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# Interactions of methoxyacetic acid with androgen receptor ☆

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ABSTRACT

Endocrine disruptive compounds (EDC) alter hormone-stimulated, nuclear receptor-dependent physiological and developmental processes by a variety of mechanisms. One recently identified mode of endocrine disruption is through hormone sensitization, where the EDC modulates intracellular signaling pathways that control nuclear receptor function, thereby regulating receptor transcriptional activity indirectly. Methoxyacetic acid (MAA), the primary, active metabolite of the industrial solvent ethylene glycol monomethyl ether and a testicular toxicant, belongs to this EDC class. Modulation of nuclear receptor activity by MAA could contribute to the testicular toxicity associated with MAA exposure. In the present study, we evaluated the impact of MAA on the transcriptional activity of several nuclear receptors including the androgen receptor (AR), which plays a pivotal role in the development and maturation of spermatocytes. AR transcriptional activity is shown to be increased by MAA through a tyrosine kinase signaling pathway that involves PI3-kinase. In a combinatorial setting with AR antagonists, MAA potentiated the AR response without significantly altering the $EC_{50}$ for androgen responsiveness, partially alleviating the antagonistic effect of the anti-androgens. Finally, MAA treatment of TM3 mouse testicular Leydig cells markedly increased the expression of *Cyp17a1* and *Shbg* while suppressing *Igfbp3* expression by ~90%. Deregulation of these genes may alter androgen synthesis and action in a manner that contributes to MAA-induced testicular toxicity.


## Introduction

Endocrine disruptive compounds (EDC) modulate hormonal signaling causing adverse physiological responses. Direct binding to steroid hormone receptors (nuclear receptors) leading to receptor activation, or to receptor antagonism, is a well-studied mode of endocrine disruption. However, recent studies have established that EDCs can interfere with hormone signaling through other, indirect mechanisms with effects on nuclear receptors that mediate steroid hormone action (Tabb and Blumberg, 2006). These indirect mechanisms include modulation of coactivator expression (Inoshita et al., 2003; Lonard et al., 2004), alteration of the rate of proteasome-dependent nuclear receptor degradation (Masuyama et al., 2002), changes in DNA methylation (Anway et al., 2005) and hormone sensitization (Jansen et al., 2004). Hormone sensitizing EDCs affect nuclear receptor activity *via* non-genomic intracellular signaling pathways, and may lead to an increase in the intrinsic transcriptional activity of the receptor without direct interactions between the EDC and the hormone or its receptor.

MAA, the toxic metabolite of the widely used industrial solvent ethylene glycol monomethyl ether (EGME) (Welsch, 2005; Bagchi and Waxman, 2008), is a hormone sensitizer that enhances the transcriptional activity of several nuclear receptors without itself being a hormone mimetic (Jansen et al., 2004). The most noticeable outcome of MAA exposure in males is testicular degradation (Li et al., 1996; Krishnamurthy et al., 1998) due to apoptosis of pachytene spermatocytes (Ku et al., 1994). The precise testicular cell target(s) of MAA and the mechanism whereby it induces germ cell apoptosis are not known. Spermatocyte apoptosis could result from direct actions of MAA on germ cells and/or could be due to indirect effects mediated through somatic cells, including Sertoli cells and/or Leydig cells, in the testis. Nuclear receptors are expressed in both germ cells and somatic cells of the testis and play an important role in spermatocyte survival and maturation. Thus, androgen receptor (AR) is required for androgen biosynthesis and spermatogenesis (Roberts and Zirkin, 1991; De Gendt et al., 2004) and estrogen receptor (ER) α is an essential mediator of the effects of estrogen on Leydig cell development (Abney, 1999). ERβ is widely expressed in testicular germ cells and somatic cells and likely contributes to germ cell maturation (O'Donnell et al., 2001). Retinoic acid receptors (RARs) also play a critical role in testes development and spermatogenesis (Vernet et al., 2006). Effects of MAA on these receptors could thus disrupt the critical physiological balance that governs nuclear receptor activity in the testes, leading to the observed germ cell toxicity.

In the present study, we investigate the effects of MAA on the transcriptional activity of AR and several other nuclear receptors. MAA is shown to enhance AR-dependent transcription without alteration of the receptor's intrinsic androgen responsiveness. This potentiation of AR activity is shown to require tyrosine kinase signaling that is

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independent of the Ras→MEK→ERK signaling pathway but requires PI3 kinase activity. Furthermore, in a combinatorial setting with AR antagonists, MAA is shown to partially alleviate the antagonistic effect of anti-androgens, particularly at androgen concentrations that induce a low, suboptimal response. Finally, MAA is shown to alter the expression of several genes associated with androgen action in studies carried out in a cultured mouse Leydig cell model.

## Materials and methods

*Chemicals.* Dimethyl sulfoxide, testosterone, 17β-estradiol, tri-iodothyronine, all trans-retinoic acid, MAA, horse serum, linuron and vinclozolin were purchased from Sigma Chemical Co. (St. Louis, MO). The synthetic progesterone R5020 (promegestone) was purchased from Perkin Elmer Inc. (Waltham, MA). U0126 and antibodies against ERK (cat. #9102), and phosphorylated ERK (cat. #9101), were purchased from Cell Signaling Technology, Inc. (Danvers, MA). SB203580, SP600125, bisindolylmaleimide, LY294002, and PP2 were obtained from Calbiochem (San Diego, CA) and PD98059 from BioSource International (Camarillo, CA). DMEM and DMEM-F12 culture medium, fetal bovine serum (FBS) and TRIzol reagent were purchased from Invitrogen Corp. (Carlsbad, CA). Bicalutamide was a gift from AstraZeneca Pharmaceuticals (Waltham, MA).

*Plasmids.* The human AR expression plasmid pSG5-hAR and the androgen-responsive reporter plasmid pProbasin-Luc, which contains two naturally occurring androgen response elements (AREs), were obtained from Dr. Ollie Janne (Institute of Biomedicine, Univ. of Helsinki, Finland). The human ERα and ERβ expression plasmids, pcDNA3.1-hERα and pcDNA3.1-hERβ, were obtained from Dr. Myles Brown (Dana-Farber Cancer Institute, Boston, MA). The ER-C3-luciferase reporter plasmid was obtained from Dr. Kevin Gaido (CIIT Centers for Health Research, Research Triangle Park, NC). Thyroid hormone receptor (TR)-β expression plasmid, pCDM8-TR, a luciferase reporter vector containing three copies of a thyroid hormone-responsive DR4 element, pTK-TR$(DR4)_3$, and the retinoic acid reporter plasmid pGL3-β-RARE-luc were obtained from Dr. David Moore (Baylor College of Medicine, Houston Texas). The retinoic acid receptor (RAR) expression vectors pSG-mRARα, pTL-mRARβ and pTL-mRARγ were provided by Dr. Pierre Chambon (Institut de Génétique et de Biologie Moléculaire et Cellulaire, Strasbourg, France). Expression plasmids for the coactivators GRIP1, p300 and CARMI were obtained from Dr. Michael R. Stallcup (Univ. of Southern California, Los Angeles, CA). Human progesterone receptor expression plasmid (hPR-B) and its reporter plasmid were gifts from Dr. Donald P. McDonnell (Duke University Medical Center, Durham, NC). pSV-βgal vector, containing the SV40 early promoter and enhancer upstream of bacterial *lacZ* gene, encoding β-galactosidase, was purchased from Promega Corp. (Madison, WI).

*Cell culture and transient transfection.* tsA201 cells, a derivative of the cell line HEK293, were obtained from Dr. J. Larry Jameson (Northwestern Univ. Medical School, Chicago, IL) and grown in DMEM containing 10% FBS. TM3 mouse Leydig cells and TM4 mouse Sertoli cells (American Type Culture Collection, Manassas, VA) were grown in DMEM-F12 containing 5% horse serum and 2.5% FBS. HepG2 human hepatocellular carcinoma cells (American Type Culture Collection) were grown in Eagle's Minimum Essential Medium containing 10% FBS. For transfection studies, ~$3\times10^4$ cells/well (tsA201, HepG2 and TM3 cells) were plated in 48-well tissue culture plates. 24 h later, the cells in individual wells were transfected with a total of 250 ng DNA/well using 0.3 μl of TransIT-LT1 transfection reagent (Mirus Bio, Madison, WI) and salmon sperm DNA as a carrier. TransIT-LT1 (0.3 μl/well) was pre-incubated with DMEM without serum, following which DNA was added to the TransIT-DMEM mix and incubated for 15 min at room temperature. This cocktail was then aliquoted into each well to be transfected. To improve the transfection efficiency of TM3 cells, Hoechst dye 33258 (Molecular Probes, Eugene, OR) was included in the transfection mix at 100 μM final concentration. DNA to be transfected was added to the serum free medium followed by addition of Hoechst 33258 and incubated for 30 min at room temperature. TransIT-LT1 was pre-incubated with serum free medium for 5 min and added to the Hoechst dye-DNA mix and further incubated for 15 min. The mix was then added equally to each well to be transfected (Shipley and Waxman, 2006). Transfections were performed using the following amounts of plasmid DNA per well of a 48-well tissue culture plate: 10 ng of receptor plasmid (AR, ERα, ERβ, TRβ, PR-B, RARα, RARβ or RARγ), 90 ng of reporter plasmid (pPB-luc, ERC3-luciferase, pTK-TR$(DR4)_3$, progesterone receptor reporter plasmid or pGL3-β-RARE-luc), 20 ng of pSV-βgal (for normalization of luciferase activity) and 130 ng salmon sperm DNA. For coactivator experiments, transfections were performed using 1 ng or 15 ng AR, 90 ng of pProbasin-luc reporter plasmid, 20 ng of pSV-βgal, 50 ng of coactivator plasmids (10 ng GRIP1, 20 ng p300 and 20 ng CARM1) and 80 ng salmon sperm DNA.

*Reporter assay.* Transfected cells were stimulated for 24 h (or 6 h for the inhibitor studies) with the nuclear receptor ligands testosterone, 17β-estradiol (E2), tri-iodothyronine (T3), R5020 or all trans-retinoic acid dissolved in DMSO (10 nM each, except for the dose-response curves shown in Fig. 5) in the presence or absence of 5 mM MAA, as specified in each experiment. Each treatment (with vehicle, hormone, etc.) was administered to transfected cells in three separate wells. MAA stock solution was prepared by diluting MAA in culture medium (without serum), followed by adjustment of the pH to 7.4 with 10 N NaOH, to yield a final MAA concentration of 5 mM. Cells were treated with 0.1% DMSO as a vehicle control. For inhibitor experiments, transfected cells were pre-treated with the inhibitor for 30 min before addition of testosterone and/or MAA to the culture medium. Following treatment, the cultured medium was removed, and 100 μl passive lysis buffer (Promega) was added to each well for 15 min to lyse the cells. Firefly luciferase and β-galactosidase activities were respectively assayed using luciferase assay reagent from Promega Corp. (Madison, WI) and Galacto-Light Plus™ beta-Galactosidase Reporter Gene Assay System from Applied Biosystems (Foster City, CA) and a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA). The resultant firefly luciferase activity was divided by the measured β-galactosidase activity to normalize for transfection efficiency. For each transfection experiment, triplicate sets of analyses were carried out for each data point on every bar graph and line graph. The $n=3$ reported for each transfection experiment thus indicates normalized reporter activities obtained from three separate platings of cells from the same passage, each of which was transfected and treated individually and processed for reporter assays, in parallel. Reporter gene assays (luciferase and β-galactosidase) were carried out on each of the triplicate cell samples and normalized reporter activities were calculated. The normalized activity obtained from cells stimulated with ligand alone was set=1.0 and activities corresponding to all other treatments were expressed in relation to this value. Data shown are mean±SD values, based on the triplicate analyses. Furthermore, in all cases, the experimental results were verified using at least two or three other passages of the cell line, each of which was carried out as a set of three independent transfections (i.e., $n=3$), as outlined above.

*Determination of $EC_{50}$ values.* tsA201 cells transfected with AR expression plasmid and pPB luciferase reporter plasmid were treated for 24 h with six or more concentrations of testosterone alone or in combination with MAA, and in the presence or absence of an AR antagonist. Controls included vehicle alone (DMSO, 0.1%), MAA alone (5 mM), AR antagonist alone (10 μM), and MAA+antagonist combined. Normalized reporter activities determined as described above were expressed relative to the DMSO control. $EC_{50}$ values were determined by non-linear regression analysis using GraphPad Prism software, version 4.0 (GraphPad, San Diego, CA).

*Western blotting.* tsA201 cells were seeded in 6-well plates at ~$1.5\times10^5$ cells/well and allowed to grow overnight, following which the standard culture medium was replaced by starvation medium (DMEM without phenol red+0.5% charcoal stripped serum). Twenty four hours later, the cells were treated for time periods ranging from 2 to 15 min with 5 mM MAA, with culture medium alone (negative control) or with culture medium+10% FBS (positive control). Where indicated, 10 μM U0126 was added to the cells 30 min prior to the addition of MAA or stimulation of the cells with FBS. Cells lysates (20 μg protein/lane) were analyzed on Western blots probed with anti-phospho-ERK and anti-ERK antibodies according to the manufacturers' protocol. Membranes were incubated in 5% non-fat milk blocking buffer for 1 h at room temperature, washed 3× with 10 mM Tris, 150 mM NaCl, and 0.1% v/v Tween 20 and then incubated with primary antibody (1:1000 dilution) overnight at 4 °C. The membrane was subsequently washed and incubated with HRP-conjugated secondary antibody and developed using Amersham ECL™ detection reagent (GE Healthcare, Piscataway, NJ).

*qPCR analysis.* TM3 cells were seeded in 6-well plates, cultured overnight to ~60% confluence and then treated with 5 mM MAA or with culture medium alone for 3 h or 24 h. Total RNA was prepared using TRIzol reagent following the manufacturer's protocol. RNA samples were treated with RQ1 RNAse-free DNAse for 1 h at 37 °C followed by heating at 75 °C for 5 min. cDNA synthesis and real-time qPCR analysis using SYBR Green I-based chemistry were as described (Holloway et al., 2006). Dissociation curves were examined after each qPCR run to ensure amplification of a single, specific product at the correct melting temperature. qPCR primers were designed using Primer express software (Applied Biosystems) (Table 1). Relative RNA levels were calculated after normalization to the 18S rRNA content of each sample and

**Table 1**
Mouse qPCR Primer sets and Genbank accession numbers.

| Gene | Oligo No. | Accession | Ampli-con (nt) | Forward primer | Reverse primer |
|---|---|---|---|---|---|
| *AR* | 1762/1763 | NM_013476 | 51 | AACTCGATCGCATCATTGCA | TTGAGCAGGATGTGGGATTCT |
| *Shbg/ABP* | 1742/1743 | NM_011367 | 65 | CTGGCCAGCTTGAAATCCA | CCGAGGACCAAAGCCTACTGT |
| *Igfbp3* | 1764/1765 | NM_008343 | 54 | CATATGCCTGAGGCTCATGGT | GCCAGGCCCTTATTCAGAGAT |
| *StAR* | 2028/2029 | NM_011485 | 53 | TTCATCCGCAGTGCCATTT | CCCACACGATAAGGGACAGAA |
| *Cyp19a1* | 2030/2031 | NM_007810 | 51 | CCGAGCCTTTGGAGAACAATT | GCCCGTCAGAGCTTTCATAAA |
| *Cyp17a1* | 1408/1409 | NM_007809 | 51 | TGCCCCTGGTGGGTAGTCTA | CATGCATATGACCACGTCTGG |