

Two faces of the estrogen metabolite 2-methoxyestradiol *in vitro* and *in vivo*

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Abstract. 2-Methoxyestradiol (2-ME), an endogenous metabolite of 17 β -estradiol (E2), interacts with estrogen receptors (ERs) and microtubules, however, 2-ME has a low affinity for ERs. Furthermore, 2-ME has been identified as a potential novel antitumor agent, combining its anti-proliferative effects on a variety of tumor cell types with its anti-angiogenic action. Therefore, 2-ME is of interest due to its potential anticancer therapeutic effects. In the current study, the estrogenic effect of 2-ME on CaBP-9k, ER α , and progesterone receptor (PR) mRNA levels in the absence and presence of E2 and progesterone (P4) in *in vivo* and *in vitro* models was examined. In GH3 cells, the mRNA level of CaBP-9k was induced in the E2 treatment group (concentration, 10⁻⁹ M), and the expression of CaBP-9k was also upregulated in the 2-ME-treated group (concentration, 10⁻⁷ M). Uterine lactoferrin (Ltf) mRNA expression was also increased in the 2-ME group [dose, 40 mg/kg body weight (BW)], which was comparable to the response with E2 (dose, 40 μ g/kg BW) observed in mice. As inhibitors of ER and PR activity, ICI 182,780 and mifepristone (RU486) were observed to reverse the E2 or 2-ME mediated increase of CaBP-9k and Ltf mRNA expression. In addition, it was found that 2-ME significantly decreased the levels of ER α and increased PR transcripts. Consistent with the *in vitro* results, the mRNA levels revealed decreased ER α and increased PR in *in vivo* treatment of E2 and 2-ME. These findings demonstrate that the expression of estrogenic markers, CaBP-9k and Ltf, is regulated by 2-ME in *in vitro* and *in vivo* models, therefore, estrogenic activities of 2-ME may be increased in females during the estrous cycle via the ER and/or PR-mediated signaling pathway.

Introduction

2-Methoxyestradiol (2-ME) is a metabolite of the endogenous estrogen hormone, 17 β -estradiol (E2) and the oral contraceptive agent 17-ethylestradiol, which is produced by sequential hepatic hydroxylation and methylation from the parent compounds (1). 2-ME is generated by catechol-O-methyltransferase, an enzyme expressed in various mammalian tissues, including the liver, kidneys, brain and red blood cells (2). 2-ME is the major catechol estrogen produced by the cytochrome P450 system in the liver (3). Estrogens act as important regulators of cell proliferation, cell survival, and differentiation in a variety of organs and tissues. In addition, estrogens have been implicated in the etiology of a variety of malignant cancers and benign tumors (4).

The estrogen metabolite, 2-ME, is an anti-proliferative and anti-angiogenic molecule that effectively induces apoptosis in actively proliferating cells *in vitro* and *in vivo* (5,6). 2-ME was found to act as a potent inhibitor of estrogen-induced proliferation in F344 rat pituitaries (7). Various mechanisms of 2-ME activity have been proposed, such as exerting effects on tubulin polymerization and depolymerization (8,9) or inducing mitochondrial apoptotic signaling (10). In breast cancer, however, higher baseline levels of estrogen and certain estrogen metabolites (total estradiol, bioavailable estradiol, estrone and estrone sulfate) were associated with higher breast cancer risk in post-menopausal females, other forms of the estrogen metabolite, 2-ME were also known as antitumor reagents (11). Estrogens may cause *de novo* breast cancer through either receptor-dependent or independent mechanisms, and through actions mediated by its receptor, estradiol, which enhances cell proliferation, a factor causally associated with breast cancer development (12). Furthermore, 2-ME is recognized for its unique and profound actions on various tumor cell lines and cancer cells, independent of the hormone receptor status (13). Regardless of differences in function, 2-ME has an affinity for estrogen receptors (ERs) (14,15), however, the underlying mechanisms of 2-ME via ERs have not been fully elucidated.

Previous studies have shown that CaBP-9k, a cytosolic calcium binding protein, is expressed in a variety of mammalian tissues, including the uterus, placenta, the intestine, kidneys, and pituitary glands (16,17). In addition, the CaBP-9k gene is well understood in the uterus and the levels of

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CaBP-9k mRNA and protein, which are induced by endocrine disruptors (EDs), were considered to be facilitative during the screening of environmental estrogenic compounds in an immature rat model (18). In addition, CaBP-9k expression was strongly regulated by sex-steroid hormones in the uterus and pituitary glands of mice (16,19). Uterine lactoferrin (Ltf), an iron-binding glycoprotein that is present in the majority of exocrine secretions and in the secondary granules of leukocytes, is regulated by estrogen in the reproductive tract (20). In previous studies, Ltf was localized in uterine epithelial cells and was shown to fluctuate in a mature mouse, during the estrous cycle, in response to the rise and fall of estrogen levels (20,21). The GH3 cell line, a well-established pituitary cell line, is sensitive to estrogenic stimulation (22). GH3 cells possess an ER that correlates with cell proliferation (23). Yang *et al* reported that treatment with sex-steroids and EDs induces CaBP-9k expression in GH3 cells (24).

The estrogenic or non-estrogenic activity of estrogen metabolites and EDs has been evaluated in recent studies (25,26). However, the residual effect of high dose 2-ME administered to cancer patients on physiological conditions remains to be fully elucidated. In the current study, it was hypothesized that the estrogenicity of 2-ME may increase the expression of estrogen-mediated genes and contribute to the induction of endocrine disturbance. Therefore, estrogen response genes were investigated using CaBP-9k, Ltf, and mRNA expression levels of steroid hormone receptors in *in vitro* and *in vivo* models.

Materials and methods

Chemicals. E2, progesterone (P4) and mifepristone (RU486) were obtained from Sigma-Aldrich (St. Louis, MO, USA). 2-ME and ICI 182,780 were purchased from Tocris Bioscience (Bristol, UK).

Cell culture. GH3 cells were obtained from The Korean Cell Line Bank (Seoul, Republic of Korea). The cells were grown as a monolayer culture in Dulbecco's modified Eagle's medium (DMEM; Gibco Life Technologies, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS; Gibco Life Technologies), 100 IU/ml penicillin and 100 µg/ml streptomycin (Gibco Life Technologies) at 37°C in a humidified atmosphere of 5% CO₂. The GH3 cells were plated on 6-well plastic tissue culture dishes (NUNC, Roskilde, Denmark) at a density of 3×10⁵ cells/well and grown until 70–80% confluence was reached. The media was replaced with phenol red-free DMEM supplemented with 5% charcoal/dextran (CD)-stripped FBS and 100 U/ml penicillin-streptomycin for seven days to ensure depletion of the steroid hormones in the cells. The cells used in the present study were grown normally throughout the investigation. After seven days, the cells were exposed to a single dose of E2 (10⁻⁹ M), P4 (10⁻⁶ M), 2-ME (10⁻⁸, 10⁻⁷ or 10⁻⁶ M). Each chemical was dissolved in 100% dimethyl sulfoxide (DMSO) and added to phenol red-free DMEM-5% CD-FBS (starvation media) with a final DMSO concentration of 0.1%. The starvation media with 10⁻⁹ M E2 served as a positive control and starvation media alone served as the negative control [vehicle (VE)]. The GH3 cells were harvested 24 h after treatment for measurement of the mRNA

levels. To examine the mechanism of CaBP-9k induction by these chemicals, the cells were pre-treated with 10⁻⁷ M ICI 182,780 and 10⁻⁶ M RU486 for 30 min prior to exposure to E2 and 2-ME (27,28). Following treatment with ICI 182,780 and RU486, the cells were treated with E2 (10⁻⁹ M) and 2-ME (at 10⁻⁸, 10⁻⁷ or 10⁻⁶ M). After 24 h, whole cells were harvested for mRNA analysis. All of the experiments were performed in duplicate.

Animals and treatments. Female Institute for Cancer Research mice (postnatal day 14) were obtained from Koatech Co., Ltd. (Gyeonggi, Republic of Korea) and maintained in the animal facility at the College of Veterinary Medicine, Chungbuk National University (Chungbuk, Republic of Korea). The use of the animals and the experimental procedures were approved by the Institutional Animal Care and Use Committee of Chungbuk National University. All of the animals were housed in polycarbonate cages and used after acclimation to an environmentally controlled room (temperature, 23±2°C; relative humidity, 50±10%; frequent ventilation and a 12:12 h light-dark cycle). The mice were fed soy-free pellet food (Dyets Inc., Bethlehem, PA, USA) and had access to water *ad libitum* throughout the study. The 35 mice were divided into seven groups (n=5 per group), and each group was administered subcutaneously with 24% DMSO, 38% ethanol and 38% sterile saline (VE), 40 µg/kg body weight (BW) E2 (a physiological dose level), or 4, 40 or 80 mg/kg BW 2-ME for three days. The mice were euthanized 24 h after the final injection. To investigate the effect of antagonism, 10 rats were injected subcutaneously with 10 mg/kg BW ICI 182,780 and 10 mg/kg BW RU486 30 min prior to injection with 40 mg/kg BW 2-ME for three days. The rats were euthanized 24 h after the final injection.

Uterine wet weight. Mice were sacrificed by cervical dislocation. The uterus was excised completely and trimmed free of fat. The body of the uterus was cut just above the junction with the cervix. The wet weight of the mouse uterus was recorded.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted using TRI reagent (Ambion Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions and the concentration of total RNA was determined by measuring the absorbance at 260 nm. Total RNA (1 µg) was reverse transcribed into first-strand cDNA using M-MLV Reverse Transcriptase (Invitrogen Life Technologies, Carlsbad, CA, USA) and 9-mer random primers (Takara Bio, Inc., Otsu, Japan).

RT-qPCR was performed using a 7300 Real-Time PCR system (Applied Biosystems Life Technologies, Foster City, CA, USA) with 1 µl cDNA template added to 10 µl 2X SYBR Premix Ex Taq (Takara Bio, Inc.) containing specific primers at a concentration of 10 pM each. The reactions were performed for 40 cycles and the cycling parameters were as follows: Denaturation at 95°C for 30 sec, annealing at 58°C for 30 sec and extension at 72°C for 30 sec. The fluorescence intensity was measured on termination of the extension phase of each cycle and the threshold value for the fluorescence intensity of all samples was set manually. The reaction cycle at which the PCR products exceeded this fluorescence intensity threshold

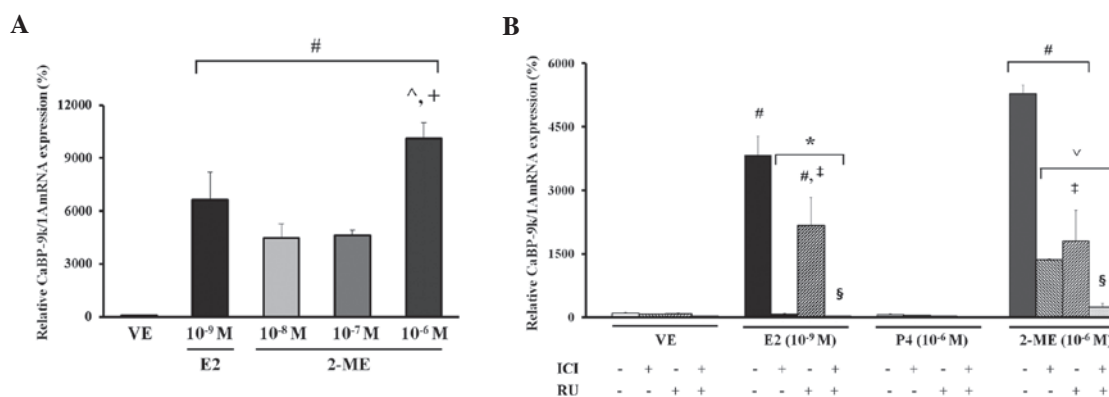


Figure 1. mRNA expression of CaBP-9k under E2, P4 and 2-ME treatment or co-treatment with/without ICI 182,780 or RU486 in GH3 cells. (A) The concentration-dependent effect of 2-ME and E2 on CaBP-9k mRNA expression. (B) Inhibition effects of E2, P4 and 2-ME on CaBP-9k mRNA expression. [#]P<0.05 compared with VE; ^{*}P<0.05 compared with E2 (10⁻⁹ M); [^]P<0.05 compared with 2-ME (10⁻⁸ M); ⁺P<0.05 compared with 2-ME (10⁻⁷ M); [∨]P<0.05 compared with 2-ME (10⁻⁶ M); [§]P<0.05 compared with steroids with ICI (10⁻⁷ M); [§]P<0.05 compared with steroids with RU (10⁻⁶ M). Each gene was normalized with cytochrome c oxidase subunit 1 gene (1A) and served as the internal control for the mRNA concentration. Data are presented as the mean ± standard error of the mean of triplicate samples. VE, vehicle; E2, 17 β -estradiol; 2-ME, 2-methoxyestradiol.

in the exponential phase of PCR amplification was taken as the threshold cycle (CT). The PCR product of cytochrome c oxidase subunit 1 (1A, a ubiquitously expressed housekeeping gene) (16) served as a control for mRNA concentrations in the RT-qPCR reactions. The relative expression level of each gene was quantified using RQ study software (Applied Biosystems Life Technologies). The quantity of transcript present was inversely associated with the observed CT and, for every two-fold dilution in the quantity of transcript, CT was expected to increase by one. Relative expression (*R*) was calculated using the equation $R = 2^{-(\Delta CT_{\text{sample}} - \Delta CT_{\text{control}})}$. To determine a normalized arbitrary value for each gene, every data point was normalized to the control gene (1A), as well as to the respective controls. The primers were as follows: Sense, 5'-CCAGGGTTTGGGAATTATTTTC-3' and antisense, 5'-GAA GATAAACCTTAAGGCTC-3' for rat and mouse 1A; sense, 5'-AAGAGCATTTCCTCAAAAATA-3' and antisense, 5'-GTC TCAGAATTTGCTTTATT-3' for rat CaBP-9k; sense, 5'-GAC TTGAATCTCCACGATCA-3' and antisense, 5'-CTTCAA GGTGCTGGATAGAA-3' for rat ER α ; sense, 5'-TGTGTC CAGCTACAAACCAATG-3' and antisense, 5'-CATCATGCC CACTTCGTAACA-3' for mouse ER α ; sense, 5'-GAAGAG CAAACCTCGAGCAC-3' and antisense, 5'-AGCAGAAAA CCGGAATCTT-3' for rat PR; sense, 5'-AATCCCACAGGA GTTTGTCA-3' and antisense, 5'-GGACAACCCCTTCT GTCTT-3' for mouse PR; and sense, 5'-CTTTAAGGTGTC TGGCTGAGAAG-3' and antisense, 5'-AGTTCCTTAGCCT CAGTCACAGGTT-3' for mouse Ltf.

Statistical analysis. The results are presented as the mean ± standard error of the mean. P-values were calculated using one-way analysis of variance, followed by Tukey's test for multiple comparisons of columns. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of 2-ME on regulation of CaBP-9k mRNA expression in GH3 cells. 2-ME was applied to GH3 cells at three concentrations (10⁻⁸, 10⁻⁷ and 10⁻⁶ M). CaBP-9k mRNA levels

were then analyzed as an estrogen marker of rat pituitary GH3 cells using RT-qPCR (25). As shown in Fig. 1A, a significant increase in expression of CaBP-9k mRNA was observed at concentrations of 10⁻⁸, 10⁻⁷ and 10⁻⁶ M 2-ME, and 10⁻⁹ M E2 (P<0.02). Compared with the VE (negative) controls, the CaBP-9k mRNA level increased by ~66-fold in the E2-treated (10⁻⁹ M) positive control group and by 44.4- to 100-fold in the 2-ME-treated group. The expression of CaBP-9k demonstrated a dose-dependent response to 2-ME; treatment with 2-ME at a dose of 10⁻⁶ M resulted in a 2.2-fold increase, compared with the 10⁻⁸ or 10⁻⁷ M treatment group (Fig. 1A). For identification of the steroid hormone receptor signaling pathway involved in transcriptional upregulation of CaBP-9k expression by 2-ME, the GH3 cells were pretreated with the ER antagonist, ICI 182,780 or with the PR antagonist, RU486 (28), which determined that co-treatment with 2-ME (10⁻⁶ M) induced the highest expression of CaBP-9k mRNA among the 10⁻⁸, 10⁻⁷ and 10⁻⁶ M 2-ME doses, and compared with the E2 (10⁻⁹ M)-, P4 (10⁻⁶ M)- or VE (0.1% DMSO)-treated groups (Fig. 1B).

CaBP-9k mRNA levels were analyzed using RT-qPCR. As shown in Fig. 1B, induction of CaBP-9k mRNA expression by E2 and 2-ME was completely abolished by co-treatment with ICI 182,780 at a concentration of 10⁻⁷ M. Results of co-treatment of the PR antagonist, RU486 (10⁻⁶ M) with E2 (10⁻⁹ M) and 2-ME (10⁻⁶ M) induced a significant increase in expression of CaBP-9k mRNA when compared with the VE control (P<0.0001). Notably, CaBP-9k mRNA expression in the E2- or 2-ME-treated groups following co-treatment with ICI 182,780 (10⁻⁷ M) and RU486 (10⁻⁶ M) was significantly decreased when compared with co-treatment with RU486 (10⁻⁶ M) alone (P<0.0002; Fig. 1B).

Effect of 2-ME on mRNA expression of steroid hormone receptors in GH3 cells. 2-ME was applied to GH3 cells at three concentrations (10⁻⁸, 10⁻⁷ or 10⁻⁶ M) for evaluation of the potential impact of 2-ME. In addition, the mRNA levels of ER α between ER α and PR were measured to evaluate the estrogenicity of estrogen metabolite, 2-ME (27). GH3 cells were treated with 2-ME (10⁻⁸, 10⁻⁷ and 10⁻⁶ M), E2 (10⁻⁹ M) or the VE (0.1% DMSO). Compared with the VE control, E2

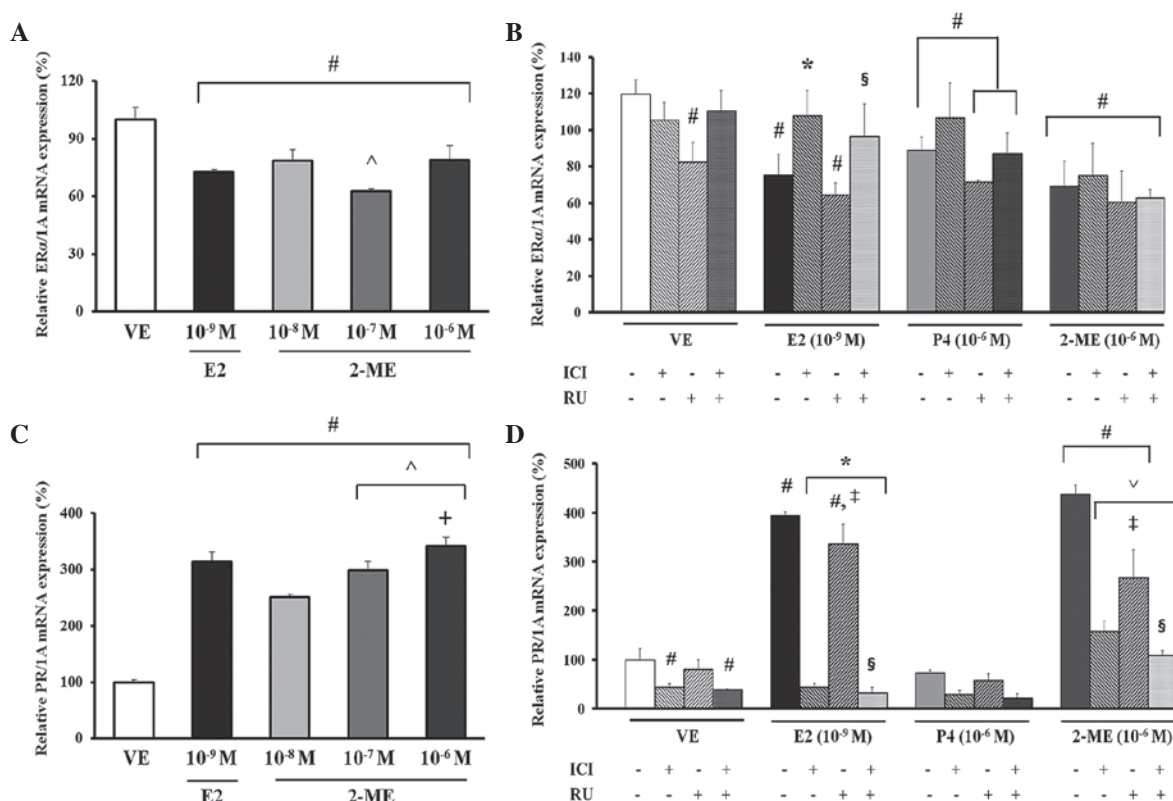


Figure 2. mRNA expression of ER α and PR under E2, P4 and 2-ME treatment or co-treatment with/without ICI 182,780 or RU486 in GH3 cells. (A) The concentration-dependent effect of 2-ME and E2 on ER α mRNA expression. (B) Inhibition effects of E2, P4 and 2-ME on ER α mRNA expression. (C) The concentration-dependent effect of 2-ME and E2 on PR mRNA expression. (D) Inhibition effects of E2, P4 and 2-ME on PR mRNA expression. #P<0.05 compared with VE; *P<0.05 compared with E2 (10^{-9} M); ^P<0.05 compared with 2-ME (10^{-8} M); +P<0.05 compared with 2-ME (10^{-7} M); §P<0.05 compared with 2-ME (10^{-6} M); vP<0.05 compared with steroids with ICI (10^{-7} M); §P<0.05 compared with steroids with RU (10^{-6} M). Each gene was normalized with cytochrome *c* oxidase subunit 1 gene (1A) and served as the internal control for the mRNA concentration. Data are presented as the mean \pm standard error of the mean of triplicate samples. VE, vehicle; E2, 17 β -estradiol; 2-ME, 2-methoxyestradiol; ER α , estrogen receptor α ; PR, progesterone receptor.

induced a decrease of ~ 1.4 -fold and treatment with all doses of 2-ME resulted in decreased expression of ER α mRNA by ~ 1.2 - to 1.3 -fold (Fig. 2A). The dose-dependent response of 2-ME on ER α gene expression was observed in the 10^{-7} M treatment group compared with the 10^{-8} M 2-ME treatment group. In addition, the 2-ME treatment group experienced a similar response to that of the E2 treatment group by demonstrating a decrease in ER expression levels (Fig. 2A).

For identification of the steroid hormone receptor signaling pathway involved in transcriptional upregulation of CaBP-9k mRNA expression by 2-ME, the GH3 cells were pretreated with the ER antagonist, ICI 182,780 or with the PR antagonist, RU486 (28). In the case of ER α mRNA expression compared with the VE control, E2 induced a decrease of ~ 1.5 -fold and treatment with ICI 182,780 and/or RU486 induced 2-ME resulted in a decrease of ~ 1.7 - to 1.9 -fold (Fig. 2B). Notably, ER α mRNA expression in the E2 treatment group, as a result of co-treatment with ICI 182,780 (10^{-7} M) and RU486 (10^{-6} M), was markedly increased compared with co-treatment with RU486 (10^{-6} M) alone (Fig. 2B; P<0.005). In the case of PR mRNA expression, the positive control, E2, and all doses of 2-ME induced a significant increase in expression of PR mRNA compared with the VE (Fig. 2C; P<0.0003).

For identification of the steroid hormone receptor signaling pathway involved in transcriptional regulation of PR mRNA expression by 2-ME, GH3 cells were pretreated with the ER

antagonist, ICI 182,780 or with the PR antagonist, RU486 (28). Compared with VE, the E2 and 2-ME treatment groups showed a significantly increased pattern of PR expression (P<0.0001). Of particular interest, PR mRNA expression resulting from co-treatment with ICI 182,780 (10^{-7} M) and RU486 (10^{-6} M) in the E2 or 2-ME treatment groups was markedly decreased compared with co-treatment with RU486 (10^{-6} M) alone (Fig. 2D; P<0.0001). Thus, the magnitude and pattern of induction of PR mRNA by 2-ME were very similar to the enhanced expression of CaBP-9k following treatment with 2-ME (Fig. 1A and 2C; Fig. 1B and 2D).

Effects of 2-ME on uterine wet weight. A uterotrophic assay was used to identify an estrogen-like effect (26). The effects were determined by subcutaneous injection with 2-ME (4, 40 or 80 mg/kg BW) or E2 (40 μ g/kg BW). ICI 182,780 or RU486 were treated with 2-ME (40 mg/kg BW) for determining the steroid hormone receptor signaling pathway involved in 2-ME. Compared with the VE, an ~ 3.9 -fold increase in uterine wet weight was observed upon treatment with 40 μ g/kg BW E2, and ~ 4.1 - to 5.0 -fold increase at 4, 40 and 80 mg/kg BW 2-ME. However, no significant dose-dependent result was observed for any dose of 2-ME (Fig. 3A; P<0.05). Due to demonstrating the greatest increase in uterine wet weight among the 4, 40 and 80 mg/kg BW doses of 2-ME, the steroid hormone receptor antagonist treatment of 40 mg/kg BW 2-ME, became the focus

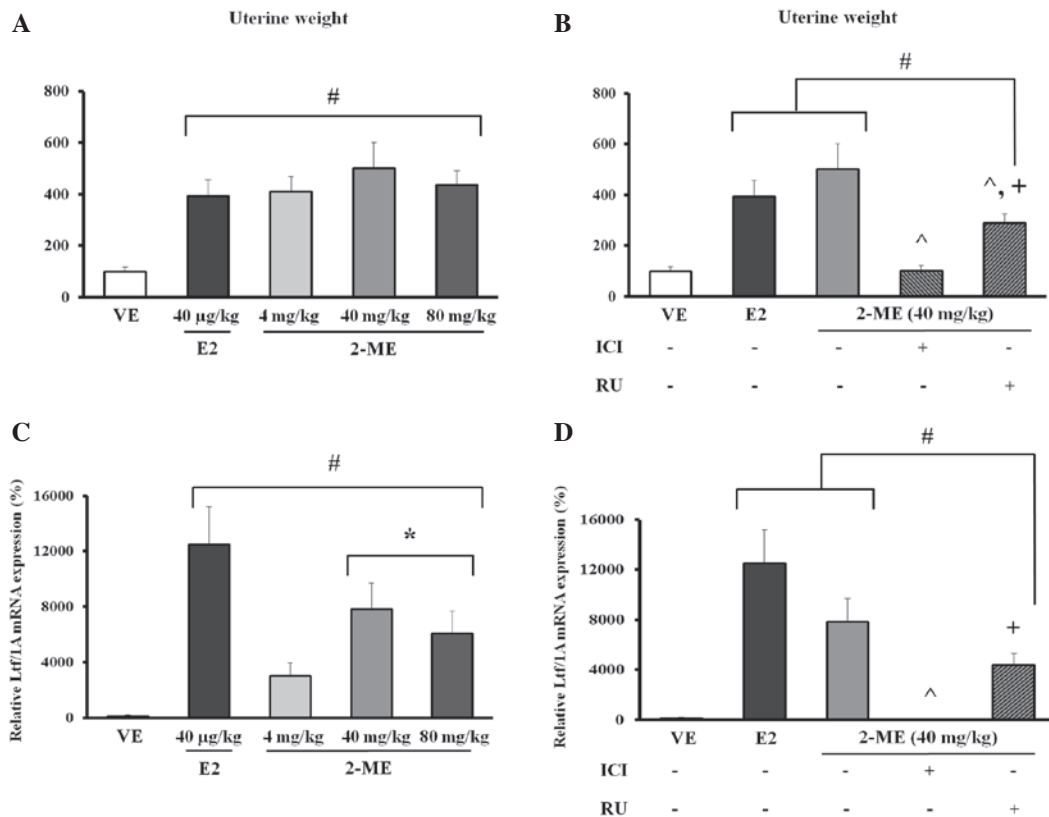


Figure 3. Uterine weight and mRNA expression of Ltf under E2 and 2-ME treatment or co-treatment with/without ICI 182,780 or RU486 on 2-ME induced mice. (A) The concentration-dependent effect of 2-ME and E2 on mice uterus weight. (B) Inhibition effects of 2-ME on mice uterus weight. (C) The concentration-dependent effect of 2-ME and E2 on Ltf mRNA expression. (D) Inhibition effects of 2-ME on Ltf mRNA expression. #P<0.05 compared with VE; *P<0.05 compared with 2-ME (4 mg/kg BW); ^P<0.05 compared with 2-ME (40 mg/kg BW); +P<0.05 compared with 2-ME (40 mg/kg BW) with ICI (10 mg/kg BW). Each gene was normalized with cytochrome c oxidase subunit 1 gene (1A) and served as the internal control for the mRNA concentration. Data is presented as the mean \pm standard error of the mean of duplicate samples. VE, vehicle; E2, 17 β -estradiol; 2-ME, 2-methoxyestradiol; Ltf, uterine lactoferrin.

(Fig. 3A). Co-treatment with ICI 182,780 (10 mg/kg BW) on 2-ME (40 mg/kg BW) induced a 5-fold decrease compared with 2-ME alone (P<0.0001). In addition, RU486 treatment on 2-ME (40 mg/kg BW) induced a significant 1.7-fold decrease compared with 2-ME alone. Notably, co-treatment with RU486 in the 2-ME treatment group resulted in a 2.8-fold increase when compared with ICI 182,780 co-treatment (Fig. 3B).

Effects of 2-ME on the regulation of Ltf gene mRNA expression in the uterus of mice. To evaluate the potential impact of 2-ME, Ltf mRNA levels were analyzed as an estrogen marker of rodents using RT-qPCR (29). When compared with the VE control, E2 at 40 µg/kg BW induced an ~125-fold increase in Ltf mRNA expression and 2-ME at 40 mg/kg BW induced an ~78.3-fold increase. Furthermore, treatment with 2-ME at 80 mg/kg BW resulted in an ~60.7-fold increase in Ltf mRNA expression. In particular, the dose of 40 mg/kg BW 2-ME demonstrated the most similarity with E2 among the three doses of 2-ME, as shown by the increased Ltf gene expression level (Fig. 3C).

For identification of the steroid hormone receptor signaling pathway involved in transcriptional upregulation of Ltf gene expression by 2-ME, mice were pre-injected with the ER antagonist, ICI 182,780, at 10 mg/kg BW or with the PR antagonist, RU486, at 10 mg/kg BW (28). E2 at 40 µg/kg BW and 2-ME at 40 mg/kg BW were analyzed and co-treatment

of RU486 (10 mg/kg BW) with 2-ME (40 mg/kg BW) induced significantly increased Ltf gene expression when compared with the VE (P<0.004). In addition, 2-ME (40 mg/kg BW) was completely abolished by treatment with ICI 182,780 (10 mg/kg BW). Notably, co-treatment of RU486 with 2-ME resulted in a significant 470-fold increase when compared with co-treatment of ICI 182,780 and 2-ME (Fig. 3D; P<0.003).

Effect of 2-ME on mRNA expression of steroid hormone receptors in the uterus of mice. For evaluation of the potential impact of 2-ME, the mRNA levels of ER α and PR, which respond to materials similar to natural and synthetic estrogen were determined (27). Mice received a subcutaneous injection of 2-ME (4, 40 and 80 mg/kg BW), E2 (40 µg/kg BW) or VE (24% DMSO, 38% ethanol and 38% sterile saline). Treatment with 2-ME (4, 40 and 80 mg/kg BW) or E2 (40 µg/kg BW) resulted in a decreasing pattern of ER α when compared with the VE, however, the differences were not considered to be significant, apart from 2-ME in the 80 mg/kg BW treatment group. In addition, no dose-dependent response on ER α expression was observed at any of the doses of 2-ME (Fig. 4A).

For identification of the steroid hormone receptor signaling pathway involved in transcriptional upregulation of Ltf mRNA expression by 2-ME, the mice were pretreated with the ER antagonist, ICI 182,780 or with the PR antagonist, RU486. In the case of ER α , co-treatment with ICI 182,780 (10 mg/kg

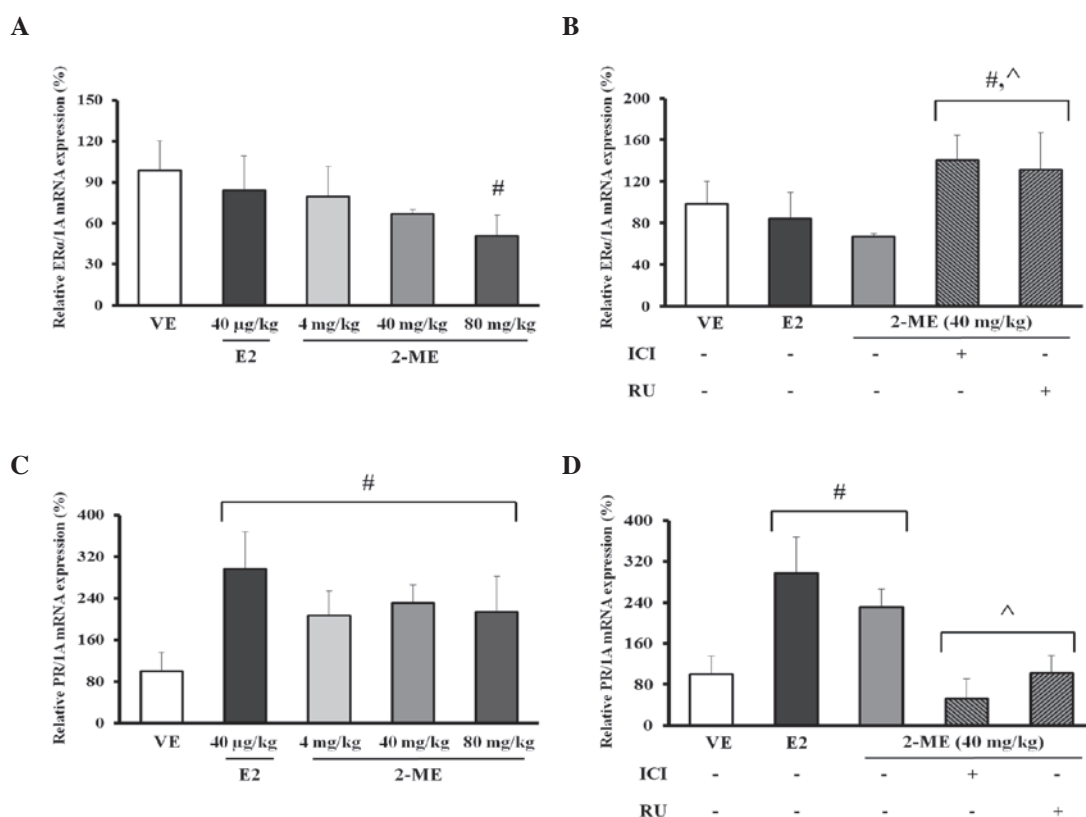


Figure 4. mRNA expression of ER α and PR under E2 and 2-ME treatment or co-treatment with/without ICI 182,780 or RU486 on 2-ME induced mice. (A) The concentration-dependent effect of 2-ME and E2 on ER α mRNA expression. (B) Inhibition effects of 2-ME on ER α mRNA expression. (C) The concentration-dependent effect of 2-ME and E2 on PR mRNA expression. (D) Inhibition effects of 2-ME on PR mRNA expression. [#]*P*<0.05 compared with VE, [^]*P*<0.05 compared with 2-ME (40 mg/kg BW). Each gene was normalized with cytochrome *c* oxidase subunit 1 gene (1A) and served as the internal control for the mRNA concentration. Data are presented as the mean \pm standard error of the mean of duplicate samples. VE, vehicle; E2, 17 β -estradiol; 2-ME, 2-methoxyestradiol; ER α , estrogen receptor α ; PR, progesterone receptor.

BW) or RU486 (10 mg/kg BW) on 2-ME (10⁻⁶M) induced a marked increase in ER α mRNA expression of 1.42-fold when compared with 2-ME alone (Fig. 4B; *P*<0.02). In the case of PR mRNA expression, PR showed an increase of ~2.94-fold in the E2 treatment group when compared with the VE and an increase of ~2.1- to 2.3-fold was observed for all doses in the 2-ME (4, 40 and 80 mg/kg BW) treatment group; however, a dose-response effect of 2-ME was not observed (Fig. 4C).

For identification of the steroid hormone receptor signaling pathway involved in transcriptional upregulation of Ltf mRNA expression by 2-ME, mice were pretreated with the ER antagonist, ICI 182,780 or with PR antagonist, RU486. When compared with the VE control, treatment with E2 and 2-ME resulted in a marked increase in PR mRNA expression. Co-treatment with ICI 182,780 (10 mg/kg BW) or RU486 (10 mg/kg BW) on 2-ME resulted in a significant decrease in PR mRNA expression when compared with administration of 2-ME (40 mg/kg BW) alone (*P*<0.002). In addition, co-treatment with RU486 on 2-ME resulted in an increase in PR mRNA expression when compared with co-treatment with ICI 182,780; however, the increase was not significant (Fig. 4D).

Discussion

Previously, the effect of pharmacological levels of 2-ME on normal physiological systems has not been adequately

described. Although the various possible mechanisms of 2-ME were not fully elucidated, 2-ME has been administered as an antitumor therapy. For example, *in vitro*, 2-ME has been shown to affect angiogenesis and proliferation of malignant cells, and *in vivo*, 2-ME was demonstrated to inhibit the growth of tumors arising from a subcutaneous injection in mice (30). To the best of our knowledge, the estrogenic activity *in vitro* and *in vivo* of the estradiol metabolite, 2-ME has not been fully determined. The present study indicates that 2-ME exhibits estrogenicity under *in vitro* and *in vivo* conditions.

The CaBP-9k gene is known to be controlled by sex hormones. Expression of the CaBP-9k gene was shown to be upregulated by estrogen and downregulated by P4 during the estrous cycle and early pregnancy in a rat uterus (31,32). In the current study, CaBP-9k expression in GH3 cells was demonstrated as a useful marker for evaluating the estrogenic potential of environmental chemicals, such as parabens (27). Thus, the present findings, showing induction of CaBP-9k gene mRNA expression in GH3 cells demonstrate that 2-ME may have an estrogenic affect. In addition, 2-ME shares the effects of estradiol, such as increasing uterine weight, and the data obtained in the current study indicated the presence of circulating estrogens, which was indicated by the antagonism of uterine wet weights by ICI 182,780 (33). In mice, the Ltf gene expression caused by exposure to estrogen was shown to be associated with an estrogen-stimulated mouse uterus (29).

During the present study, it was demonstrated that 2-ME mediates the estrogen biomarker gene. Changes in biomarker expression could be expected to result from this ER-dependent action that would be matched by E2-induced effects. ER α is expressed in the uterus, mammary glands, testes, pituitary gland, liver, kidneys and heart (34). ER β is found in the prostate and ovaries (35-37). According to Akbas *et al.*, the ER α mRNA expression of the bilateral ovariectomized uterus of adult mice was decreased by treatment with E2 when compared with untreated mice (38). The present result indicated that the effect of 2-ME on ER α mRNA expression may partly result from reduced binding to and signaling through the ER α pathway, which is blocked by pure antiestrogen. Estrogenic chemicals, such as parabens, induce an increase in PR gene expression, which is known to be highly sensitive to, and enhanced by, ER-mediated transcriptional signaling. Therefore, almost all ER-dependent signaling may contribute significantly to the enhanced expression of CaBP-9k and PR genes by specific paraben compounds in *in vitro* models (39). In the present study, the effect of 2-ME on Ltf and PR expression may partly result from binding to and signaling through the PR pathway, which is blocked by pure anti-P4.

2-ME is known as a potential novel antitumor agent combining its anti-proliferative activity on a wide range of tumor cell types with anti-angiogenic action (40). Combination treatment with agents that possess anti-proliferative and anti-angiogenic activity results in antitumor synergy and reduces the likelihood of antitumor drug resistance (41). For example, *in vitro*, 2-ME demonstrated the strongest inhibitory effect of all of the estrogen metabolites tested, with a half-maximal inhibitory concentration of $\sim 0.15 \mu\text{M}$ (30). In addition, it exhibited a highly similar growth-inhibitory effect in human breast cancer cell lines, and this effect was not altered by the presence or absence of exogenous E2 (42). Similarly, *in vivo*, it was reported that a 100-mg/kg dose of 2-ME inhibited tumor growth in various models with dramatic suppression of longitudinal bone growth in ovary-intact young, growing rats (43). However, low levels of 2-ME may have an influence on females during pregnancy. Preeclampsia, hypoxia and placental defects lead to deficiency in placenta-derived hydroxyestradiols, which in turn may result in a further decrease in the 2-ME level (44).

2-ME was found to be rapidly removed from the plasma and was below the limit of detection (11 ng/ml) 1 h after administration of a 10-mg/kg dose. Following, intravenous administration of 2-ME, its half-life in plasma was ~ 14 min (45). Despite numerous studies on the effect of pharmacological levels of 2-ME on normal physiological systems, it remains to be described fully.

In conclusion, the results of the present study (based on uterine wet weight gain, and increasing levels of estrogen biomarkers in *in vitro* and *in vivo* models) demonstrate that all experimental doses of 2-ME, which were lower than anticancer therapeutic concentrations, exerted an estrogenic effect. In addition, the estrogenic activity of 2-ME was observed to be blocked by the ER and PR antagonists, ICI 162,780 and RU486, respectively, in the *in vitro* and *in vivo* models. Since 2-ME shows proliferative effects, it is necessary that potential risk of 2-ME should be further investigated as cancer therapeutic reagents, which may affect endocrine homeostasis

and/or function. Overall, the present results reveal that 2-ME may impact the ER and PR, and maintain the body in a state of disturbance of the endocrine system.

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