METABOLISM OF ESTRADIOL, ETHYNYLESTRADIOL, AND MOXESTROL IN RAT UTERUS, VAGINA, AND AORTA: INFLUENCE OF SEX STEROID TREATMENT

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(Received September 1, 2000; accepted September 20, 2000)

This paper is available online at http://dmd.aspetjournals.org

ABSTRACT:

Estrogen replacement therapy for postmenopausal women consists of an estrogenic and a progestagenic compound. The treatment has a positive estrogenic effect on bone, the cardiovascular system, and vagina but is dependent of the estrogen-progestagen balance in uterus to prevent unwanted proliferation. We were interested in the influence of estrogens and progestagens on estrogen metabolism in target tissues of estrogen replacement therapy. Therefore, we studied the metabolism of estradiol, 17α-ethynylestradiol, and moxestrol (11β-methoxy-17α-ethynylestradiol) in rat uterus, vagina, and aorta. In uterus and vagina, estradiol was converted to estrone, estradiol-3-glucuronide, and estrone-3-glucuronide. These metabolites demonstrate the presence of 17β-hydroxyysteroid dehydrogenase (17β-HSD) and UDP-glucuronosyl transferase (UDP-GT) in uterus and vagina. We found that the conversion of estradiol by 17β-HSD in uterus was increased in animals treated with estradiol or with a combination of estradiol and progesterone. The conversion of estradiol in uterus by UDP-GT was estradiol-induced and in contrast, progesterone-suppressed. In the vagina, steroid hormone treatment had no effect on estradiol conversion by 17β-HSD or UDP-GT. Ethynylestradiol was glucuronidated only, and this was not affected by steroid treatment. Moxestrol was not converted in any of the three organs that were studied, indicating that the 11β-methoxy substituent renders it a poor substrate for glucuronidation. Overall, the estrogen metabolism, and its regulation by sex steroids, in rat uterus is different compared with human uterus. Therefore, the rat may not be the best-suited model to investigate uterine effects of estradiol-progestagen combined treatment.

The target cell sensitivity of steroid hormones is determined by the concerted action of steroid hormone receptors and steroid-inactivating enzymes or steroid-activating enzymes (for review, see Roy, 1992). For estrogen target tissues, this phenomenon has been studied mostly in the human uterus. In the human uterus, inactivation of the most potent natural estrogen, estradiol, to a weakly active estrogen, estrone, is catalyzed by 17β-hydroxyysteroid dehydrogenase (17β-HSD)1 EC 1.1.1.62. In addition, estrogen sulfotransferase (EC 2.8.2.15) converts estradiol to its inactive sulfoconjugate (Liu and Tseng, 1979). In the human uterus, both enzymes are induced by progesterone and their activity is highest during the secretory phase of the menstrual cycle, whereas estradiol receptor levels are lowest during this phase. Together, the lower estradiol receptor levels and the increased intracellular inactivation of estradiol play a role in the arrest of estrogen-mediated cell proliferation and the transition to the differentiated function of the endometrium during the secretory phase.

The estrogen-inactivating capacity of estrogen-sensitive tissues may also have a pharmacological role in estrogen replacement therapy (ERT) of postmenopausal women. Estrogen replacement therapy with (synthetic) estrogens can effectively treat symptoms and osteoporosis that are related to the marked decline in plasma estradiol levels after menopause (Horsman et al., 1983). Organs that are positively affected by ERT include bone, vagina, and the cardiovascular system. The apparent cardiovascular protection by estrogens, for example, is thought to be mediated in part by a favorable alteration in the plasma lipid profile (Walsh et al., 1991), but growing evidence suggests that direct effects of estrogen on the blood vessel wall play a major role (Mikkola et al., 1998). However, unwanted estrogenic effects in other organs accompany the beneficial estrogenic effects on bone and the cardiovascular system. In the uterus, for example, unopposed estrogen treatment can frequently lead to an increased risk of developing uterine cancers. Therefore, ERT consists of a combined estrogen and progestagen treatment. Because ERT thus ideally would act estrogenic in certain organs (e.g., bone, cardiovascular system), and not in others (e.g., uterus), it is of interest whether, and to what extent, inactivation of administered estrogen occurs in these tissues, and whether it is influenced by the progestagen coadministered in ERT.

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1 Abbreviations used are: 17β-HSD, 17β-hydroxyosteroid dehydrogenase; ERT, estrogen replacement therapy; E2, estradiol, 1,3,5[10]-estratriene-3,17β-diol; EE, ethynylestradiol, 17α-ethynylestradiol, and moxestrol (11β-methoxy-17α-ethynylestradiol) for ERT treatment; 11β-methoxy-ethynylestradiol, (11β,17α)-17-ethynyl-11-1,3,5[10]-estratriene-3,17β-diol; ovx, ovariectomized; P4, progesterone; UDP-GT, UDP-glucuronosyl transferase; HPLC, high performance liquid chromatography; E2-3,3'-diol, estradiol-3-[3,3'-diol]; E1-3,3'-diol, estrone-3-[3,3'-diol]; E1-3-G, estrone-3-glucuronide.

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what has been observed in postmenopausal women (Turner et al., 1994). Whether the estrogenic effects in other organs that may be affected by ERT can also be reliably studied in this model will depend, among other factors, on the similarity in estrogen metabolism in these organs in rat and human. Therefore, we were interested in the estrogen-inactivating capacity of rat uterus, vagina, and aorta. It is known that 17β-HSD activity is present in human and rat uterus, but in contrast to the human uterus, its activity in the rat can be induced by estradiol treatment and is highest during proestrus and estrus of the estrous cycle (Kreitmann et al., 1980; Wahawisan and Gorell, 1980; Liu et al., 1990). In this study, we assessed the ability of uterus, vagina, and aorta tissue to regulate local estrogen levels through metabolism. Also the influence of estrogen and progestagen treatment on the estrogen-metabolizing potency of these three organs was investigated. Furthermore, we studied the effect of different substituents at positions 11 and 17 of the steroid structure on its conversion. Therefore, we investigated the metabolism of 17β-estradiol (E2), 17α-ethynylestradiol (EE), and moxestrol (MOX; 11β-methoxy-17α-ethynylestradiol) in uterus, vagina, and aorta tissue from ovariectomized (ovx) rats and ovx rats treated with E2, progesterone (P4), or a combination of E2 and P4.

To our knowledge, no reports exist on the metabolism of EE and MOX in uterus, vagina, or aorta. Preliminary experiments in our laboratory indicated the presence of 17β-HSD and UDP-glucuronosyltransferase (UDP-GT, EC 2.4.1.17) activity in uterus and vagina tissue.

Materials and Methods

**Chemicals.** [2,4,6,7-3H]Estradiol (specific activity 3110 GBq/mmol), [6,7-3H]ethynylestradiol (specific activity 1820 GBq/mmol), and [11β-methoxy-3H]moxestrol (specific activity 3130 GBq/mmol) were purchased from NEN Life Science Products, Hoofddorp, The Netherlands. All other chemicals were obtained from local commercial sources and were of analytical grade.

**Animals.** Mature female Wistar rats, strain HSd/Cpd:Wu (Harlan, Horst, The Netherlands), with a weight between 225 and 250 g were fed with standard dry food and tap water ad libitum. The animals were subjected to a 14:10 h light/dark daily cycle. The Animal Ethics Committee approved all animal procedures.

**Animal Treatment.** All animals were ovariectomized and randomly distributed over four experimental groups, each group consisting of five animals. After 1 week the animals were treated as follows: a sham control group (ovx) received a silastic implant (catalog no. 602-265, 0.062-inch i.d. and 0.095-inch o.d., 1 cm in length; Dow Corning, Wiesbaden, Germany) filled with cholesterol. The animals were anesthetized with diethylether and the implants were placed subcutaneously on the back. The next group received an estradiol implant (silastic implants filled with a mixture of estradiol and cholesterol of 1:50 (ovx + E2)). In a pilot experiment, the amount of estradiol in the implant was previously tested to give rise to plasma levels of approximately 25 pg/ml. The third group received daily s.c. progesterone injections (two times per day, 5 mg/kg) for 7 days (ovx + P4), and the last group received the estradiol implant plus daily s.c. progesterone injections (two times per day, 5 mg/kg) on the last 3 days (ovx + E2 + P4). After 7 days of treatment, the animals were anesthetized with ether, and blood was collected from the abdominal aorta in a heparinized tube for E2 and P4 determination by radioimmunoassay. The uterus, vagina, and aorta were removed and prepared for the incubations.

To determine whether the endogenous E2 was influencing radiolabeled E2 substrate concentrations, the endogenous E2 concentration was determined in uterus and vagina tissue after E2 treatment. In a parallel experiment the organs were removed and frozen in liquid nitrogen for storage and subsequent E2 determination by radioimmunoassay.

**Tissue Incubation.** The dissected organs were minced into pieces of ca. 1 mm³, and 25 mg of uterus and vagina tissue or 50 mg of aorta tissue was transferred to vials containing 3 ml of Leibowitz medium (L-15) (buffered with HEPES at pH 7.4 and supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin) and 3H-labeled steroid dissolved in 50 μl of propylene glycol to give a final concentration of 10 nmol/l. The fragments were incubated at 37°C for 24 h in a shaking water bath. Cooling on ice stopped the incubation, and the samples were stored at −20°C until analysis.

**Extraction.** After separation from the medium, the tissue fragments were extracted twice with 3 ml of ethanol. The ethanol extract was dried under a stream of nitrogen, redissolved in 300 μl of methanol, and combined with the medium. To this mixture, 2.5 μl of unlabeled E2, EE, or MOX was added as carrier. The mixture was then applied to an activated Sep-Pak C18 column and eluted with 5 ml of water, hexane, and methanol consecutively. The radioactivity in each eluate was determined by liquid scintillation counting (Tri-Carb 1900 TR; Canberra Packard, Groningen, The Netherlands), and the methanol eluates were used for HPLC analysis.

The solid phase extraction procedure gave good recoveries for all three steroids. The percentages of radioactivity recovered in the methanol eluates were 85 ± 7.5, 91.9 ± 9.8, and 85.3 ± 12.0% for estradiol, ethynylestradiol, and moxestrol, respectively. Only minor amounts (less than 1%) of radioactivity were found in both the hexane and aqueous eluates of ethynylestradiol and moxestrol incubations. The aqueous eluates from the estradiol incubations contained 4.0 ± 1.2% of total radioactivity.

**HPLC Analysis of Estrogen Metabolites and Enzymatic Hydrolysis.** Separation of the estrogens and their metabolites was performed using a Waters spherisorb S5 ODS-2 column (4.6 × 250 mm) (Phase Separations B.V., Emmen, The Netherlands) with a guard column (4.6 × 10 mm) and a gradient of ammonium sulfate buffer (20 mM) (solvent A) and methanol (solvent B). The analytes were eluted with 40% solvent B for 5 min, followed by a linear gradient of 40 to 75% solvent B in 35 min. After 5 min of elution with 75% solvent B, initial values were reestablished in 5 min. The flow rate was 1.0 ml/min.

The solvent was delivered by a Bio-Rad series 800 liquid chromatograph (Bio-Rad Laboratories B.V., Veenendaal, The Netherlands) and the column effluent was monitored by a Bio-Rad model 1706 UV detector set at a wavelength of 280 nm, and a Flo-one model A200 on-line radioactivity detector (Canberra Packard).

Immediately before analysis, the methanol Sep-Pak eluates were dried under a stream of nitrogen and redissolved in 250 μl of 40% (v/v) acetonitrile/water. An aliquot of 100 μl was injected on the column.

The remainder of an HPLC sample was evaporated to dryness and redissolved in 0.75 ml of a phosphate buffer solution (4 mM, pH 6.8) of type VII-A glucuronidase (100 U/ml) (Sigma, Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands). The sample was incubated at 37°C for 2 h, evaporated to dryness, and redissolved in 250 μl of 40% (v/v) acetonitrile, of which 100 μl was injected on the HPLC column.

The HPLC method was selective in separating estradiol and estrone from most of their sulfates and glucuronides, as shown by typical retention times for estradiol-3,17-disulfate (6.5 min), estrone-3-glucuronide (13.9 min), estradiol-3-glucuronide (E2-3-G) (14.8 min), estradiol-17-glucuronide (16.7 min), estrone-3-sulfate (18.9 min), estradiol-3-sulfate (20.0 min), estrone (34.8 min), and estradiol (35.9 min). Using E2-3-G and estradiol-3-sulfate (E1-3-S) as substrates for the enzymatic hydrolysis of steroid conjugates, the specificity of our procedure for deglucuronidation could be demonstrated. As is evident from Figure 1, all E2-3-G was deconjugated into E2, whereas there was no formation of E1 from deconjugation of E1-3-S.

**Statistics.** All graphs and tables show means ± S.E.M. with n = 5. Significant differences between multiple treatment groups in one tissue were analyzed by ANOVA, followed by Tukey’s honestly significant difference test (p < 0.05). Groups sharing the same underscore in the legends do not differ significantly. Correlation between two variables was calculated using Spearman correlation. Statistical analysis was performed with SPSS 8.0 for Windows (SPSS Inc., Chicago IL).

**Results**

Effect of Steroid Treatment on Estradiol and Progesterone Levels in Plasma, on Estradiol Levels in Uterus and Vagina, and on Uterus Weight. Plasma estradiol and progesterone levels of the four treatment groups are summarized in Table 1. Hormonal treatment apparently resulted in estradiol and progesterone plasma levels that...
are only slightly higher (Smith et al., 1975) than normal maximal values during the estrous cycle.

As shown in Table 1, estradiol treatment after ovariectomy resulted in an increased uterus wet weight. Progesterone treatment alone caused no significant uterus weight increase, whereas the combined estradiol/progesterone treatment had an intermediate effect. Tissue estradiol concentrations after estradiol treatment increased significantly from 3.9 ± 0.4 to 13.8 ± 2.0 fmol/mg for uterus, and from 4.9 ± 0.5 to 29.9 ± 5.1 fmol/mg for vagina.

Metabolism of Estradiol: Effect of E2 and P4 Treatment. Upon incubation of tritium-labeled estradiol with the rat tissues as described under Materials and Methods, radioactive metabolites of estradiol were found. These radioactive metabolites of 3H-labeled estradiol could be identified by cochromatography with unlabeled steroids such as estrone, estrone-3-glucuronide (E1-3-G) and E2-3-G. Furthermore, the E1-3-G and E2-3-G could be selectively deglucuronidated by glucuronidase treatment. No sulfates of E2 or E1 were detected.

Representative chromatograms for ovx E2 + P4-treated animals are shown in Fig. 1. As is also apparent from these chromatograms, the ratio E2-3-G/E1-3-G correlates well (r² = 0.860, slope = 0.95 ± 0.07) with the E2/E1 ratio. Therefore, we decided to define 17β-HSD activity as the amount of E1 and E1-3-G formed per milligram of tissue in 24 h. Similarly, we defined UDP-GT activity as the amount of E1-3-G and E2-3-G formed per milligram of tissue in 24 h. Next, we evaluated the influence of steroid hormone treatment on both enzyme activities.

Only in the uterus tissue fragment incubations, 17β-HSD activity was changed by steroid treatment. The uterine 17β-HSD activity was significantly increased 10.1-fold in the E2-treated animals compared with the ovx animals, and increased 23.5-fold in the combined E2/P4-treated animals. No significant difference between the uterine 17β-HSD activity of ovx and P4-treated animals was found. The
UDP-GT activity was decreased, although not significantly, by E2 treatment. Its activity was however significantly higher in the P4-treated animals (Fig. 2). In fact, UDP-GT activity was higher than 17β-HSD activity in this group. In vagina tissue fragment incubations, no significant change in 17β-HSD or UDP-GT activity between the treatment groups was observed. In the incubations of vagina tissue, 17β-HSD activity was higher than UDP-GT activity.

A concentration of unlabeled estradiol in the tissue range of, or exceeding the concentration of tritiated estradiol in the incubation medium might interfere with the 17β-HSD and UDP-GT activity measurement by changing the substrate concentration or by radiolabel dilution. Therefore, we investigated the effect of hormonal treatment on the estradiol concentration in the uterus, which may influence the measured 17β-HSD and UDP-GT activity. However, the tissue concentrations of unlabeled estradiol after estradiol treatment were approximately 500 to 600 times lower than the amount of labeled substrate. Therefore, we may neglect the problem of unequal substrate concentrations or radiolabel dilution between different treatment groups.

Considering the 17β-HSD and UDP-GT activity in individual samples, a difference in the ratio of the two enzyme activities between uterus and vagina incubations became apparent. As is illustrated in Fig. 3, there is a good correlation ($r^2 = 0.782, p < 0.001$) between 17β-HSD activity and UDP-GT activity in vagina tissue fragment incubations. In uterus incubations, however, higher UDP-GT activity occurred only with low 17β-HSD activities, corresponding to the hormonal treatment. Apparently, both enzyme activities are intrinsically correlated in vagina, regardless of hormonal treatment. There was no detectable metabolism of estradiol in aorta tissue fragment incubations in any of the treatment groups.

**Metabolism of Ethynylestradiol and Moxestrol: Effect of E2 and P4 Treatment.** Upon incubations of uterine and vaginal tissue fragments with 3H-labeled ethynylestradiol, only one peak appeared as a metabolite in the HPLC radiochromatograms (Fig. 4). Selective deglucuronidation shifted its retention time to that of ethynylestradiol. In uterus tissue incubations there was no significant difference in glucuronidation between any of the steroid-treated groups and the ovx group (Fig. 4).

There was no detectable metabolism of ethynylestradiol in aorta tissue fragment incubations in any of the treatment groups. From incubations of tissue fragments with 3H-labeled moxestrol, no metabolites could be analyzed in any of the tissue incubations.

**Discussion**

**Estradiol Metabolism.** Our results with estradiol metabolism in rat uterus and vagina tissue show the conversion of estradiol to estrone, estradiol-3-glucuronide, and estrone-3-glucuronide. This demonstrates the presence of 17β-HSD and UDP-GT activity in both tissues. It was also shown that there is a clear increase of 17β-HSD activity in ovariectomized rat uterus after estradiol treatment and after combined estradiol/progesterone treatment. Furthermore, it was demonstrated that there is a progesterone-increased and estradiol-suppressed glucuronidation of estradiol in rat uterus.

The 17β-HSD enzyme catalyzes the interconversion of 17β-hydroxy- and 17-ketosteroids. The several isoforms that are known display a preference for catalyzing either in the reductive or oxidative direction and show an isoform-specific tissue distribution (Miettinen et al., 1996). In rat, two 17β-HSD isoforms have been described (Akinola et al., 1996). In uterus, 17β-HSD type 2 is the predominant isoform, preferentially catalyzing the conversion of estradiol to estrone. The induction by estradiol treatment of 17β-HSD activity that
we observed in ovariectomized rat uterus is in agreement with previous observations, reporting that in rat, 17β-HSD activity is stimulated by E2 or diethylstilbestrol (Liu et al., 1990), and is highest during the proestrus and estrus phase of the estrous cycle (Kreitmann et al., 1980; Wahawisan and Gorell, 1980). This study reports for the first time the glucuronidation of estradiol in rat uterus and vagina. The progesterone-enhancement and estradiol suppression of UDP-GT is remarkable since the other estrogen-inactivating enzyme, 17β-HSD, is regulated almost in the opposite way.

If we compare these results from rat uterus to the estrogen-inactivating enzymes in the human uterus, we see that in the human uterus both estrogen-inactivating enzymes (17β-HSD and estradiol sulfotransferase) are induced (Wahawisan and Gorell, 1980; Fuentes et al., 1990; Liu et al., 1990) by progesterone, whereas in the rat one enzyme is induced by estradiol treatment (17β-HSD) and the other (UDP-GT) is suppressed by estradiol. In human endometrium and in endometrium of ovariectomized pigs, 17β-HSD activity is induced by progesterone treatment (Kaufmann et al., 1995). We can speculate that the differences in induction of 17β-HSD are related to the fact that human and swine display spontaneous corpus luteum maintenance (spontaneous pseudopregnancy) and therefore have a pronounced luteal phase with corresponding high serum progesterone levels. In rat, maintenance of corpus luteum is induced by mating, leading to pseudopregnancy. In fact, the highest 17β-HSD activity in rat uterus was found at day 7 of pregnancy (Kreitmann et al., 1980). This correlates well with our finding that combined estradiol- and progesterone-treated rats show the highest 17β-HSD activity.

In summary, both human and rat uterus have the ability to regulate local estradiol levels through 17β-HSD activity and conjugation. We demonstrated in this study that there is no estradiol sulfation, but rather glucuronidation of estradiol to E1-3-G and E2-3-G in rat uterus. The preference for glucuronidation over sulfation is supported by the fact that in earlier studies no rat sulfotransferase mRNAs could be detected in rat uterus (Dunn and Klaassen, 1998). Furthermore, no estradiol sulfates were formed in incubations of uterus homogenates with radiolabeled estradiol and 3'-phosphoadenosine 5'-phosphosulfate as a cofactor, whereas incubations with uridine 5'-diphosphoglucuronic acid as a cofactor did yield E2-3-G (data not shown). Comparing our results with data from experiments with human uterus, there seems to be dissimilarity between the rat and the human uterus as far as the regulation by steroid hormones of estradiol 17β-HSD and conjugating activities is concerned.

In contrast to the uterus, in vagina tissue, the hormone treatments in the rat did not influence 17β-HSD or UDP-GT activity. Although the interindividual variance for the 17β-HSD and UDP-GT activity is much higher in vagina, there is always a good correlation between 17β-HSD and UDP-GT activity, regardless of the hormonal treatment (Fig. 3). We therefore conclude that, although estradiol metabolism in rat uterus is under steroidal control, in vagina it is not. No metabolism of estradiol could be demonstrated in rat aorta tissue incubations, which indicates that this tissue has no appreciable capacity for inactivating estradiol.

**Ethynylestradiol and Moxestrol Metabolism.** Ethynylestradiol was shown in this study to be metabolized to a glucuronide in rat uterus and vagina, but not in aorta tissue. In contrast to estradiol glucuronidation in uterus, the hormonal treatments that were applied had no significant effect on ethynylestradiol conjugation in rat uterus. Estradiol and ethynylestradiol may thus be conjugated by different UDP-GT isoforms. Micronosmal UDP-GTs are a family of isoenzymes that glucuronidate endogenous as well as xenobiotic compounds and drugs. They exhibit broad and sometimes overlapping substrate specificity toward, for example, steroids (Mackenzie et al., 1996). It is known that human liver UDP-GT is able to catalyze the conversion of ethynylestradiol to ethynylestradiol-3-glucuronide (Ebner et al., 1993). Also, both expressed rat and expressed human UGT1A1, which are considered orthologous enzymes, are able to conjugate ethynylestradiol (King et al., 1996). This isoform also catalyzed the glucuronidation of estradiol, although at a lower level. Besides human UGT1A1, also human UGT1A4, UGT1A8, and UGT1A9 are able to glucuronidate ethynylestradiol (Tukey and Strassburg, 2000). It is not clear which rat UDP-GT isomorf(s) catalyzes the conversion of ethynylestradiol in uterus. If in rat uterus, estradiol is glucuronidated by UGT1A1, maybe another isomorf than UGT1A1 is more important for the conversion of ethynylestradiol, which could explain the lack of regulation by hormonal treatment.
No metabolism of moxestrol could be detected in any of the tissues examined, which reveals an interesting structure-function relationship for the glucuronidation of 17α-ethynylated estrogens. Apparently, the 11β-methoxy substituent renders it a poor substrate for glucuronidation.

Overall, we demonstrated the ability of rat uterus and vagina to metabolize estradiol and ethynylestradiol, but not moxestrol. Only in uterus, the metabolism of estradiol is influenced by hormonal treatment with E2 and P4. This fact should be taken into account when evaluating ERT in an ovariectomized rat model, if the treatment consists of estradiol and a progestagen. The higher estradiol-metabolizing capacity of rat uterus after combined estradiol/progestagen treatment may enhance the antiestrogenic effects in uterus of the progestagen component of a combined ERT by reducing the concentration of estradiol in the tissue. No such effects are to be expected in vagina or aorta tissue. The ovariectomized rat may thus be a poor model to study endometrial effect of estradiol/progestin combinations in view of the differences between rat and human in uterine estradiol metabolism and its regulation by sex steroids.

References