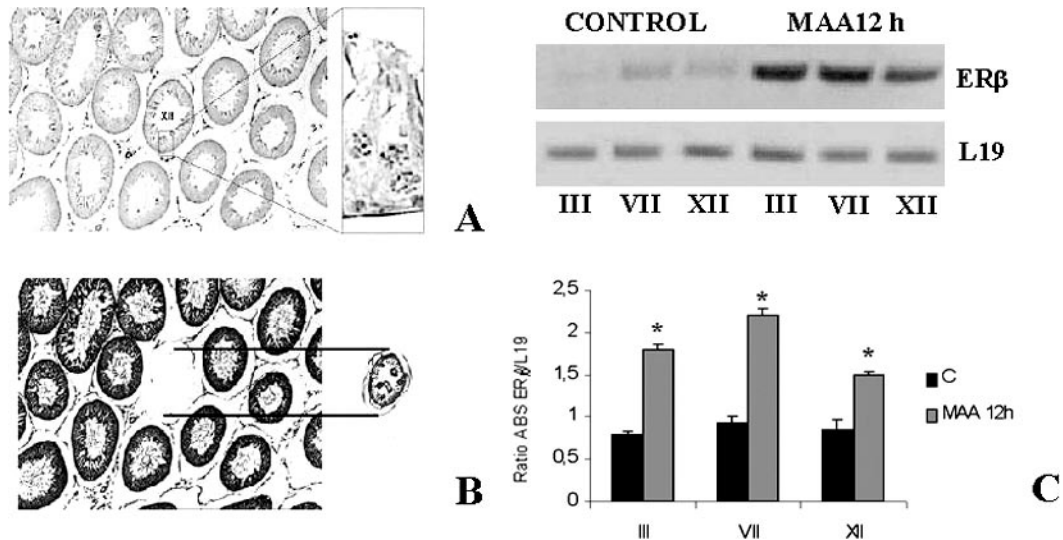

Figure 5. Analysis of the stage specific ERβ mRNA expression by LCM in the testis of control rats and after 12 hours of MAA administration. The stages of the seminiferous epithelium were identified in cryostat sections, fixed in ethanol, and stained with H and E **(A)**, and the stages of interest (III, VII, and XII) were harvested by LCM **(B)**. The mRNA isolated from selected tubules was retrotranscribed and amplified using specific primers for ERβ and L19. A representative blot and the mean and standard deviation values of the ERβ/L19 ratios obtained from the 3 controls and 3 MAA-exposed rats at 12 hours are illustrated **(C)**. Note that the expression of ERβ was low but detectable in stages VII and XII of control testis and increased significantly after the MAA administration. ERβ mRNA represent both Sertoli and germ cell levels, because not attempts were made to separate Sertoli cells from the targeted and captured material.
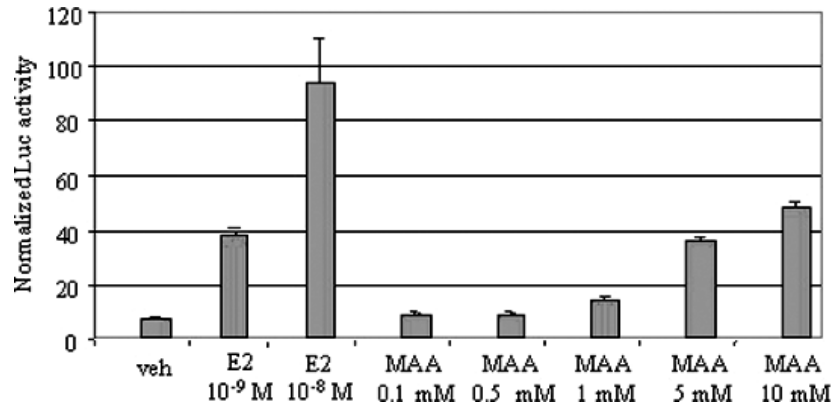
Figure 6. Stimulation of ERβ-mediated transactivation of a reporter gene by MAA. HepG2 cells were cotransfected with 2 μg of the luciferase reporter gene driven by 3 copies of a consensus estrogen response element (3× ERE TATA Luc), 500 ng pRST7-ERβ, 100 ng pCMV-β-galactosidase, and 400 ng pBSKSII. After transfection, cells were incubated for 16 hours in phenol red–free medium that contained charcoal-stripped fetal bovine serum. Cells were then treated for 24 hours with vehicle, 17β-estradiol (E2), or increasing concentrations of MAA. Cell lysates were assayed for luciferase and β-gal activities. Luciferase activity was normalized to β-galactosidase activity as a control for transfection efficiency. Data are presented as the averages of triplicate transfections.

that MAA exhibits ERβ activity, at least in a model system engineered to demonstrate this activity. Regarding question 3, ongoing experiments and evidence from the literature favor the interpretation that elevated levels of ERβ are associated with germ cell apoptosis, especially given that we demonstrated here when it is located in the cytoplasm. First, in the present experiments, we observed repeatedly that significant ERβ immunostaining was found in scattered, isolated germ cells undergoing degeneration in control testis, which possibly suggests that ERβ has an active role in this step during normal spermatogenesis. Conversely, we showed that, in primary spermatocytes of seminiferous tubular stage VII, the stage where the germ cells are least at risk of degeneration in normal rats, TUNEL staining was absent and ERβ was nearly impossible to detect in the MAA-treated rats (Kerr, 1992). Although ERβ mRNA expression was found in stages VII–VIII of MAA-treated animals by microdissection, it is possible to speculate that this mRNA will be translated into protein at advanced stages and that the protein will accumulate in the stages found by immunohistochemistry. In addition, some of the ERβ mRNA is certainly attributable to Sertoli cells, because no attempt was made to isolate by LCM germ cells that were free of Sertoli cells. An alternative explanation for the lack of correlation between the levels of mRNA and protein, however, may be the fact that the ERβ protein could be more strictly regulated than the mRNA. In this regard, it has been shown that both ERα and β (Alarid et al, 1999; Tschugguel et al, 2003) can be rapidly regulated via a proteasome-mediated pathway.

A second piece of evidence that associates ERβ and apoptosis is the detection of a robust ERβ immunoreactivity in degenerating pachytene spermatocytes in another model of primary spermatocyte apoptosis (unpubl. data), the androgen-binding protein transgenic mouse (Selva et al, 2000). Given this apparent association of enhanced ERβ mRNA and protein with apoptotic pachytene spermatocytes, ongoing experiments using aromatase inhibitors will test directly whether the inhibition of P450 activity will produce a protective effect on MAA-induced apoptosis of pachytene spermatocytes. If our prediction holds true that aberrant ERβ expression is intimately involved in pachytene spermatocyte apoptosis, then the inhibition of estrogenic activity, regardless of ERβ levels after MAA treatment, should dramatically diminish pachytene spermatocyte apoptosis. In addition, other experiments will use antiestrogenic compounds to try and dissect out whether ERβ involvement in apoptosis works via genomic effects or whether the cytoplasmic disposition of the receptor is indicating a nongenomic action heretofore unknown in the apoptotic process.

The enhanced cytoplasmic expression of ERβ observed by immunohistochemistry and Western blot analysis, and our suggestion that it may be directly implicated in the apoptotic process, may appear to be in conflict with the ability of MAA to potentiate the genomic effects of estrogen detected in the transfection/promoter model system. As demonstrated in "Results," however, it is important to note that, even in the presence of enhanced cytoplasmic ERβ expression, nuclear disposition of the receptor was present. Thus, it is possible that MAA does exert a genomic effect and that the enhanced cytoplasmic presence of the ERβ is merely an inability of nuclear targeting of a steroid receptor in dying cells. Alternatively, as has been suggested for other nongenomic actions of steroid receptors (Beato and Klug, 2000; Moggs and Orphanides, 2001), perhaps it is now necessary to spec-

ulate that the enhanced expression of the ERβ in the cytoplasm is associated with apoptosis.

Moreover, the functional studies demonstrating elevated levels of ERβ with pachytene spermatocyte apoptosis described above are consistent with emerging concepts in the literature that link elevated ERβ levels to cellular apoptosis. For example, estradiol was shown to function either as a neuroprotective agent or as an inducer of apoptosis in cultured neurons, depending on the estrogen receptor subtype present in the cell; ERα was a neuroprotective factor, but ERβ acted as a mediator of apoptosis (Nilsen et al, 2000). Similar to our findings, ERβ immunoreactivity was increased in the cytoplasm of degenerative hippocampal neurons in patients with Alzheimer disease (Savaskan et al, 2001) and in colonocytes of a cell line that lacked ERα expression (COLO205) and degenerated by apoptosis when the cells were treated with estradiol (Qiu et al, 2002). Conversely, ERβ expression was diminished in conditions where cell survival was favored—breast, colon, ovarian, and prostate cancers (Leygue et al, 1998; Pujol et al, 1998; Foley et al, 2000; Iwao et al, 2000; Rutherford et al, 2000; Campbell-Thompson et al, 2001; Horvath et al, 2001; Pasquali et al, 2001; Roger et al, 2001) and ERβKO mice at 1 year of age develop lesions in their prostates similar to intraepithelial neoplasia (Weihua et al, 2002). All of these data support the hypothesis that ERβ can act as a negative regulator of proliferation. In addition, because the immunohistochemical localization of the ERβ seems to be different as a function of the specific domain recognized by each ERβ antibody (Rosenfeld et al, 1998) and several forms of ERβ have been described in testis (Makinen et al, 2001), it is possible that the induction of apoptosis depends on the specific ERβ variant present in the cell.

In summary, although the normal expression of ERβ in pachytene spermatocytes implicates its function in normal spermatogenic progression, clearly, whether the elevation of ERβ message and protein levels under certain conditions is an epiphenomenon of apoptosis or a causative mediator merits further investigation. At the present time, the accumulating body of evidence seems to favor the interpretation that ERβ is a direct participant in this process. Because, in general, endocrine disruptors have a high affinity for ERβ, this action may help explain the fertility problems caused by a large number of chemical compounds exhibiting estrogenic activity.

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