Published in final edited form as:

J Steroid Biochem Mol Biol. 2007 ; 107(3-5): 253–261.

Progesterone regulates catechol-O-methyl transferase gene expression in breast cancer cells: distinct effect of progesterone receptor isoforms

Salama A. Salama^{1*}, Mohammad Jamaluddin², Raj Kumar², Memy H. Hassan¹, and Ayman Al-Hendy¹

¹Department of Obstetrics & Gynecology, 301 University Blvd., University of Texas, Medical Branch, Galveston, TX, 77555, USA

²Department of Internal Medicine, 301 University Blvd., University of Texas, Medical Branch, Galveston, TX, 77555, USA

Abstract

There is strong evidence that catechol-O-methyl transferase (COMT) protects breast cells against estrogen-induced cancer by detoxifying catecholestrogens, the carcinogenic estrogen metabolites. COMT gene expression is controlled by two promoters—a proximal promoter (COMTP1) and a distal promoter (COMTP2)—that regulate the expression of soluble (S-COMT) and membrane-bound (MB-COMT) isoforms, respectively. We investigated the transcriptional regulation of the COMT gene by progesterone/progesterone receptors in breast cancer cells. Our results indicated that progesterone (P4) downregulates COMT gene expression in breast cancer cell lines. In addition, the COMTP1 and COMTP2 harbor several progesterone response elements (PREs). Electrophoretic mobility shift assay (EMSA) indicated that nuclear extracts of T47D cells bind to the identified PREs in COMTP1. Site-directed mutagenesis of PREs in COMTP1 not only reversed the P4-induced inhibition of COMTP1, but also increased its basal activity. The two progesterone receptor isoforms, PR-A and PR-B, were found to have opposite effects on the regulation of P4 in COMT expression; PR-A is associated with P4-induced upregulation of COMT, while PR-B is associated with P4-induced downregulation of COMT. In summary, our data demonstrated that P4 downregulates the COMT gene expression through multiple PREs in the COMT promoters and that different progesterone receptor isoforms have distinctive effects on COMT gene expression.

1. Introduction

Breast cancer is the most common malignancy and the second most frequent cause of cancer death among women in the United States [1]. It is well-established that prolonged exposure to estrogen is an important risk factor for genesis and promotion of breast cancer [2,3]. Epidemiological studies suggest that the addition of progestins in hormone replacement therapy increases the risk of breast cancer relative to estrogen use alone [4,5]. The World Health Organization International Agency for Research and Cancer has classified combination (estrogen and progesterone) hormone contraception and menopausal therapy as carcinogenic

*Corresponding author: Salama A. Salama, PhD, Department of Obstetrics & Gynecology, University of Texas Medical Branch, 301 University Blvd., Galveston, TX 77555-1066, USA, Tel.: +1 409 747 0492, Fax: +1 409 747 1669 E-mail address: sasalama@utmb.edu (S. Salama).

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

in humans [6]. A potential mechanism of estrogen carcinogenicity is based on its mitogenic effect and stimulation of cell division, thus increasing random errors during DNA replication [7]. Additionally, oxidative metabolism of estrogen plays a detrimental role in the initiation and/or promotion of estrogen-related cancer [8,9]. The initial step in estrogen metabolism is oxidation by cytochrome P450 (CYP450) 1A1 and 1B1 to produce 2-hydroxyestradiol (2-OHE₂) and 4-hydroxyestradiol (4-OHE₂), respectively [10–12]. 4-OHE₂ has a potent estrogenic effect and was found to be carcinogenic in animal models [13–15]. The carcinogenicity of 4-OHE₂ resides in its vulnerability for auto-oxidation to the corresponding orthoquinone derivative (3,4-OHE₂-o-quinone) [16]. These catecholestrogens-derived orthoquinones are electrophilic compounds capable of reacting with DNA to form mutagenic and carcinogenic covalent adducts [9,17]. In extrahepatic tissues, the principal pathway for detoxification of catecholestrogens is O-methylation by catechol-O-methyl transferase (COMT) [18]. COMT-mediated conjugation reactions render catecholestrogens more water soluble and easily excreted, thus impeding further oxidation into the corresponding quinones [18,19]. Consistent with the pivotal role of COMT in the development of breast cancer, genetic epidemiological studies suggest that the COMTLL genotype, which is associated with low COMT enzymatic activity, confers a significantly increased risk for breast cancer [20–22].

COMT is expressed in different mammalian tissues [23–26] where it exerts various biological functions. There are two isoforms of COMT with similar functions—soluble (S-COMT) and membrane-bound (MB-COMT). Both are encoded by a single gene located in chromosome 22q11.2 [23,25]. The expression of MB-COMT and S-COMT is regulated by two separate promoters: the proximal promoter (COMTP1), which regulates S-COMT, and the distal promoter (COMTP2), which regulates MB-COMT [26–29]. Since COMT plays a critical role in the detoxification of carcinogenic estrogen metabolites, studying the transcription regulation of COMT expression is of particular interest in the understanding of the pathobiology of estrogen-related cancer and in designing chemopreventive strategies. Previous work from our laboratory and from other groups indicates that the expression of COMT is regulated by sex steroid hormones [29–31]. The current study was undertaken to systematically investigate the potential role of progesterone/progesterone receptors in the regulation of COMT gene expression in breast cancer cell lines.

2. Materials and Methods

2.1. DNA vectors, chemicals, and reagents

The PGL3 luciferase (Luc) vector, pSV- β -galactosidase control vector, *pfu* DNA polymerase, luciferase enzyme assay system, and β -galactosidase enzyme assay system were purchased from Promega (Madison, WI). Restriction enzymes, bovine serum albumin (BSA), and *Escherichia coli* DH5 α competent cells were obtained from Life Technologies, Inc. (Gaithersburg, MD). [γ -³²P]ATP was purchased from Amersham (Buckinghamshire, United Kingdom). All of the chemicals and reagents were of the highest commercially available grade.

2.2. Cell culture

Human breast cancer cell lines MCF-7 and T47D (positive for progesterone receptors), and MDA-MB-231 (negative for progesterone receptors) were obtained from American Type Culture Collection (ATCC, Manassas, VA). MCF-7 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS). T47D cells were grown in Roswell Park Memorial Institute 1640 (RPMI 1640) medium containing 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic Acid (HEPES), 1.0 mM sodium pyruvate, 0.2 U/ml bovine insulin, 1% penicillin/streptomycin, and 10% FBS. MDA-MB-231 cells were grown in Leibovitz's L-15

medium. For treatment with progesterone or RU 486, cells were maintained in the indicated growth medium without phenol red and supplemented with 10% dextran charcoal-treated FBS.

2.3. Construction of chimeric COMT promoters-luciferase reporters

Chimeric COMTP1 and COMTP2 reporters were constructed according to the method described by Xie et al. [29] with some modification. The forward primers contained the *Mlu*I recognition site at the 5' end, and the reverse primers contained the recognition site for *Bgl*II restriction enzymes at the 5' end. The amplified COMTP1 and COMTP2 were digested with *Mlu*I and *Bgl*II enzymes and cloning into *Mlu*I and *Bgl*II linearized PGL3 luciferase reporter vector. The identity of the cloned inserts was confirmed by sequencing and comparison with published sequences.

2.4. Site-directed mutagenesis of PREs in COMTP1

To further examine the functionality of the various PREs identified in the COMTP1 (which controls the S-COMT expression), the following three putative PRE sites—PRE I (5'-TGTGCC-3', located from -48 to -53); PRE II (5'-TGTGCC-3', located from -705 to -711); and PRE III (5'-TGTTCT-3', located from -1201 to -1206)—were mutated individually or in combination using a PCR-based strategy. PRE sites were changed either from 5'-TGACCT-3' (site I and II) to 5'-CGGCCG-3' or from 5'-TGTTCT-3' (site III) to 5'-CCGATA-3'. Mutation of the PREs, individually or in combination, resulted in a total of seven constructs harboring site-directed mutations: mutant I (mutated at PRE I), mutant II (mutated at PRE II), mutant III (mutated at PRE III), mutant IV (mutated at PRE I and PRE II), mutant V (mutated at PRE I and PRE III), mutant VI (mutated at PRE II and PRE III), and mutant VII (mutated at PRE I, PRE II, and PRE III).

2.5. Mammalian cell transfection with luciferase and β -galactosidase plasmids

The effect of progesterone or RU 486 on activities of COMTP1-luciferase (COMTP1-Luc), COMTP2-Luc, or the mutated COMTP1-Luc constructs was assessed in transiently transfected MCF-7 and T47D breast cancer cell lines. In each transfection experiment, the cells (60%–70% confluent) were cotransfected with luciferase reporter constructs (10 μ g), and Promega pSV- β -galactosidase control vector (1 μ g) using FuGENE 6 Transfection Reagent (Roche Applied Science, Indianapolis, IN), according to the manufacturer's protocol. The transfected cells were treated with different concentrations of progesterone (10^{-6} , 10^{-7} , or 10^{-8} M) or the antiprogesterin RU 486 (10^{-6} M) in 0.01% (v/v) ethanol. Forty-eight hours later, the luciferase and pSV- β -galactosidase activities were determined using β -galactosidase and luciferase assay kits (Promega) according to the manufacturer's instructions. Protein concentrations were determined by the BCA protein assay kit (Pierce Co, Rockford, IL). Luciferase activity was expressed after normalization against β -galactosidase activity and protein concentration.

2.6. RNA isolation and real time reverse transcription-polymerase chain reaction

Quantitative real time reverse transcription-polymerase chain reaction (RT-PCR) was performed in triplicate for each sample on an ABI Prism 7900HT with TaqMan universal PCR master mix (Applied Biosystems, Foster City, CA), as previously described [30]. For each sample, the Δ Ct values were determined by subtracting the average of triplicate Ct values of the target gene from the average of the triplicate Ct values of the reference gene (glyceraldehyde-3-phosphate dehydrogenase, GAPDH). The quantification result was transformed into an exponential value, $2^{-\Delta\Delta C_t}$. The final results were expressed as n-fold differences in COMT expression in cells treated with progesterone compared with vehicle-treated cells.

2.7. Expression of progesterone receptor isoforms in MDA-MB231 cells using adenovirus vectors

To study the role of different progesterone receptor subtypes on COMT gene expression, the breast cancer cells MDA-MB231 (negative for progesterone receptors) were transfected with adenovirus vector expressing either progesterone receptor A (Ad-PR-A) or progesterone receptor B (Ad-PR-B). MDA-MB231 cells are typically plated in six-well plates (350,000 cells/well) and allowed to recover overnight. The cells were incubated with adenoviral vectors (multiplicity of infection [MOI] of 50 PFU/cell) in transfection media (containing 2% fetal calf serum [FCS], 1% penicillin/streptomycin) for 3 h. The cells were then replenished with regular growth media containing 10% FBS. We investigated the effect of treatment with progesterone or RU 486 on COMT expression by growing MDA-MB231 cells transfected with either Ad-PR-A or Ad-PR-B in phenol red-free DMEM containing 5% dextran-charcoal-treated FCS for 3 days, followed by treatment with various concentrations of progesterone or RU 486.

2.8. Western blot analysis

MCF-7, T47D, or MDA-MB231 cells were treated for 72 h with different concentrations of progesterone or RU 486. After treatment, the cells washed with phosphate-buffered saline (PBS) and whole cell lysates were prepared with lysis buffer. The cell lysates were solubilized in sample buffer (60 mM Tris-hydrochloride [HCl], pH 6.8; 2% sodium dodecyl sulfate [SDS]; 10% glycerol; 0.7 mol/l 2- β -mercaptoethanol; and 0.01% bromophenol blue) and subjected to SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were electroblotted onto nitrocellulose membranes. Membranes were immunoblotted with the primary antibody against COMT (AB5873; Chemicon, International, Inc. Temecula, CA), human progesterone receptor PR-A and PR-B Ab52 (sc-810) (Santa Cruz Biotechnology, Santa Cruz, CA), or β -Actin (Sigma-Aldrich Co, St. Louis, MO). After washing, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Vector Laboratories, Burlingame, CA). The intensity of each protein band was determined using a scanning densitometer (Epson 4870, Epson America, Long Beach, CA).

2.9. Binding of progesterone receptors to PRE in COMT gene promoter region

The human breast cancer cell line T47D was grown, as mentioned previously. The cells were then treated with 10^{-7} M of progesterone in serum-free medium and incubated at 37°C for 1 h. Nuclei were prepared from the treated cells using a nuclear extraction kit (Active Motif, Carlsbad, CA), following manufacturer's protocol. The nuclear preparation was stored at -70°C and thawed only on the day of the study to avoid the loss of DNA-binding activity. Single-stranded oligomers were annealed with their complementary strands. These oligomers contained half-palindromic PRE I sequences (underlined) from human COMTP1 corresponding to nucleotide (-39 to -61) (P1) and -1193 to -1213 upstream from the translation start site of COMTP1: P1: 5'-CTGGGGCCTGTGCCTTATCGGC-3' and P2: 5'-TGCTTCCCTGTTCTTCTGCT-3'. The double-stranded oligomers were end-labeled with [γ - 32 P]ATP using T4 polynucleotide kinase (10 U) to generate double-stranded P1 and P2 probes. Electrophoretic mobility shift assay (EMSA) was carried out as described by Xie et al. [29]. T47D cell nuclear extracts (2 μ g) were incubated with 32 P-labeled probes (60,000 cpm) in a buffer containing Tris-HCl (20 mM, pH 7.8), potassium chloride (60 mM), magnesium chloride (5 mM), and dithiothreitol (5 mM) in a final volume of 20 μ l at room temperature for 30 min. The protein-DNA complexes were separated in a 4% nondenaturing polyacrylamide gel, and the gel was dried and autoradiographed. To determine the specificity of the PRE-bound complex, we performed competitive EMSA by adding 100-fold excess of unlabeled DNA duplex. To identify progesterone receptors in the shifted complex, we preincubated nuclear extracts for 60 min in ice with anti-PR antibodies (NeoMarker Inc., Fremont, CA) prior to the addition of radio-labeled probes.

2.10. Statistical analysis

Data are presented as the mean $\pm$ standard error of the mean (SEM), where $n = 3$ for each experiment. Statistical significance between different treatments was determined by one-way analysis of variance (ANOVA) followed by posthoc comparison using Student's t test. A p value $< .05$ was considered statistically significant.

3. Results

3.1. Progesterone downregulated COMT protein expression in breast cancer cell lines

Western blot analyses of COMT expression in MCF-7 and T47D cells indicated that progesterone reduced the COMT protein levels in a concentration-dependent manner. As shown in Figure 1A and 1B, treatment of MCF-7 cells with progesterone (10^{-8} , 10^{-7} , or 10^{-6} M) reduced the expression of both MB-COMT (by 20%, 46%, and 49%, respectively, compared with the untreated control) and S-COMT (by 33%, 39%, and 55%, respectively, compared with the untreated control). Treatment with progesterone receptor antagonist, RU 486 (10^{-6} M) in combination with progesterone (10^{-7} M), reversed the inhibitory effect of progesterone in COMT expression. A similar effect of progesterone on COMT protein expression was observed in T47D cells. Progesterone (10^{-8} , 10^{-7} , or 10^{-6} M) reduced the expression of both MB-COMT (by 24%, 49%, and 60%, respectively, compared with the untreated control) and S-COMT (by 36%, 43%, and 56%, respectively, compared with the untreated control). The progesterone receptor antagonist, RU 486, reversed the progesterone (10^{-7} M)-induced inhibition of COMT expression (Fig. 1C, 1D).

3.2. Progesterone induced inhibition of COMT mRNA expression in MCF-7 and T47D cells

To verify whether the effect of progesterone on the COMT expression occurs at the transcriptional level, the expression of COMT mRNA was analyzed in progesterone-treated MCF-7 and T47D cells by real-time RT-PCR. The real time RT-PCR assay indicated that progesterone significantly downregulated COMT mRNA expression in a concentration-dependent manner. Progesterone (10^{-8} , 10^{-7} , 10^{-6} M) reduced the COMT mRNA expression in MCF-7 cells by 30%, 60%, and 80%, respectively, compared with the vehicle-treated control (Fig. 2A). Under the same treatment conditions, the COMT mRNA expression in T47D cells was reduced by 40%, 70%, and 80%, respectively, compared with the vehicle-treated control (Fig. 2B). The effect of progesterone (10^{-7} M) on COMT mRNA expression in both cell lines was reversed by cotreatment with RU 486 (10^{-6} M) (Fig. 2A, 2B), which suggests that the effect of progesterone on COMT expression is mediated by progesterone receptors.

3.3. Progesterone downregulated COMT promoter-reporter expression in breast cancer cells

For further verification that downregulation of COMT expression by progesterone occurs at the transcriptional level, luciferase reporter vectors under the transcriptional control of COMTP1 or COMTP2 were transiently transfected into MCF-7 and T47D cells. Progesterone decreased both COMTP1- and COMTP2-Luc activities in a concentration-dependent manner in MCF-7 cells and T47D cells (Fig. 3A, 3B). In MCF-7 cells, treatment with progesterone (10^{-8} , 10^{-7} , or 10^{-6} M) decreased the COMTP1-Luc activity by 25%, 49%, and 57%, respectively, compared with the corresponding control; and the COMTP2-Luc activity by 26%, 55%, and 67%, respectively, compared with the corresponding control (Fig. 3A). Treating T47D cells with progesterone (10^{-8} , 10^{-7} , or 10^{-6} M) decreased the COMTP1-Luc activity by 22%, 54%, and 63%, respectively, compared with the corresponding control; the COMTP2-Luc activity decreased by 29%, 48%, and 59%, respectively, compared with the corresponding control (Fig. 3B). This effect of progesterone on COMT promoter luciferase activities was reversed by the addition of RU 486 (Fig. 3A, 3B), indicating that downregulation of COMT promoter activities by progesterone is mediated by classical progesterone receptors.

3.4. Identification of progesterone response elements in COMT promoters

Computer-assisted search homology analysis indicated that the COMTP1 (−1475 to +1) harbors three half-consensus putative PREs—PRE I (5′-TGTGCC-3′, located at −48 to −53); PRE II (5′-TGTGCC-3′, located at −705 to −711), and PRE III (5′-TGTTCT-3′, located at −1201 to −1206) upstream from the translation start codon of S-COMT. In COMTP2, five half-consensus PRE sites were identified: PRE1 (5′-GGGACA-3′, located at −20,837 to −20,834); PRE 2 (5′-AGAACT-3′, located at −21,025 to −21,030); PRE3 (5′-TGTACA-3′, located at −21,180 to −21,623); PRE4 (5′-AGAACA-3′, located at 21,618 to −21,623); and PRE5 (5′-TGTTCT-3′, located at −22,006 to −22,011) upstream from the translational start codon of MB-COMT. In this study, we focused on the COMTP1, which controls the expression of the S-COMT isoform—the most abundant isoform in MCF-7 and T47D breast cancer cell lines. We examined the role of these putative PRE motifs in controlling the expression of COMT gene by mutating the three putative PRE motifs in COMTP1 individually or in combination. Site-directed mutations were introduced into the various PREs (described in the Materials and Methods section) to produce the single mutants (mutant I, mutant II, mutant III); the double mutants (mutant IV, mutant V, mutant VI); and the triple mutant construct (mutant VII). The wild-type or mutant constructs were then transiently transfected into T47D cells, and luciferase activities were determined after treatment with progesterone (100 nM) for 48 h. When the three putative PRE sites were mutated individually or in combination, the basal COMTP1-Luc activities increased rather than decreased compared with the wild-type COMTP1-Luc construct (Fig. 4). In contrast to its effect on the wild-type COMTP1-Luc promoter activity, progesterone increased the activities of COMTP1-Luc with individual or combined mutations in the PRE. Thus, in T47D cells transfected with different COMTP1-Luc mutants (mutant I, II, III, IV, V, VI, or VII) and treated with 10^{-7} M of progesterone, the luciferase activity increased 1.6-, 1.3-, 1.1-, 1.6-, 1.3-, 1.9-, and 2-fold, respectively, compared with the untreated control (Fig. 4).

3.5. Progesterone receptor binding to the PREs in COMT promoters

We demonstrated the specificity of the progesterone receptor and its relative binding affinity for the newly identified PREs in COMT promoters by executing gel mobility shift competition assays (Fig. 5). Nuclear extracts from T47D cells (treated with 10^{-7} M of progesterone), and either P1 or P2 radio-labeled oligonucleotides, were used for the gel mobility shift assay. Our results demonstrate that a single DNA-protein complex was formed by T47D nuclear extracts and P1 probe (lane 3); whereas, no complex was detected in the absence of nuclear extracts (lane 2, negative control). The formation of DNA-protein complex was significantly impaired by the addition of unlabeled P1 probe ($100 \times$ molar excess of labeled P1, lane 4), which suggests that the binding was specific. To demonstrate whether this DNA-protein complex was specific to the progesterone receptor, we carried out supershift assay. T47D cell nuclear extracts were incubated with progesterone receptor-specific antibody (PgR Ab-5, NeoMarkers), and applied to the gel mobility shift assay; the results are shown in lane 5. We expected to observe a supershift in the DNA-protein complex as seen in lane 3. However, we did not observe any supershift in this assay (lane 5) compared with cell nuclear extracts that were not incubated with the progesterone receptor-specific antibody (lane 3). Adding $100 \times$ excess of unlabeled P1 blocked the DNA-protein complex (lane 6). Similarly, DNA-protein complex was formed by PRE2 (P2) and T47D nuclear extract (lane 7). The formation of this P2-protein complex was significantly impaired by the addition of unlabeled P2 probe ($100 \times$ molar excess of labeled P2, lane 9). Similar to the effect observed with P1, the addition of PR antibody did not cause a supershift in the DNA-protein complex (lane 9). Since the gel shift assay was carried out on the native gel, there was a possibility that the antigen sites were not exposed due to some kind of folded conformation of the progesterone receptor; therefore, no antibody reaction could take place. In one set of experiments, we sought to confirm these possibilities by using nuclear extracts from the MDA-MB231 cell, which does not express the progesterone receptor. When

gel shift assays were carried out using these nuclear cell extracts and the P1 probe, we did not see any DNA-protein complex form (data not shown); this suggests that the DNA-protein complex that formed, as assessed by gel mobility shift assay, involved the progesterone receptor. The absence of supershift may be due to the antibody failing to cross-react with the progesterone receptor protein in the cell extract. Similar results were obtained when T47D cell nuclear extracts were applied for gel shift assay on the P2 probe.

3.6. Distinct effect of PR-B and PR-A on COMT expression in breast cancer cells

We further evaluated the role of different progesterone receptor subtypes in mediating the effect of progesterone in COMT expression by examining the effect of progesterone in progesterone receptor-negative breast cancer cells (MDA-MB231). Progesterone or RU 486 did not change the expression level of COMT at any given concentration used (data not shown). We investigated the role of PR-A and PR-B in mediating the effect of progesterone on COMT expression by transiently transfecting MDA-MB231 cells with adenovirus vectors expressing either PR-A or PR-B. MDA-MB231 cells transfected with adenovirus (MOI, 50 PFU/cell), expressing either PR-A or PR-B, resulted in expression of PR-A or PR-B protein, respectively (Fig. 6A). In MDA-MB231 cells expressing PR-A alone, treatment with progesterone (10^{-8} to 10^{-6} M) resulted in a concentration-dependent increase in COMT gene expression compared with the vehicle-treated control (Fig. 6B). In contrast, progesterone treatment downregulated COMT in MDA-MB231 cells expressing only PR-B (Fig. 6C). These results suggest a distinctive function of PR-A and PR-B on COMT promoter activity—progesterone represses COMT promoter activity via PR-B and increases it via a PR-A—dependent mechanism.

4. Discussion

In view of the pivotal role of COMT in detoxification of carcinogenic estrogen metabolites, it is important to understand the mechanisms that govern its expression by identification of cis-acting elements and their cognate transcription factors involved in the basal expression of COMT. Our previous work indicated that the COMT promoter regions harbor multiple progesterone half-site response elements [30], suggesting that progesterone/progesterone receptors are among those transcription factors involved in the regulation of COMT expression in breast cells. Our data clearly indicated that progesterone downregulates COMT expression at the transcriptional level. We further investigated the mechanism of downregulation of COMT gene expression by progesterone by examining the involvement of the identified putative PRE motifs in mediating the effect of progesterone/progesterone receptors on COMTP1 promoter activity. The site-directed mutagenesis study showed that mutation in the three putative PRE sites in COMTP1 (mutated individually or in combination) resulted in not only reversing the inhibitory effect of progesterone on COMPT1-luc activity but also caused an increase in the basal COMTP1-Luc activities. Progesterone-induced increase in COMTP1-Luc activity after disruption of the PREs might be attributed to the non-genomic effect of progesterone or due to the cross-talk between the progesterone receptors and other transcription factors involved in the regulation of COMT expression.

Similarly, the EMSA showed that PREs can bind to nuclear extracts from PR-positive cells, but not to nuclear extracts from PR-negative cells. Thus, results from EMSA and site-directed mutagenesis led us to conclude that PREs mediate the effects of progesterone on COMT expression. Regulation of COMT expression by progesterone is consistent with our previous report that progesterone regulates COMT expression in uterine leiomyomas cells. Contrary to its effect in leiomyoma cells, progesterone downregulated COMT expression in breast cancer cell lines. This differential effect of progesterone on COMT expression in leiomyomas and breast cancer cell lines may be due to different receptor or cofactor expression levels, the presence of different receptor isoforms, and different components of the intracellular signaling

machinery [32,33]. It is well known that the effect of progesterone on gene expression is tissue specific [34–36].

One of the most interesting findings of this study is that the two progesterone receptor isoforms, PR-A and PR-B, have distinct effects on progesterone-induced COMT expression. While PR-A is associated with progesterone-induced COMT upregulation, PR-B is associated with downregulation of COMT expression. The distinctive effect of PR-B and PR-A on COMT expression is consistent with other observations that PR-A and PR-B have an opposing effect on the expression of many genes [37–40]. The underpinning mechanism of the distinct activities of PR-A and PR-B in gene expression is not well understood, although it has been shown that distinct coregulator recruitment and usage are involved at some levels [41]. Exploring the distinctive role of PR-A and PR-B in the regulation of COMT expression in breast epithelial cells is fundamental to understanding the role of each subtype and how the balanced expression of PR-A and PR-B contributes to the development of breast cancer. In addition, this information will help reveal the tissue-specific effect of progesterone in COMT expression. Analysis of the selective roles of the individual PR isoforms in the transcription regulation of COMT expression will have important clinical implications in developing improved PR subtype-specific ligands for reproductive management and hormone replacement therapy.

The results from our study provide a plausible explanation of the recent results of the Women's Health Initiative trial and the Million Women Study, which indicated that women taking estrogen combined with progesterone have an increased risk of developing breast cancer [42]. Thus, it is possible that downregulation of COMT by progesterone will impede the metabolic detoxification of catecholestrogens. When the metabolic clearance of catecholestrogens is slow or incomplete, they undergo oxidation into the corresponding orthoquinone derivative, which can initiate and promote breast cancer development.

To the best of our knowledge, this is the first study to examine the role of progesterone/progesterone receptors on COMT expression in breast cancer. However, further studies are needed to examine whether the effect of progesterone in COMT expression is progesterone-type specific or common to all types of progestins. Such information will be valuable to the selection of appropriate progestins for hormone replacement therapy and other clinical applications.

Acknowledgments

This work was supported by UTMB NIEHS Center Pilot Project #E506676 to S.A. Salama.

Reference List

1. American Cancer Society. Cancer facts and figures. 2003.
2. Cavalieri E, Chakravarti D, Guttenplan J, Hart E, Ingle J, Jankowiak R, Muti P, Rogan E, Russo R, Santen R, Sutter T. Catechol estrogen quinones as initiators of breast and other human cancers: Implications for biomarkers of susceptibility and cancer prevention. *Biochim. Biophys. Acta* 2006;1766(1):63–78. [PubMed: 16675129]
3. Kaaks R, Berrino F, Key T, Rinaldi S, Dossus L, Biessy C, Secreto G, Amiano P, Bingham S, Boeing H, Bueno de Mesquita HB, Chang-Claude J, Clavel-Chapelon F, Fournier A, van Gils CH, Gonzalez CA, Gurra AB, Critselis E, Khaw KT, Krogh V, Lahmann PH, Nagel G, Olsen A, Onland-Moret NC, Overvad K, Palli D, Panico S, Peeters JR, Quiros JR, Roddam A, Thiebaut A, Tjonneland A, Chirlaque MD, Trichopoulou A, Trichopoulos D, Tumino R, Vineis P, Norat T, Ferrari P, Slimani N, Riboli E. Serum sex steroids in premenopausal women and breast cancer risk within the European Prospective Investigation into Cancer and Nutrition (EPIC). *J Natl. Cancer Inst* 2005;97(10):755–765. [PubMed: 15900045]

4. Ross RK, Paganini-Hill A, Wan PC, Pike MC. Effect of hormone replacement therapy on breast cancer risk: estrogen versus estrogen plus progestin. *J Natl. Cancer Inst* 2000;92(4):328–332. [PubMed: 10675382]
5. Schairer C, Lubin J, Troisi R, Sturgeon S, Brinton L, Hoover R. Menopausal estrogen and estrogen-progestin replacement therapy and breast cancer risk. *JAMA* 2000;283(4):485–491. [PubMed: 10659874]
6. Schneider HP, Mueck AO, Kuhl H. IARC monographs program on carcinogenicity of combined hormonal contraceptives and menopausal therapy. *Climacteric* 2005;8(4):311–316. [PubMed: 16390765]
7. Prokai-Tatrai K, Prokai L. Impact of metabolism on the safety of estrogen therapy. *Ann. N. Y. Acad. Sci* 2005;1052:243–257. [PubMed: 16024767]
8. Cavalieri, E.; Frenkel, K.; Liehr, JG.; Rogan, E.; Roy, D. *J Natl. Cancer Inst. Monogr* (27). 2000. Estrogens as endogenous genotoxic agents--DNA adducts and mutations; p. 75-93.
9. Cavalieri EL, Stack DE, Devanesan PD, Todorovic R, Dwivedy I, Higginbotham S, Johansson SL, Patil KD, Gross ML, Gooden JK, Ramanathan R, Cerny RL, Rogan EG. Molecular origin of cancer: catechol estrogen-3,4-quinones as endogenous tumor initiators. *Proc. Natl. Acad. Sci. U. S. A* 1997;94(20):10937–10942. [PubMed: 9380738]
10. Hayes CL, Spink DC, Spink BC, Cao JQ, Walker NJ, Sutter TR. 17 beta-estradiol hydroxylation catalyzed by human cytochrome P450 1B1. *Proc. Natl. Acad. Sci. U. S. A* 1996;93(18):9776–9781. [PubMed: 8790407]
11. Yager, JD. *J Natl. Cancer Inst. Monogr* (27). 2000. Endogenous estrogens as carcinogens through metabolic activation; p. 67-73.
12. Zhu BT, Conney AH. Functional role of estrogen metabolism in target cells: review and perspectives. *Carcinogenesis* 1998;19(1):1–27. [PubMed: 9472688]
13. Zhu BT. Medical hypothesis: hyperhomocysteinemia is a risk factor for estrogen-induced hormonal cancer. *Int. J Oncol* 2003;22(3):499–508. [PubMed: 12579301]
14. Liehr JG, Fang WF, Sirbasku DA, Ari-Ulubelen A. Carcinogenicity of catechol estrogens in Syrian hamsters. *J Steroid Biochem* 1986;24(1):353–356. [PubMed: 3009986]
15. Li JJ, Li SA. Estrogen carcinogenesis in Syrian hamster tissues: role of metabolism. *Fed. Proc* 1987;46(5):1858–1863. [PubMed: 3030825]
16. stack DE, Byun J, Gross ML, Rogan EG, Cavalieri EL. Molecular characteristics of catechol estrogen quinones in reactions with deoxyribonucleosides. *Chem. Res. Toxicol* 1996;9(5):851–859. [PubMed: 8828920]
17. Chakravarti D, Mailander PC, Li KM, Higginbotham S, Zhang HL, Gross ML, Meza JL, Cavalieri EL, Rogan EG. Evidence that a burst of DNA depurination in SENCAR mouse skin induces error-prone repair and forms mutations in the H-ras gene. *Oncogene* 2001;20(55):7945–7953. [PubMed: 11753677]
18. Zhu BT, Conney AH. Is 2-methoxyestradiol an endogenous estrogen metabolite that inhibits mammary carcinogenesis? *Cancer Res* 1998;58(11):2269–2277. [PubMed: 9622057]
19. Al-Hendy A, Hortelano G, Tannenbaum GS, Chang PL. Correction of the growth defect in dwarf mice with nonautologous microencapsulated myoblasts--an alternate approach to somatic gene therapy. *Hum. Gene Ther* 1995;6(2):165–175. [PubMed: 7734517]
20. Lin WY, Chou YC, Wu MH, Jeng YL, Huang HB, You SL, Chu TY, Chen CJ, Sun CA. Polymorphic catechol-O-methyltransferase gene, duration of estrogen exposure, and breast cancer risk: a nested case-control study in Taiwan. *Cancer Detect. Prev* 2005;29(5):427–432. [PubMed: 16191465]
21. Goodman MT, McDuffie K, Kolonel LN, Terada K, Donlon TA, Wilkens LR, Guo C, Le Marchand L. Case-control study of ovarian cancer and polymorphisms in genes involved in catecholesterogen formation and metabolism. *Cancer Epidemiol. Biomarkers Prev* 2001;10(3):209–216. [PubMed: 11303589]
22. Lavigne JA, Helzlsouer KJ, Huang HY, Strickland PT, Bell DA, Selmin O, Watson MA, Hoffman S, Comstock GW, Yager JD. An association between the allele coding for a low activity variant of catechol-O-methyltransferase and the risk for breast cancer. *Cancer Res* 1997;57(24):5493–5497. [PubMed: 9407957]

23. Lundstrom K, Tenhunen J, Tilgmann C, Karhunen T, Panula P, Ulmanen I. Cloning, expression and structure of catechol-O-methyltransferase. *Biochim. Biophys. Acta* 1995;1251(1):1–10. [PubMed: 7647086]
24. Ulmanen I, Peranen J, Tenhunen J, Tilgmann C, Karhunen T, Panula P, Bernasconi L, Aubry JP, Lundstrom K. Expression and intracellular localization of catechol O-methyltransferase in transfected mammalian cells. *Eur. J Biochem* 1997;243(1–2):452–459. [PubMed: 9030772]
25. Bertocci B, Miggiano V, Da Prada M, Dembic Z, Lahm HW, Malherbe P. Human catechol-O-methyltransferase: cloning and expression of the membrane-associated form. *Proc. Natl. Acad. Sci. U. S. A* 1991;88(4):1416–1420. [PubMed: 1847521]
26. Tenhunen J, Heikkilä P, Alanko A, Heinonen E, Akkila J, Ulmanen I. Soluble and membrane-bound catechol-O-methyltransferase in normal and malignant mammary gland. *Cancer Lett* 1999;144(1):75–84. [PubMed: 10503880]
27. Tenhunen J, Salminen M, Lundstrom K, Kiviluoto T, Savolainen R, Ulmanen I. Genomic organization of the human catechol O-methyltransferase gene and its expression from two distinct promoters. *Eur. J Biochem* 1994;223(3):1049–1059. [PubMed: 8055944]
28. Mannisto PT, Kaakkola S. Catechol-O-methyltransferase (COMT): biochemistry, molecular biology, pharmacology, and clinical efficacy of the new selective COMT inhibitors. *Pharmacol. Rev* 1999;51(4):593–628. [PubMed: 10581325]
29. Xie T, Ho SL, Ramsden D. Characterization and implications of estrogenic down-regulation of human catechol-O-methyltransferase gene transcription. *Mol. Pharmacol* 1999;56(1):31–38. [PubMed: 10385681]
30. Salama SA, Ho SL, Wang HQ, Tenhunen J, Tilgmann C, Al Hendy A. Hormonal regulation of catechol-O-methyl transferase activity in women with uterine leiomyomas. *Fertil. Steril* 2006;86:259–262. [PubMed: 16730007]
31. Jiang H, Xie T, Ramsden DB, Ho SL. Human catechol-O-methyltransferase downregulation by estradiol. *Neuropharmacology* 2003;45(7):1011–1018. [PubMed: 14573393]
32. Aranda A, Pascual A. Nuclear hormone receptors and gene expression. *Physiol Rev* 2001;81(3):1269–1304. [PubMed: 11427696]
33. Vegeto E, Shahbaz MM, Wen DX, Goldman ME, O'Malley BW, McDonnell DP. Human progesterone receptor A form is a cell- and promoter-specific repressor of human progesterone receptor B function. *Mol. Endocrinol* 1993;7(10):1244–1255. [PubMed: 8264658]
34. Jacobsen BM, Schittone SA, Richer JK, Horwitz KB. Progesterone-independent effects of human progesterone receptors (PRs) in estrogen receptor-positive breast cancer: PR isoform-specific gene regulation and tumor biology. *Mol. Endocrinol* 2005;19(3):574–587. [PubMed: 15563544]
35. Richer JK, Jacobsen BM, Manning NG, Abel MG, Wolf DM, Horwitz KB. Differential gene regulation by the two progesterone receptor isoforms in human breast cancer cells. *J Biol. Chem* 2002;277(7):5209–5218. [PubMed: 11717311]
36. Jacobsen BM, Richer JK, Sartorius CA, Horwitz KB. Expression profiling of human breast cancers and gene regulation by progesterone receptors. *J Mammary. Gland. Biol. Neoplasia* 2003;8(3):257–268. [PubMed: 14973372]
37. Yudit MR, Berrodin TJ, Jelinsky SA, Hanna LA, Brown EL, Chippari S, Bhat RA, Winneker RC, Zhang Z. Selective and opposing actions of progesterone receptor isoforms in human endometrial stromal cells. *Mol. Cell Endocrinol* 2006;247(1–2):116–126. [PubMed: 16442706]
38. Brayman MJ, Julian J, Mulac-Jericevic B, Conneely OM, Edwards DP, Carson DD. Progesterone receptor isoforms A and B differentially regulate MUC1 expression in uterine epithelial cells. *Mol. Endocrinol.* 2006
39. Ni X, Hou Y, Yang R, Tang X, Smith R, Nicholson RC. Progesterone receptors A and B differentially modulate corticotropin-releasing hormone gene expression through a cAMP regulatory element. *Cell Mol. Life Sci* 2004;61(9):1114–1122. [PubMed: 15112058]
40. Cheng KW, Cheng CK, Cheng PC. Differential role of PR-A and -B isoforms in transcription regulation of human GnRH receptor gene. *Mol. Endocrinol* 2001;15(12):2078–2092. [PubMed: 11731610]

41. Giangrande PH, Kimbrel EA, Edwards DP, McDonnell DP. The opposing transcriptional activities of the two isoforms of the human progesterone receptor are due to differential cofactor binding. *Mol. Cell Biol* 2000;20(9):3102–3115. [PubMed: 10757795]
42. Beral V. Breast cancer and hormone-replacement therapy in the Million Women Study. *Lancet* 2003;362(9382):419–427. [PubMed: 12927427]

Fig. 1.

Effect of progesterone or RU486 on COMT protein expression in MCF-7 and T47D breast cancer cell lines. The cells were treated with different concentrations of progesterone (10^{-8} – 10^{-6} M) or progesterone (10^{-7} M) combined with RU486 (10^{-6} M) for 72 hours. (A) Western blot analysis of COMT protein in MCF-7 cells treated with different concentrations of progesterone or/and progesterone combined with RU486. (B) The average percentage changes of COMT protein expression in MCF-7 cells by progesterone or/and RU486. (C) Western blot analysis of COMT protein in T47D cells treated with progesterone or/and progesterone combined with RU486. (D) The average percentage changes of COMT protein expression in T47D cells treated with progesterone or/and RU486. The values represent mean $\pm$ SEM of three independent experiments (* $P < 0.05$ as compared with control)

Fig. 2. Quantitative analysis of COMT mRNA expression levels with real time RT-PCR after treatment of MCF-7 cells (A) or T47D cells (B) with different concentrations of progesterone (10⁻⁸–10⁻⁶ M) or progesterone (10⁻⁷M) combined with RU486 (10⁻⁶M) for 48 hours. Expression levels are displayed as the mean ratio of target-to-reference gene $\pm$ SEM relative to the vehicle-treated control. Results are expressed as mean $\pm$ SEM of three independent experiments (**P* < 0.05 as compared with control)

Fig. 3.

Effect of progesterone and RU 486 on COMT promoter luciferase activities in MCF-7 cells (A) and T47D cells (B). The cells were cotransfected with COMTP1-luc or COMTP2-Luc luciferase reporter constructs and Promega pSV- β -galactosidase control vector (as a control for transfection efficiency). The transfected cells were treated with different concentrations of progesterone (10^{-8} – 10^{-6} M) or progesterone (10^{-7} M) combined with RU486 (10^{-6} M) for 48 hours. The luciferase and pSV- β -galactosidase activities were measured and the protein concentrations were determined. Luciferase activity was expressed after normalization against β -galactosidase activity and protein concentration. Results are expressed as mean $\pm$ SEM of three independent experiments (* P < 0.05 as compared with control)

Fig. 4.

Effect of progesterone on wild-type COMTP1-Luc or COMTP1-Luc with individual or combined mutations in PREs. The T47D cells were cotransfected with pSV- β -galactosidase (1 μ g) and the wild-type COMTP1-Luc or COMTP1-Luc with mutation in different PREs ((10 μ g) and then treated with progesterone (10^{-7} M) for 48 hours. The luciferase activity was determined and normalized against the protein concentration and β -galactosidase activity. The data are presented as the mean value $\pm$ SEM of three separate experiments.

Fig. 5.

EMSA of a half-palindromic PRE sequence from human COMT promoter by the progesterone receptor. T47D cell nuclear extracts (NE) were incubated with ^{32}P -labeled oligonucleotides containing a half-palindromic PRE. After incubation, the DNA-protein complexes were separated by electrophoresis on a nondenaturing polyacrylamide gel. Lane 1, NE only; lane 2, ^{32}P -PRE1 only; lane 3, NE + ^{32}P -PRE1; lane 4, NE + ^{32}P -PRE1 + $100 \times$ PRE1 (cold); lane 5, NE + ^{32}P -PRE1 + PR antibody; lane 6, NE + ^{32}P -PRE1 + PR antibody + $100 \times$ PRE1 (cold); lane 7, NE + ^{32}P -PRE2; lane 8, NE + ^{32}P -PRE2 + PR antibody; lane 9, NE + ^{32}P -PRE2 + $100 \times$ PRE2 (cold); and lane 10, NE + ^{32}P -PRE2 + PR antibody + 2 (cold). Free probe is shown at the bottom of the gel.

Fig. 6. SDS-PAGE/Western blot analysis of COMT expression in MDA-MB231 cells expressing either PR-A (panel B) or PR-B (panel C). The expression of PR-A or PR-B was attained by transfecting MDA-MB231 cells with adenovirus expressing PR-A or PR-B (panel A). The cells were treated with progesterone (10^{-8} – 10^{-7} M) or progesterone (10^{-7} M) in combination with RU486 (10^{-6} M) for 72 hours. The cell lysates were harvested and used for western blot analysis of COMT.