Steroids and steroid analogues for Hormone Replacement Therapy; Metabolism in Target Tissues

Steroiden en steroid analogen voor Hormoon Vervangings Therapie; Metabolisme in doelweefsels

(Met een samenvatting in het Nederlands)

Proefschrift

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C o n t e n t s

Abbreviations

17β-HSD	17-beta hydroxysteroid dehydrogenase
20α-HSD	20-alpha hydroxysteroid dehydrogenase
3α-HSD	3-alpha hydroxysteroid dehydrogenase
3β-HSD	3-beta hydroxysteroid dehydrogenase
AR	androgen receptor
ER	estrogen receptor
HEK293	human embryonic kidney (cells)
HPLC	high pressure liquid chromatography
HPTLC	high performance thin layer chromatography
HRT	hormone replacement therapy
LSC	liquid scintillation counting
ovx	ovariectomized
PR	progesterone receptor
RBA	relative binding affinity
TLC	thin layer chromatography
UDP-GT	UDP-glucuronosyl transferase

CHAPTER 1

General Introduction

The use of estrogens and progestagens in Hormone Replacement Therapy for postmenopausal women.

The goal of Hormone Replacement Therapy (HRT) is to treat the symptoms that are related to the marked decline in plasma levels of estradiol in postmenopausal women. Although originally considered a reproductive hormone, the influence of estradiol extends beyond the reproductive organs to a variety of non-reproductive target organs. These organs include the bone, the cardiovascular system and the central nervous system.

One of the most drastic effects of the lowered estrogen levels in women after menopause is the increase of bone resorption that may lead to osteoporosis. Replacement of estrogens has been long recognized as an effective treatment (Horsman et al., 1983), but unfortunately, the estrogen replacement therapy also increases the incidence of endometrial and breast cancer (Jacobs, 2000). This unwanted effect of unopposed estrogen replacement therapy seems to be related to the estrogen-induced proliferation in the endometrium and the breast, two organs that are very sensitive to estrogens. Therefore, Hormone Replacement Therapy usually consists of a combination of an estrogen and a progestagen, which are administered in a continuous or sequential fashion. The progestagen is added to suppress proliferative action on the endometrium.

The effect of progestagens on breast cancer is still a matter of debate. In a large case-control study, there was an increased relative breast cancer risk of estrogen / progestagen combined HRT compared to estrogen-only HRT (Pike and Ross, 2000). Several large prospective trials have been initiated to study long term effects of HRT (for a review, see Wren, 1998). The first of these studies, the Postmenopausal Estrogen / Progestagen Intervention (PEPI) trial which is still to be completed, was insufficiently powerful to determine the influence of HRT on the incidence of breast cancer. The PEPI trail, however, did show that the combined estrogen / progestagen treatment, given sequentially or continuously, protected the endometrium from hyperplastic changes associated with estrogen-only therapy (The Writing Group for the PEPI, 1996). The outcome of the other large prospective trails still has to be awaited, but it can be anticipated that probably in all HRT regimens, progestagens will continue to be administered in combination with estrogens. The nature of the progestagens used in combined estrogen / progestagen HRT includes the natural sex steroid progesterone, its derivative medroxy-progesterone acetate (MPA) and 19-nor-progestagens such as norethisterone (17α-ethynyl-19-nor-testosterone).

Another strategy to achieve the combined estrogen / progestagen balance that is desired in the target tissues of HRT is the use of a single steroid that shows combined estrogenic and progestagenic properties *in vivo*. Org OD14 ([7α,17α]-17-hydroxy-7-methyl-19-norpregn-5(10)-en-20-yn-3-one) is such a steroid. Org OD14, also known as *Tibolone* or *Livial*, causes an increase in bone mineral density, and a reduction of vaginal atrophy. However, it has no estrogenic effect on the endometrium in postmenopausal women (for a review, see Moore, 1999)

Tissue selective action of steroids and the role of steroid metabolism in target tissues.

An ideal HRT should display tissue selective effects on target tissues. It should cause estrogen agonistic effects on the bone and other tissues that benefit from estrogen replacement. However, it should not cause estrogen induced proliferation in the endometrium or the breast. So what are the possible mechanisms that underlie the tissue selective action of steroids?

The effect of steroids is mediated by their interaction with steroid receptors, which are members of the nuclear transcription factor family (Mangelsdorf et al., 1995). The steroid hormone action on target tissues is considered to be composed of three components, as proposed by Katzenellenbogen *et al.* (Katzenellenbogen et al., 1996). These authors conceptualized a tripartite action of steroid hormones, which involves the ligand, the receptor and receptor co-regulating proteins. In this model, tissue selective action of circulating steroids can be generated at different levels. Firstly, the ligand needs to be absorbed from the plasma and can be metabolized in the target tissue. Secondly, the composition of the receptors (concentration, subtypes, isoforms, and variants) determines the response to the ligand present. And finally, the transactivation of target genes by the liganded receptors is modulated by the presence of nuclear receptor co-activators and co-repressors. Thus, the first step that triggers the action of a circulating steroid hormone is the uptake and the eventual metabolism in a target tissue. The metabolism of a steroid in a target tissue may lead to its activation or to its inactivation. This is a physiologically important mechanism to control the action of natural circulating steroids on target tissues (for a review, see Roy, 1992). Good examples of metabolic activation are the metabolism of testosterone to the more potent androgen 5α-dihydrotestosterone in the prostate and the conversion of C19 androgens to estrogens by aromatase in e.g. adipose tissue. The classic example for the role of steroid inactivating enzymes is the enzyme 11β-hydroxysteroid dehydrogenase type 2. This enzyme is expressed in mineralocorticoid target cells and converts cortisol to cortisone, which has only a very weak affinity for the mineralocorticoid receptor (MR). Since glucocorticoids and mineralocorticoids have almost identical affinities for the MR, 11β-hydroxysteroid dehydrogenase type 2 protects the MR inside the mineralocorticoid target cells from occupation by cortisol.

Steroids used in HRT may also be subject to metabolism in the target tissues. This metabolism influences their effect on target tissues of HRT, as was demonstrated for Org OD14. Metabolism of Org OD14 occurs in the endometrium, generating a progestagenic metabolite that is implicated to suppress the estrogenic action in this tissue (Tang et al., 1993). However, while substantial information has been gathered on the systemic metabolism of estrogens and progestagens, there is little information on their metabolism in extrahepatic tissues. Therefore, this thesis will concentrate on the metabolism by target tissues of some selected steroids that are used in HRT.

Estrogen and progestagen metabolism in target tissues of HRT; activation or inactivation?

Although no guarantee for transactivation and further downstream signaling, binding to a steroid receptor is a prerequisite for the action of a steroid. Comparing the steroid receptor binding properties of a steroid and its metabolites can therefore indicate which changes in the molecular structure of the steroid may influence that steroids' action.

The steroids that were studied in this thesis are analogues of three typical steroids used in HRT. Estradiol and norethisterone are representative of the estrogen and progestagen, respectively, that are used in combined HRT, while Org OD14 is used in single-steroid HRT.

Let us now consider some changes in the structure of these three steroids which have an important effect on the binding to sex steroid receptors. The hormone-binding site of sex steroid receptors consists of a hydrophobic pocket that is generally in precise contact with the A ring of the steroid molecule. Contact with the D ring occurs with greater structural flexibility. Thus, structural changes in the steroid A and D ring may have profound effects on the binding of steroids to their receptors.

Estradiol

It is widely recognized that the 3β-OH group of the phenolic A ring is essential for high affinity binding to the estrogen receptor (ER). However, the planarity of the aromatic A ring does not seem to be essential (Jordan, 1997). Also the 17β-OH group appears to be important for high affinity receptor binding. It is therefore not surprising that the binding of estradiol to the estrogen receptor is nullified by sulfo-conjugation of the 3β- or 17β-OH group. The oxidation of the 17β-OH group results in the formation of estrone, which displays a reduced affinity for the ER (60% compared to estradiol).

Norethisterone

The Δ4, 3-keto configuration of the A ring is a requirement for the binding to the progesterone receptor (PR). In table 1 it is demonstrated for norethisterone that 5α-reduction leads to a decrease in PR binding affinity, but may confer some androgen receptor binding in combination with the 3-keto group. The 3β-reduction of the 3-keto group also decreases PR binding, and introduces some ER binding, especially in combination with 5α-reduction. The effect of 3α-reduction is similar, but less potent.

TABLE 1

Relative Binding Affinity (RBA) of norethisterone and its metabolites.

steroid	Relative Binding Affinity (%)		
	PR (Org 2058= 100%)	**AR** (5α-DHT=100%)	**ER** (E2=100%)
norethisterone	21.5	3.2	nb
3β-OH-norethisterone	2.0	0.3	11.4
5α-norethisterone	3.0	5.1	0.2
5α, 3β-OH norethisterone	0.2	nb	7.8
5α, 3α-OH-norethisterone	0.2	0.1	0.5

RBA values to the progesterone (PR), androgen (AR) and estrogen receptor (ER) in human MCF-7 cell cytosol. nb= no binding at 10^{-6} M. Adapted from (Schoonen et al., 2000)

Org OD14

Org OD14, a 7α-methyl derivative of norethynodrel, displays weak ER and PR binding. Table 2 shows the effect on ER and PR binding of several modifications to the A ring of Org OD14. Rearrangement of the Δ5-10 double bond to a Δ4 double bond creates a 3-keto, Δ4 configuration and results in a 10 fold increase in the affinity for the PR. Both the 3α- and the 3β-reduction of Org OD14 result in ablation of the PR binding and cause an increased ER binding.

TABLE 2

Relative binding affinity to the estrogen receptor (ER) and progesterone receptor (PR) for Org OD14 and its derivatives.

Steroid	Relative Binding Affinity (%)	
	ER (E2=100%)	**PR** (Org 2058= 100%)
Org OD14	<1	1
Δ4-Org OD14	<1	10
3β-OH-Org OD14	2	nb
3α-OH-Org OD14	2	nb

nb = no binding. Adapted from (Markiewicz and Gurpide, 1990)

The rat as a model to study the pharmacology of steroids in HRT

Since the primary target of HRT is to prevent bone loss in postmenopausal women, a reliable model that predicts the effectiveness of new HRT agents on bone is needed. Because of the similarities between rats and humans in their skeletal response to estrogen deficiency, the mature ovariectomized (ovx) rat is considered to be a good model to investigate the pathogenesis and treatment of postmenopausal bone loss (Kalu et al., 1989). It is less clear whether the effect of HRT on non-skeletal target tissues in the ovx rat is also similar to that in postmenopausal women. Since the metabolism of steroids is an important factor in the tissue response to these hormones, the metabolism of steroids used in HRT in target tissues other than the bone was investigated in rat and postmenopausal women.

Aim of the thesis

The aim of this thesis was to investigate the metabolism of estrogens and progestagens in tissues that are targets of HRT. Two aspects were of special interest:

How does the target tissue metabolism of steroids in rat compare to that in the human?

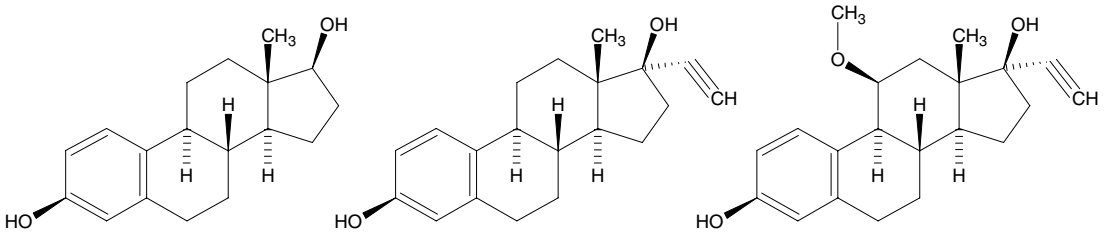
The effect of steroids for HRT on bone can be effectively studied in the ovariectomized rat as a model for postmenopausal bone loss. We wanted to investigate whether the rat can also be a model to study steroid metabolism in HRT target tissues of postmenopausal women. The uterus, vagina (rat and human) and the aorta (rat) were chosen as target tissues of HRT. The uterus was chosen as a target where no estrogen effect of HRT is desired. The vagina on the other hand is an organ where an estrogenic effect might be beneficial to relieve vaginal atrophy associated with menopause. The aorta (in rat) was chosen as a non-reproductive tissue that is a target of HRT. Of particular interest is the bio-transformation of steroids that would lead to local (in)activation or would generate metabolites that bind to other steroid receptors than the mother-compound.

What is the influence of substitutions at the 7 , 11 and 17 position of the steroid skeleton on this metabolism in both species?

The 7, 11 and 17 position on the steroid skeleton have long been recognized as powerful modulators of estrogen receptor binding (Zeelen and Bergink, 1980). Steroids that are substituted with small lipophilic groups such as a methyl or ethyl group at the 7α, 11β and 17α position are known to maintain good ER binding. But what is the effect of these substitutions on their target tissue metabolism?

Outline of the thesis

This chapter (Chapter 1) introduces the subject of this thesis. In Chapter 2, the metabolism of estrogens in rat uterus, vagina and aorta was studied. Steroids that were studied are estradiol (E2), ethynylestradiol (EE) and moxestrol (MOX) (figure 1). The structural difference between estradiol and the synthetic estrogens is a 17α-ethynyl substitution (EE), or a 17α-ethynyl and an 11β-methoxy substitution (MOX). Furthermore the influence of estrogen / progestagen treatment on the estrogen metabolism was studied.



estradiol ethynylestradiol moxestrol

Figure 1: *structure of the estrogens studied in chapter 2.*

In Chapter 3, the metabolism of four progestagens in rat uterus, vagina and aorta was investigated (figure 2). The progestagens are norethisterone and three derivatives of norethisterone, which differ from norethisterone only in a 7α-methyl (Org OM38), an 11β-ethyl (Org 4060) or both, a 7α-methyl and an 11-ethylidene substitution (Org 34694).

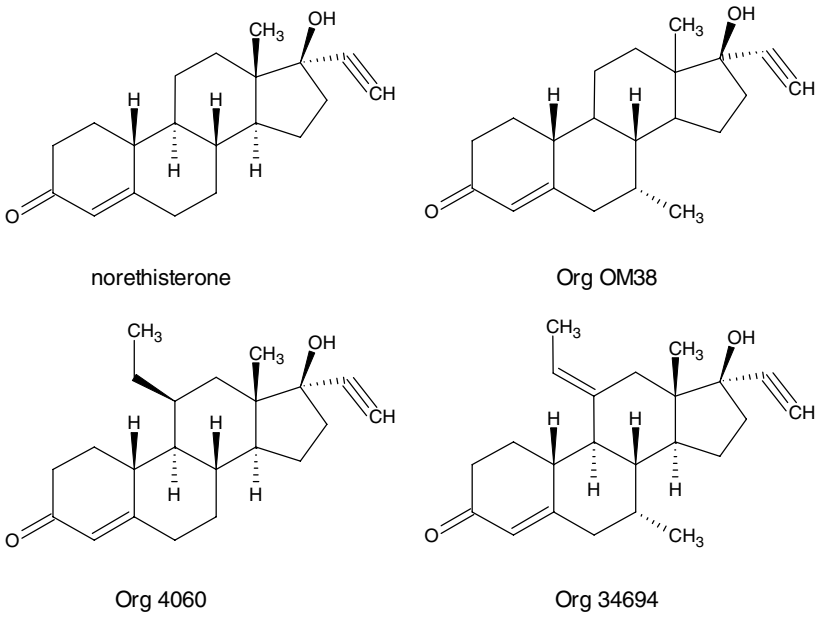


Figure 2*: structure of the progestagens studied in chapter 3 and 4.*

In Chapter 4, the metabolism in uterus and vagina of postmenopausal women was investigated of the same norethisterone derivatives.

In Chapter 5, the metabolism of Org OD14 and two of its derivatives was studied. The derivatives are the 3β-reduced (Org 30126) and the 3α-reduced (Org 4094) form of Org OD14 (figure 3). Their metabolism was studied in the uterus, vagina and aorta of the rat, and in the uterus and vagina from postmenopausal women. The metabolic routes and the enzymes involved in the biotransformation of OD 14 in these tissues were compared in the two species.

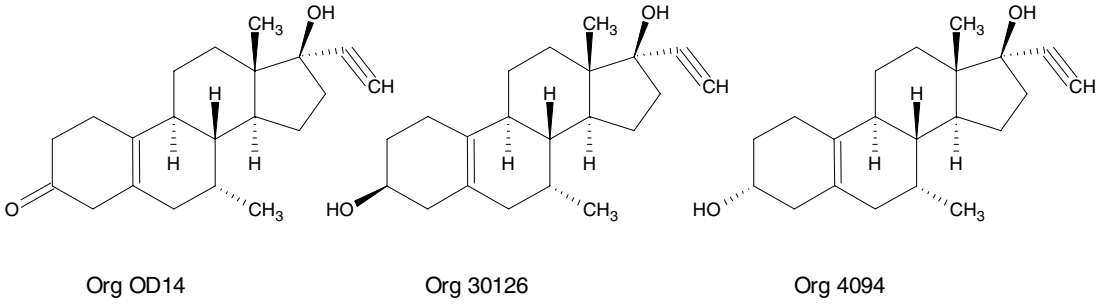


Figure 3*: structure of the steroids studied in chapter 5.*

Chapter 6 summarizes and discusses the results of the studies in this thesis. The metabolic routes in both species of the investigated species are compared, and the implications hereof are discussed for the rat as a model to study steroid metabolism in human HRT target tissues other than bone. The implications of the metabolism in target tissue for the use of the investigated steroids is discussed. Furthermore, some suggestions are made for further elucidation of the role of target tissue metabolism of this class of steroids and *in vitro* testing of new compounds.

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CHAPTER 2

Metabolism of estradiol, ethynylestradiol and moxestrol in rat uterus, vagina and aorta; the influence of sex steroid treatment.

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Abstract

Estrogen Replacement Therapy (ERT) 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 ERT.

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-hydroxysteroid 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 neither 17β-HSD nor 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 to human uterus. Therefore, the rat may not be the best-suited model to investigate uterine effects of estradiol-progestagen combined treatment.

Introduction

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 a 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-hydroxysteroid dehydrogenase (17β-HSD, EC 1.1.1.62). In addition, estrogen sulfotransferase (EST, 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, while 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 mayor 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 e.g., 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 if, and to what extent, inactivation of administered estrogen occurs in these tissues, and if it is influenced by the progestagen co-administered in ERT.

The effect of ERT is widely studied in ovariectomized rats, since ovariectomy in rodents results in an increased bone turnover similar to 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, amongst 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 uterine, vaginal 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-hydroxysteroid dehydrogenase activity (17β-HSD, EC 1.1.1.62) and UDP-glucuronosyl transferase (UDP-GT, EC 2.4.1.17) activity in uterus and vagina tissue.

Materials and methods

Chemicals

[2,4,6,7-^{3}H]Estradiol, (specific activity 3110 GBq/mmol), [6,7-^{3}H]ethynylestradiol (specific activity 1820 GBq/mmol) and [11β-methoxy-^{3}H]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, The Netherlands) with a weight between 225 and 250 g , were fed with standard pelleted diet (RMH-B; Hope Farms BV, Woerden, The Netherlands), and tap water *ad libitum*. The animals were subjected to a 14h-light/10h-dark daily cycle. The Animal Ethics Committee approved all animal procedures.

Animal treatment

*A*ll animals were ovariectomized (ovx) and randomly distributed over four experimental groups, each group consisting of 5 animals. After one week the animals were treated as follows: a sham control group (ovx) received a silastic implant (Down Corning, cat. no. 602-265, 0.062 inch I.D. and 0.095 inch O.D., 1 cm long) filled with cholesterol. The animals were anaesthetized 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 seven days (ovx+P4), and the last group received the estradiol implant plus daily s.c. progesterone injections (two times per day 5mg/kg) on the last three days (ovx+E2+P4). After seven 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 (RIA). 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 RIA.

Tissue incubation.

The dissected organs were minced in to pieces of ca. 1 mm^3, and 25 mg of uterine and vaginal tissue or 50 mg of aortic tissue, respectively, were 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 [H^3]-labeled steroid dissolved in 50µl propyleneglycol to give a final concentration of 10 nmol/l. The fragments were incubated at 37°C for 24 hours 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 ethanol. The ethanol extract was dried under a stream of nitrogen, redissolved in 300 µl methanol and combined with the medium. To this mixture, 2.5 µg of unlabeled E2, EE or Mox, respectively 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 x 250 mm) (Phase Separations B.V, Emmen, The Netherlands) with a guard column (4.6 x 10 mm) and a gradient of ammonium sulfate buffer (20mM) (solvent A) and methanol (solvent B). The analytes were eluted with 40% of solvent B for 5 minutes, followed by a linear gradient of 40-75 % solvent B in 35 minutes. After 5 minutes of elution with 75% solvent B, initial values were re-established in 5 minutes. 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 40% (v/v) acetonitrile/water. An aliquot of 100 µl was injected on the column.

The remainder of a 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 two hours, evaporated to dryness and

redissolved in 250 µl 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 (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 estradiol-3-glucuronide (E2-3-G) and estrone-3-sulfate (E1-3-S) as substrates for the enzymatic hydrolysis of steroid conjugates, the specificity of our procedure for de-glucuronidation 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 ± SEM with n=5. Significant differences between multiple treatment groups in one tissue were analyzed by ANOVA, followed by Tukey's HSD 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, USA).

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, respectively.

TABLE 1

Effect of hormonal treatment on plasma levels of estradiol and progesterone and on uterus proliferation.

Treatment	E2 in plasma (pmol/l)	P4 in plasma (nmol/l)	Uterus weight (mg)
Ovx	32.7 ± 18.2	21.6 ± 6.6	108.2 ± 5.4
Ovx+E2	392.0 ± 172.9 *	47.5 ± 12.6	506.5 ± 35.2 ***
Ovx+P4	27.4 ± 5.4	193.8 ± 41.8 **	129.5 ± 6.9
Ovx+E2+P4	595.0 ± 105.3 **	336.3 ± 60.2 ***	376.6 ± 27.4 ***

Treatment groups with values marked with asterisks are significantly (=p<0.05, **=p<0.01, ***=p<0.001) different from the ovx treatment using a Dunnett test.*

Metabolism of Estradiol; effect of E2 and P4 treatment.

Upon incubation of tritium labeled estradiol with the rat tissues as described in the Materials and Methods section, radioactive metabolites of estradiol were found. These radioactive metabolites of ^{3}H-labeled estradiol could be identified by co-chromatography with unlabeled steroids such as estrone, estrone-3-glucuronide (E1-3-G) and estradiol-3-glucuronide (E2-3-G). Furthermore, the E1-3-G and E2-3-G could be selectively de-glucuronidated by glucuronidase treatment. No sulfates of E2 or E1 were detected. Representative chromatograms for Ovx+E2+P4 treated animals are shown in figure 1. As is also apparent from these chromatograms, the ratio E2-3-G/E1-3-G correlates well (r^2=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 mg tissue in 24 h. Similarly, we defined UDP-GT activity as the amount of E1-3-G and E2-3-G formed per mg tissue in 24 h. Next, we evaluated the influence of steroid hormone treatment on both enzyme activities.

Only in the uterine 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 to 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, although not significantly, decreased 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.

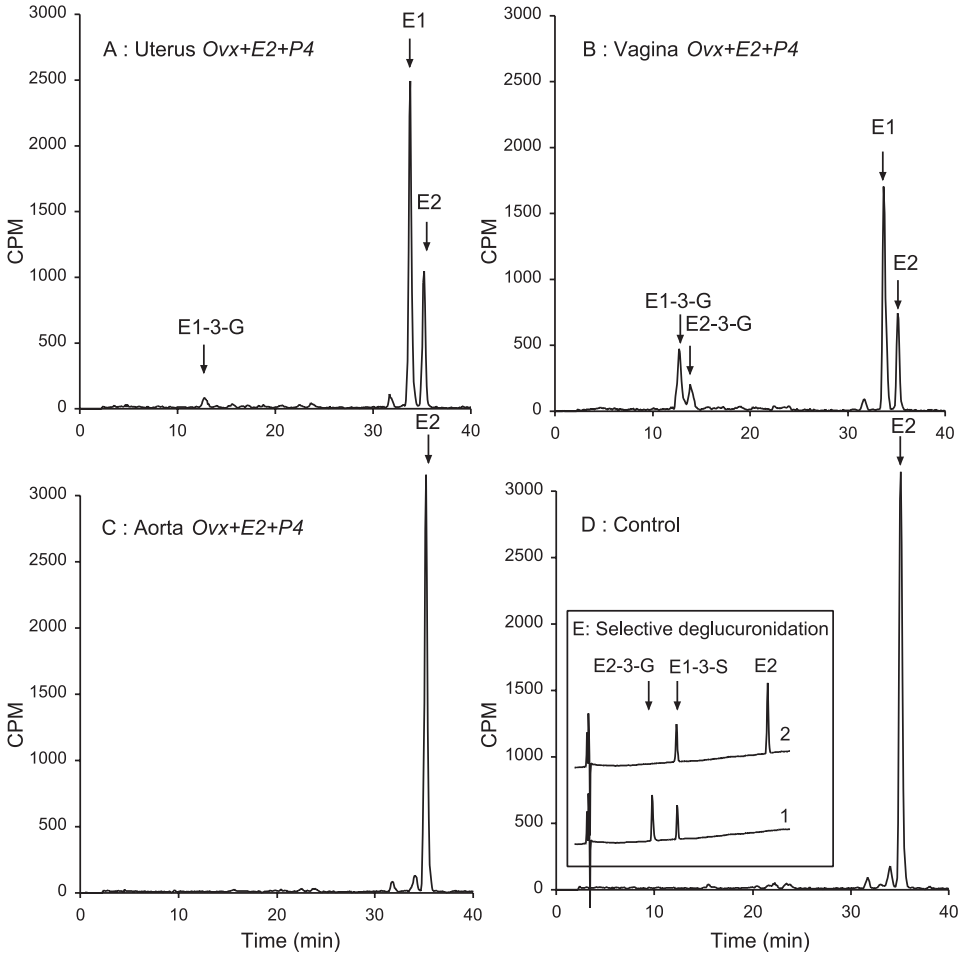

Figure 1: *Estradiol metabolism in uterus, vagina and aorta of ovariectomized rats treated with estradiol and progesterone (Ovx+E2+P4). Representative radiochromatograms are shown from tritiated estradiol incubations of uterus (A), vagina (B), aorta (C) and a control incubation without tissue (D). The formation of estrone-3-glucuronide (E1-3-G), estradiol-3-glucuronide (E2-3-G) and estrone (E1) from estradiol (E2) is indicated in the chromatograms with arrows. Panel D (insert) shows the selective deconjugation of estrone-3-glucuronide. Chromatograms of 2 µg of estradiol-3-glucuronide (E2-3-G) and estrone-3-sulfate (E2-3-S) before (1) and after (2) hydrolysis with glucuronidase are shown. While E2-3-G was completely converted into estradiol (E2), there was no conversion of E1-3-S into estrone.*

In vaginal 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.

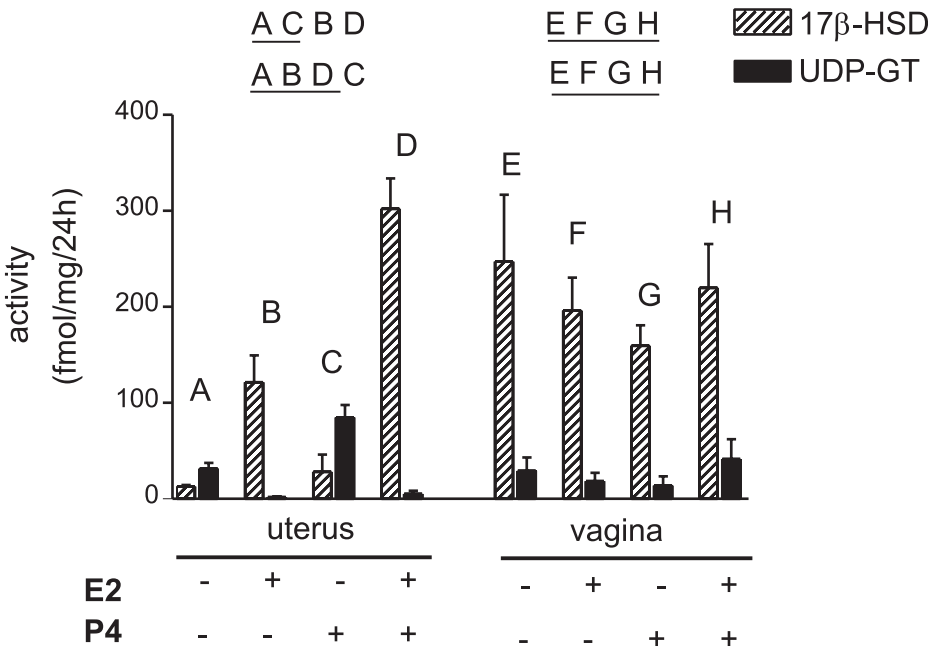

Figure 2:*Influence of steroid hormone treatment on estradiol metabolism in uterus and vagina. Ovariectomized rats received a placebo treatment (A,E), were treated with estradiol (B,F), progesterone (C,G) or received a combined estradiol/progesterone treatment(D,H). The tissues were incubated with tritiated estradiol. The activity of 17β-hydroxysteroid dehydrogenase (17β-HSD) was estimated by quantifying the amount of estrone and estrone-3-glucuronide that was formed per mg of tissue after a 24 hour incubation and the activity of UDP-Glucuronosyl transferase (UDP-GT) was estimated by quantifying the amount of estadiol-3-glucuronide and estrone-3-glucuronide that was formed per mg of tissue after a 24 hour incubation. Groups sharing the same underscore in the legend do not differ significantly ($p<0.05$).*

A concentration of unlabeled estradiol in the tissue in the 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 500-600 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.

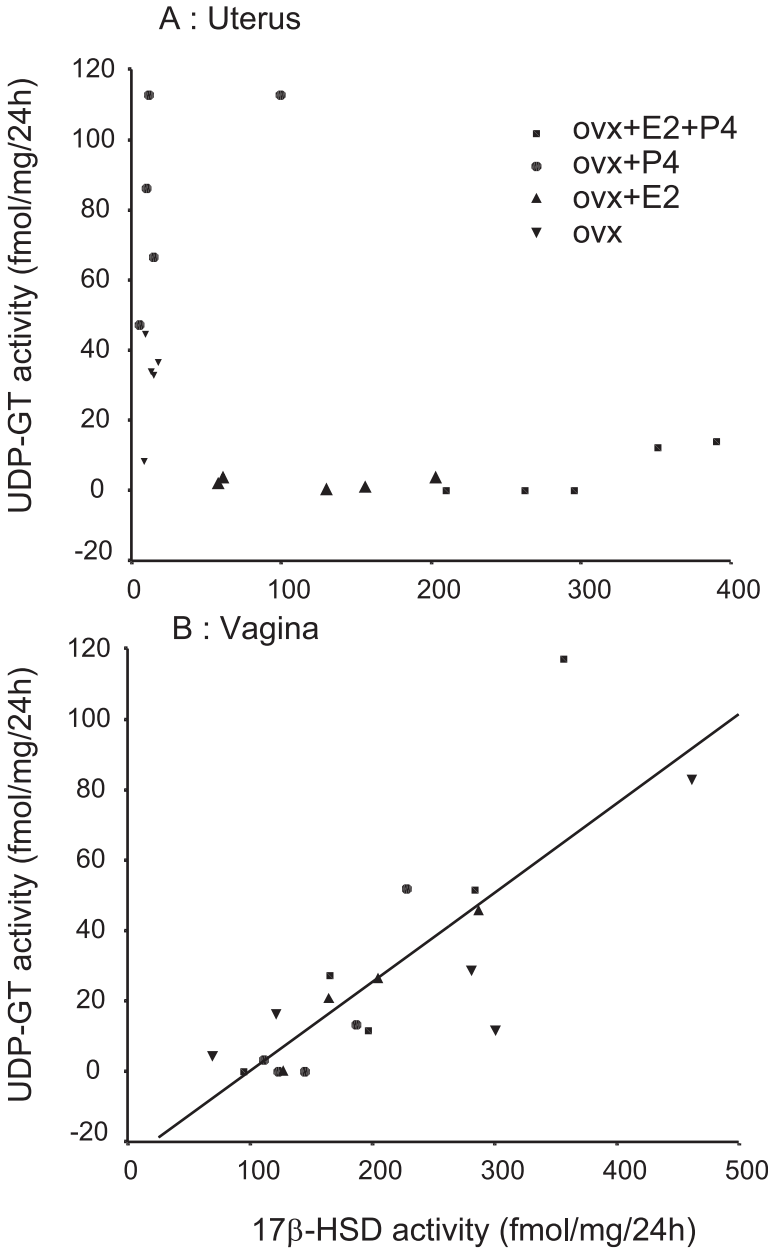

Figure 3: *Correlation between 17β-hydroxysteroid dehydrogenase (17β-HSD) activity and UDP-glucuronosyl transferase (UDP-GT) activity in uterus and vagina of all treatment groups. There was a good correlation (r^2 =0.782; p<0.001) between 17β-HSD activity and UDP-GT activity in vagina but not in uterus.*

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 figure 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 intra-individually 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 ^{3}H-labeled ethynylestradiol, only one peak appeared as a metabolite in the HPLC radiochromatograms (figure 4b). Selective de-glucuronidation 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. (Figure 4a).

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

Discussion

Estradiol metabolism

Our results with estradiol metabolism in rat uterine and vaginal 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 catalyses 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 earlier observations, reporting that in rat, 17β-HSD activity is stimulated by E2 or DES (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.

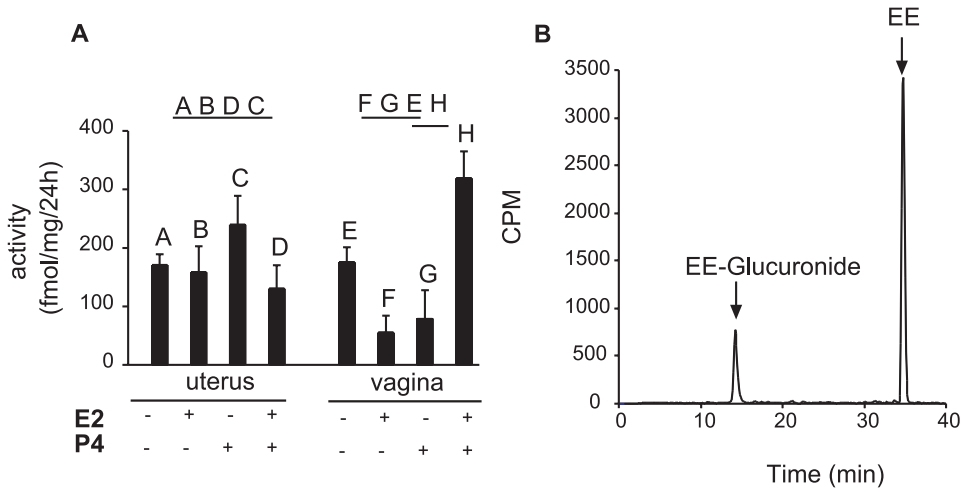

Figure 4: *Influence of steroid hormone treatment on ethynylestradiol metabolism in uterus and vagina (panel A). Ovariectomized rats received a placebo treatment (A,E), were treated with estradiol (B,F), progesterone (C,G) or received a combined estradiol/progesterone treatment(D,H). The tissues were incubated with tritiated ethynylestradiol. The activity of UDP-Glucuronosyl transferase (UDP-GT) was estimated by quantifying the amount of ethynylestradiol-glucuronide that was formed per mg of tissue after a 24 hour incubation. Groups sharing the same underscore in the legend do not differ significantly ($p<0.05$).*
A representative radiochromatogram is shown (panel B) from an incubation of vagina tissue with tritiated ethynylestradiol. The formation of ethynylestradiol-glucuronide from ethynylestradiol (EE) is indicated in the chromatograms with arrows.

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 both induced (Wahawisan and Gorell, 1980) (Liu et al., 1990) (Fuentes et al., 1990) by progesterone, while 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, 17-βHSD activity is induced by progesterone treatment (Kaufmann et al., 1995). We can speculate that the differences in induction of 17β-HSD is 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 seven of pregnancy (Kreitmann et al., 1980). This correlate 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 (PAPS) as a cofactor, whereas incubations with uridine 5'-diphosphoglucuronic acid (UDPGA) 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 inter-individual 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, while 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 uterine and vaginal, but not in aortic 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. Microsomal UDP-GTs are a family of iso-enzymes that glucuronidate endogenous as well as xenobiotic compounds and drugs. They exhibit broad and sometimes overlapping substrate specificity towards, 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 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

isoform(s) catalyze the conversion of ethynylestradiol in uterus, If in rat uterus, estradiol is glucuronidated by UGT1A1, maybe another isoform 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 anti-estrogenic 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 vaginal or aortic tissue. The ovariectomized rat may thus be a poor model to study endometrial effect of estradiol / progestagen combinations in view of the differences between rat and human in uterine estradiol metabolism and its regulation by sex steroids.

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CHAPTER 3

Metabolism of norethisterone and norethisterone derivatives in rat uterus, vagina and aorta.

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Drug Metab. Dispos. In Press

Abstract

The 19-nor-progestagen norethisterone is used as a progestagen component in contraceptives and in continuous- and sequential combined Hormone Replacement Therapy (HRT) in postmenopausal women. Metabolism of norethisterone in HRT target tissues may play a role in its biological response. The aim of this study was to investigate which steroid metabolizing enzymes are present in rat uterus, vagina and aorta, three HRT target tissues. Next, the ability of the tissues to metabolize norethisterone was assessed. Furthermore, to investigate the effect of substituents at the 7 and 11 position, the metabolism of Org OM38 (7α-methyl-norethisterone), Org 4060 (11β-ethyl-norethisterone) and Org 34694 (7α-methyl,11-ethylidene-norethisterone) was studied. Using radiolabeled progesterone, the presence of 20α-hydroxysteroid dehydrogenase, 5α-reductase and 3α-hydroxysteroid dehydrogenase could be demonstrated in uterus, vagina and to a lesser extent in aorta. The combined action of the latter two enzymes resulted in 3α-OH,5α-H-norethisterone as the major metabolite of radiolabeled norethisterone in uterus (26.9%), vagina (37.1%) and aorta (1.4%). The norethisterone derivatives however, were metabolized to a much lesser extent (1.0 –7.6%). No formation of 5α-reduced forms of Org 4060, Org OM38 or Org 34694 was found, while formation of minor amounts of 3α-OH-Org 4060 and 3α-OH-Org OM38 could be demonstrated in both uterus, vagina and aorta. These findings confirm the role of 5α-reductase as a rate-limiting step in the metabolism of norethisterone derivatives and show important inhibitory effects of substituents at the 7α- and 11-position of the steroid skeleton on 5α-reduction.

Introduction

Hormone Replacement Therapy (HRT) with estrogens can effectively treat symptoms, such as osteoporosis and climacteric complaints, that are related to the marked decline in plasma estradiol levels after menopause (Horsman et al., 1983). Ideally, the estrogenic effect of such a therapy should be evident in HRT target tissues such as bone, the cardiovascular system, vagina and the brain. An estrogenic, proliferative response however, should be absent in the breast and in the uterus. In particular in the uterus, unopposed estrogen treatment leads to an increased risk of developing uterine cancers. Combined estrogen and progestagen treatment can prevent this risk increase. Therefore, HRT often consists of a combined estrogen and progestagen treatment, given either sequentially or continuously.

Norethisterone is a synthetic steroid that is used as a progestagen in contraception and in HRT in postmenopausal women. However, both progestagenic and anti-progestagenic effects of norethisterone have been described on uteroglobin and progesterone receptor expression in the uterus of progesterone treated prepubertal rabbits (Pasapera et al., 1995). Moreover, estrogenic activity of

norethisterone in rat uterus is mediated through 5α-reduced metabolites of norethisterone (Mendoza-Rodriguez et al., 1999). Conversion of norethisterone in e.g. uterus into more estrogenic metabolites may affect the estrogen / progestagen balance that is the key feature of a combined HRT.

The aim of the present study is to investigate whether metabolism of norethisterone can take place in organs that are targets of HRT. In this study, the steroid metabolizing capacity of the uterus, vagina and aorta was studied. To deduce which steroid metabolizing enzymes are present, tissue fragments of uterus, vagina and aorta from rats were incubated with ^{3}H-labeled progesterone. Subsequently, incubations with ^{3}H-labeled norethisterone were performed, and its metabolites were analyzed. Furthermore, the effect on this metabolism of substitutions at the 7- and 11-position on the steroid skeleton was studied. The 7α- and 11β position are known to maintain good estrogen receptor binding when they are substituted with small lipophilic groups such as a methyl- or ethyl group (Zeelen and Bergink, 1980). The norethisterone derivatives studied are Org OM38, Org 4060 and Org 34694. All three steroids are similar to norethisterone, but have an additional 7α-methyl- (Org OM38), an 11β-ethyl- (Org 4060) or both a 7α-methyl- and an 11-ethylidene substituent (Org 34694).

The radiolabeled metabolites were separated and isolated using HPLC and HPTLC. Identification of isolated metabolites was performed by cochromatography with unlabeled steroids in the different chromatographic systems.

Materials and method

Chemicals

[1,2,6,7-^{3}H]-progesterone (3589 GBq / mmol) was purchased from NEN Life Science products (Hoofddorp, The Netherlands). The radiolabeled steroids [16-^{3}H]-Norethisterone (1010 GBq / mmol), [16-^{3}H]-Org OM38 (1400 GBq / mmol), [11-^{3}H]-Org 4060 (560 GBq / mmol) and [16α-^{3}H]-Org 34694 (925 GBq / mmol) were a gift from Organon (Oss, The Netherlands). The radiolabeled, 5α-reduced metabolite of Org 4060 and the 3α-hydroxylated metabolite of Org 4060 were synthesized from [11-^{3}H]-Org 4060 by Organon and yielded [11-^{3}H]- 5α-H-Org 4060 and [11-^{3}H]-3α-OH-Org 4060 with specific activities of ca. 500 GBq / mmol. The radiolabeled, 5α,3α-reduced metabolite of Org 4060 and the 5α,3β-reduced metabolite were produced from [11-^{3}H]- 5α-Org 4060. Unlabeled norethisterone, Org 4060, Org OM38 and Org 34694 metabolites that were used as reference steroids were a gift from Organon.

All other chemicals were obtained from local commercial sources and were of analytical grade.

Animals

Mature female Wistar strain Hsd / Cpd:Wu rats (Harlan, The Netherlands), with a weight between 225 and 250 g were fed with standard pelleted diet (RMH-B; Hope Farms BV, Woerden, The Netherlands), and tap water and *ad libitum*. The

animals were subjected to a 14h-light/10h-dark daily cycle. The rats were randomly distributed in two groups. One group (n=4) was ovariectomized two weeks before sacrifice. During two weeks, the other group (n=5) were monitored daily for estrous cycle progression by vaginal smears. The animals were sacrificed on the morning of proestrus at 10.00 am, and the uterus, vagina and aorta were removed for the tissue fragment incubations.

All animal procedures were approved by the Animal Ethics Committee.

Tissue incubation

The dissected organs were minced and 50 mg of tissue were 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 100 pmol of the ^{3}H-labeled steroid dissolved in 50 µl propyleneglycol. The final concentration of ^{3}H labeled steroid in the incubation medium is 33.3 nmol/l. The fragments were incubated at 37°C for 24 hours in a shaking water bath. For each steroid, a 24 hour control incubation without tissue fragments was done. For the timecourse incubation of norethisterone, the fragments were incubated for 1, 3, 6 and 24 hours. Cooling on ice stopped the incubation and the samples were stored at -20°C until analysis.

Incubations with ^{3}H-progesterone and ^{3}H-norethisterone were performed with uterine, vaginal and aortic tissue from four proestrus animals. From the same animals, uterus tissue (n=3), vagina tissue (n=1) and aorta tissue (n=1) was also incubated with ^{3}H-Org 4060, ^{3}H-Org OM38 and ^{3}H-Org 34694. Uterus, vagina and aorta tissue from the fifth proestrus animal was incubated with [^{3}H]-5α-H-Org 4060, and uterus tissue from the same animal was used to generate a time course incubation with norethisterone.

Uterine, vaginal and aortic tissue from all four ovariectomized rats was incubated with ^{3}H-norethisterone.

Extraction

After separation from the medium, the tissue fragments were extracted twice with 3 ml ethanol. The ethanol extract was dried under a stream of nitrogen, redissolved in 300 µl methanol and combined with the medium. To this mixture, 2.5 µg of appropriate unlabeled carrier steroids was added. 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 and HPTLC analysis. The recovery of radioactivity in the methanol eluates was 101 ± 8.4%, 83.6 ± 4.8%, 86.8 ± 8.5%, 94.8 ± 6.3% and 85.1 ± 12.3% for progesterone, norethisterone, Org 4060, Org OM38 and Org 34694, respectively.

HPLC analysis of metabolite profiles

Separation of the steroids and their metabolites was performed using a Waters spherisorb S5 ODS-2 column (4.6 x 250 mm) (Phase Separations B.V, Emmen, The Netherlands) with a guard column (4.6 x 10 mm) and a gradient of Water (solvent A) and acetonitrile (solvent B). The analytes were eluted with 40% of solvent B for 5 minutes, followed by a linear gradient of 40-75% solvent B in 35 minutes. After 5 minutes of elution with 75 % solvent B, initial values were re-established in 5 minutes. The flow rate was 1.0 ml/ min. Alternatively, a second elution system with methanol as solvent B was used. In this system (system II), the analytes were eluted with 50% of solvent B for 5 minutes, followed by a linear gradient of 50-90% solvent B in 35 minutes. After 5 minutes of elution with 90% solvent B, initial values were re-established in 5 minutes.

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 215 nm (system I) or 254 nm (system II), 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 500 µl 40% (v/v) acetonitrile / water. An aliquot of 100 µl was injected on the column. Metabolite identification was assigned using a first letter designating the parent compound (N for norethisterone, F for Org 4060, FM for 5α-H-Org 4060, O for Org OM38 and T for Org 34694) plus a number indicating the retention time of the metabolite.

HPTLC analysis

A 200 µl aliquot of the methanol Sep-Pak eluate was dried under a stream of nitrogen and redissolved in 20 µl propanol. Together with appropriate standard steroids, the sample was applied to the sample concentration zone of a HPTLC plate (10x10 cm, Merck kieselgel 60 F254). The plates were first run in toluene-cyclohexane (50:50) for 10 minutes to concentrate the sample at the border of the concentration zone. After drying, the plates were developed in dichloromethane-diethylether (80:20) for 15 minutes at 4°C. the reference standards were visualized under UV light. Steroids with a Δ4-3-keto group were visible with exposure to 254 nm UV light, while the other steroids were detected at 360nm UV light, after derivatization with primulin (Wright, 1971). For detection of the radioactive compounds on the HPTLC silica plate, the plates were developed on a tritium sensitive PhosphorImager screen (Molecular Dynamics) or on X-ray film after spraying with a scintillation reagent (EN^{3}HANCE, Life Science products).

Isolation of metabolites

After generation of metabolite profiles in HPLC system I, samples of progesterone, Org OM38, Org 4060 and Org 34694 incubations with uterine, vaginal and aortic tissue from four animals were pooled, extracted and injected on HPLC

system I. Using a switching valve between the UV- and on-line radioactivity detector, metabolites were isolated. The isolates were dried and redissolved in HPLC mobile phase. An aliquot of each isolated fraction was reinjected to verify that a single radioactive peak had been isolated.

Identification of metabolites.

Progesterone

The isolated progesterone metabolites were applied to a TLC plate (10x20 cm Merck kieselgel 60 F254, with concentration zone), and developed with reference steroids in diisopropylether-chloroform-hexane (7:2:1). The radioactive metabolites were identified by cochromatography with unlabeled reference standards.

Org 4060

Metabolite profiles for Org 4060 were established in HPLC system I. This system with acetonitrile as the mobile phase was chosen so that the Org 4060, 3β-OH-Org 4060 and 3α-OH-Org 4060 internal standards could be detected at 215 nm. The isolated Org 4060 metabolites and the 5α-H-Org 4060 samples were then applied to HPLC system II . The radioactive metabolites were identified by cochromatography with radioactive reference standards of 5α-H-Org 4060, 3α-OH-Org 4060, 3α-OH,5α-H-Org 4060 and 3β-OH,5α-H-Org 4060, which were all resolved in HPLC system II. The samples and the isolated Org 4060 metabolites were also applied to a HPTLC plate (10x10 cm Merck kieselgel 60 F254, with concentration zone). The radioactive metabolites were identified by cochromatography with unlabeled reference standards (5α-H-Org 4060, 3α-OH-Org 4060, 3β-OH-Org 4060, 3α-OH,5α-H-Org 4060 and 3β-OH,5α-H-Org 4060).

Org OM38

Metabolite profiles for Org OM38 were established in HPLC system I. This system with acetonitrile as the mobile phase was chosen so that the Org OM38, 3β-OH-Org OM38 and 3α-OH-Org OM38 internal standards could be detected at 215 nm. The isolated Org OM38 metabolites were applied to a HPTLC plate (10x10 cm Merck kieselgel 60 F254, with concentration zone). The radioactive metabolites were identified by cochromatography with unlabeled reference standards (5α-H-Org OM38, 3α-OH-Org OM38, 3β-OH-Org OM38, 3α-OH,5α-H-Org OM38 and 3β-OH,5α-H-Org OM38)

Results

Progesterone

Progesterone was readily metabolized in uterus en vagina and less in aorta. Figure 1a shows a representative HPLC radiochromatogram of vaginal tissue incubation with progesterone. Uterine tissue incubations with progesterone yielded similar profiles, and the amounts of metabolites are summarized in table 1. There were no qualitative differences between uterus and vagina incubations; all peaks appeared in incubations of both tissues. In aorta however, only peaks P25, P31, P36 and P39 were formed.

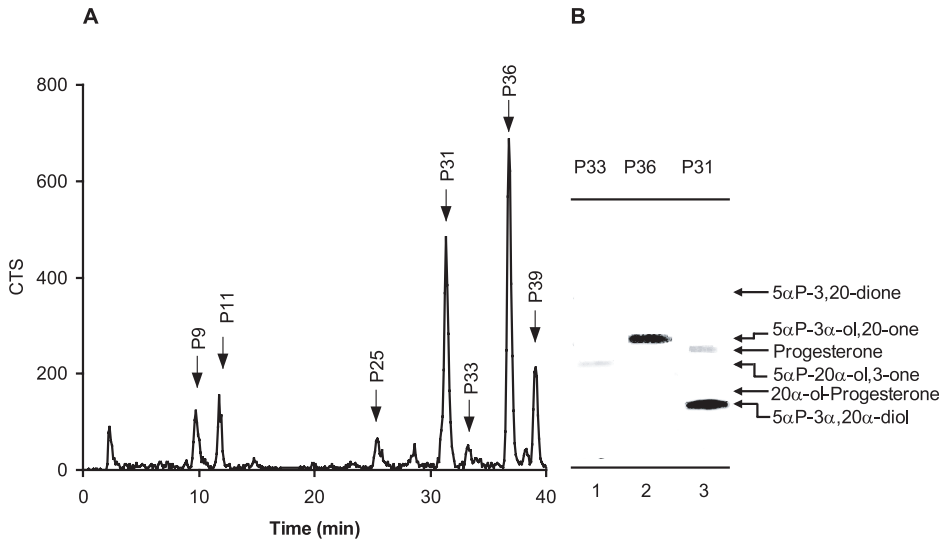

Figure 1: *Representative HPLC (system I) chromatogram showing the metabolism of ^{3}H-progesterone in vaginal tissue fragments (panel A). The arrows indicate the formation of two unknown metabolites (P9 and P11) and identified metabolites (P25, P31, P33, P36 and P39) as described in Table 1. The radioactive peaks labeled P9-P39 were isolated and identified by cochromatography on TLC with unlabeled reference standards, as is shown in panel B for P31, P33 and P36. The arrows show the co-migration with unlabeled reference progesterone metabolites of the radioactivity in metabolite peaks (P31, P33 and P36). In lane 1 and 2 P33 and P36 can be identified as 5α-pregnan-20α-ol-3-one and 5α-pregnan-3α-ol-20-one, respectively. In lane 3 it is shown that progesterone and 5α-pregnan-3α,20α-diol were not resolved in P31.*

Cochromatography on TLC of the isolated radioactive peaks with unlabeled reference steroids indicated the identity of the formed metabolites as is shown in table 1. The main metabolites were 5α-Pregnan-3α-ol-20-one (P36) and 5α-Pregnan-3α,20α-diol (P31) in uterus and vagina incubations. TLC analysis of peak P31 revealed that progesterone and 5α-Pregnan-3α,20α-diol have the same retention

time in HPLC system I. All other radioactive peaks appeared as a single band on TLC, as is shown in figure 1b for peak P36 and P33. The minor metabolites in P25, P33 and P39 were identified as 4-Pregnen-3-one-20α-ol, 5α-Pregnan-20α-ol-3-one and 5α-Pregnan-3,20-dione, respectively. The more polar radioactive peaks P9 and P11 did not cochromatograph with any of our reference steroids.

TABLE 1

Progesterone metabolites in uterus, vagina and aorta

peak ID	metabolite	% of radioactivity		
		uterus	vagina	aorta
P9	Unknown	1.7 ± 0.05	6.0 ± 2.2	nd
P11	Unknown	2.3 ± 0.6	7.3 ± 1.4	nd
P25	20α(OH)-progesterone	4.7 ± 1.4	1.0 ± 0.6	8.0
P31	5α-pregnan-3α,20α-diol progesterone	37.4 ± 5.4	26.5 ± 2.5	71.1
P33	5α-pregnan-20α-ol-3-one	nd	0.4 ± 0.1	nd
P36	5α-pregnan-3α-ol-20-one	38.1 ± 1.1	39.6 ± 2.7	9.2
P39	5α-pregnan-3,20-dione	10.7 ± 2.0	9.6 ± 0.3	2.3

nd = not detected, values are mean ± SEM

The presence of the above metabolites indicate that the uterus, vagina and aorta contain the following steroid metabolizing enzymes: 5α-reductase (5α-Red) , 20α-hydroxysteroid dehydrogenase (20α-HSD) and 3α-hydroxysteroid dehydrogenase (3α-HSD).

Norethisterone

The major metabolite of norethisterone in all three tissues was 3α-OH, 5α-H-norethisterone, as is illustrated in figure 2. In incubations of norethisterone with uterus tissue and vaginal tissue there was also formation of 5α-H-norethisterone and two unknown metabolites (N4 and N19). A time course incubation of uterine tissue with norethisterone is shown in figure 3, demonstrating the time dependent conversion of norethisterone into 3α-OH,5α-H-norethisterone and 5α-H-norethisterone The metabolism of norethisterone was not appreciably different between proestrus rats and OVX rats (student t-test, $p<0.05$). Therefore the data of the two groups were combined. Figure 4 summarizes the formation of the norethisterone metabolites in the three tissues. The metabolism of norethisterone (proestrus and Ovx animals, n=8) was lower in uterus than in vagina (table 2).

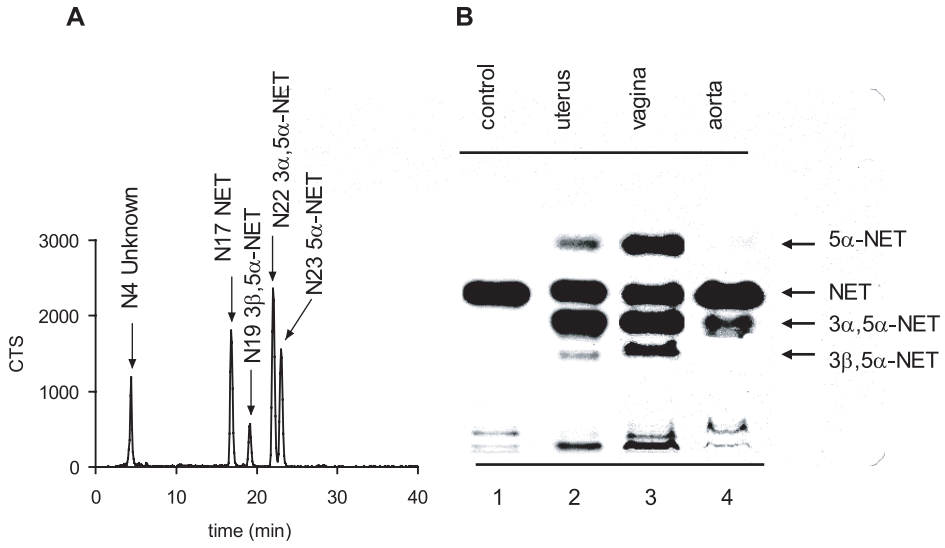

Figure 2: *Analysis of ^{3}H-norethisterone (NET) metabolites in uterus, vagina and aorta. Panel A shows a representative HPLC (system I) radiochromatogram of a 24 hour vaginal tissue incubation with ^{3}H-norethisterone. Panel B shows a representative HPTLC autoradiogram of a control incubation of ^{3}H-norethisterone without tissue (lane 1) and with uterine (lane 2), vaginal (lane 3) and aortic tissue (lane 4).*

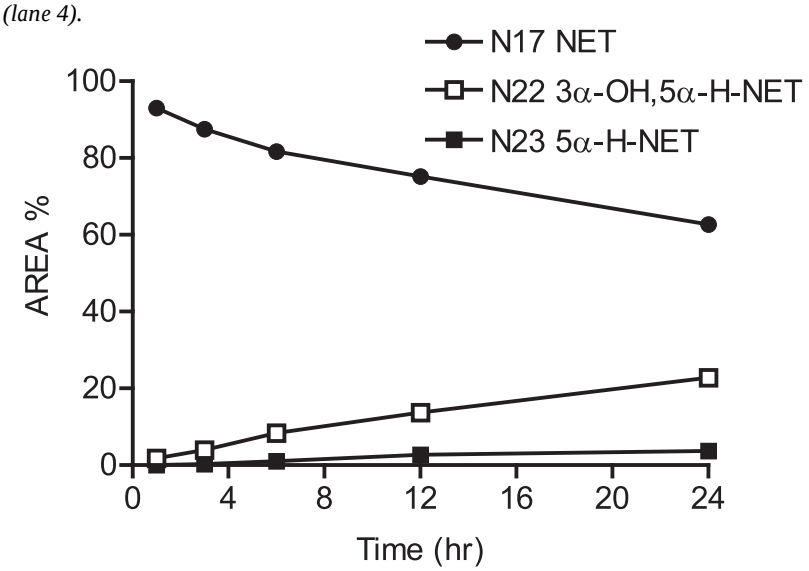

Figure 3: *Time course of norethisterone (NET) metabolism in uterine tissue fragment incubations. Uterine tissue was incubated with ^{3}H-norethisterone and analyzed by HPLC (system I) after 1, 3, 6, 12 and 24 hours of incubation. There is a steady increase of the major metabolite 3α-OH,5α-H-norethisterone up to 24 hours.*

Only 1.6% of total norethisterone was metabolized in aortic tissue. Clearly, 5α-reduction is the main metabolic pathway for norethisterone in all tissues. The 5α-H-norethisterone that is formed is then mainly metabolized by 3α-HSD, since the major metabolite in all three tissues is 3α-OH, 5α-H-norethisterone. Formation of 3β-OH, 5α-H-norethisterone occurred in uterus and vagina, but was more pronounced in vaginal tissue, demonstrating the presence of 3β-hydroxysteroid dehydrogenase (3β-HSD) activity.

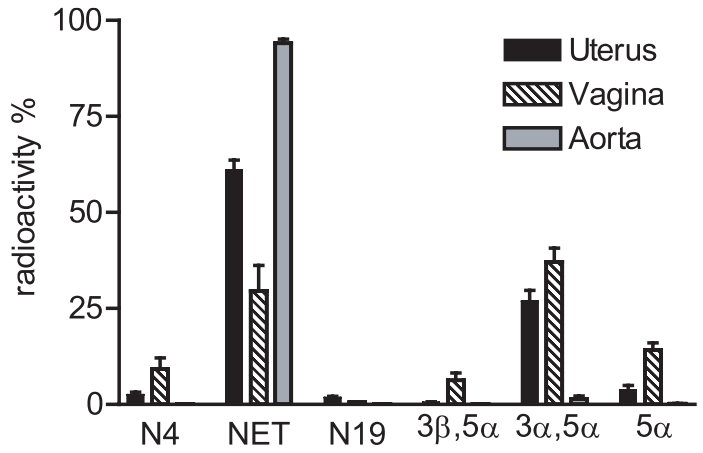

Figure 4: *Metabolism of ^{3}H-norethisterone (NET) in uterus, vagina and aorta. ^{3}H-norethisterone and its metabolites after a 24-hour incubation of uterine, vaginal and aortic tissue were analyzed using HPLC. Bars indicate average ± SEM. Major metabolites were 3α-OH,5α-H-norethisterone (3α,5α), 3β-OH,5α-H-norethisterone (3β,5α) and 5α-H-norethisterone (5α).*

Org 4060

Contrary to norethisterone, its 11β-ethyl derivative Org 4060 was poorly metabolized in uterine, vaginal and aortic tissue. A representative HPLC (system I) chromatogram is shown in figure 5a. Two radioactive peaks (F8 and F26), that were not present in the Org 4060 control incubation, appeared in uterus and vagina incubations. As is shown in table 2, these metabolites represented each less than 3% of total radioactivity in any of the tissues examined. In the aorta incubation, a radioactive peak with a retention time of 25 minutes (F25) and F26 was present. The F26 peak cochromatographed in HPLC system I with the 3α-OH-Org 4060 reference peak in the UV trace (data not shown). After isolation, peak F26 also cochromatographed with the tritiated 3α-OH-Org 4060 standard in HPLC system II (figure 6a). The formation of 5α-reduced forms of Org 4060 in the three tissues was excluded using HPTLC (data not shown).

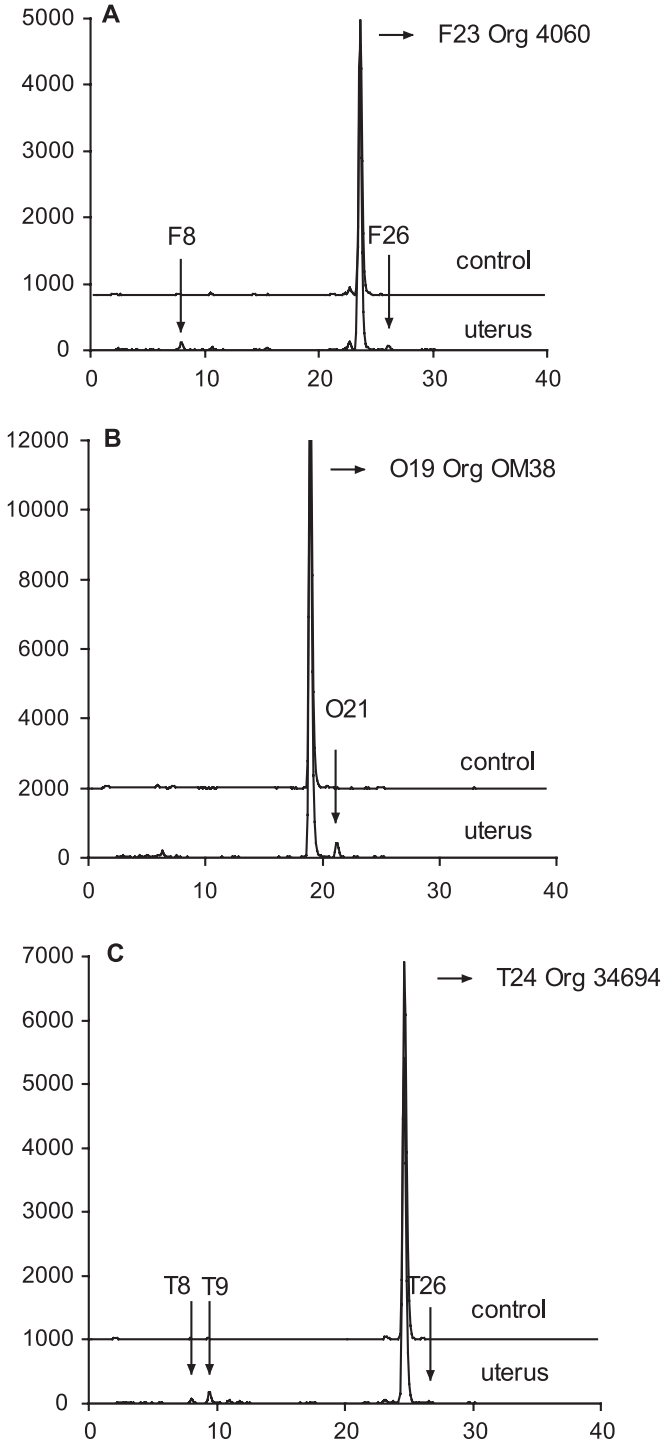

Figure 5:

Representative HPLC (system I) radiochromatograms of a 24 hour uterine tissue incubation with ^{3}H-Org 4060 (panel A), ^{3}H-Org OM38 (panel B) and ^{3}H-Org 34694 (panel C). The upper trace in each panel represents the 24-hour control incubation without tissue of the appropriate steroid. The vertical arrows indicate the metabolites that were formed.

Since the major norethisterone metabolites were identified as 5α-reduced forms of norethisterone, the three tissues were also incubated with 5α-H-Org 4060. Incubations of uterus and vagina with radiolabeled 5α-H-Org 4060 showed good conversion into two radioactive peaks FM18 and FM37. The major metabolite (FM37) in uterus and vagina represented 40.4% and 35.8%, respectively (table 2). The FM37 peak cochromatographed with 3α-OH,5α-H-Org 4060. In aorta incubations, 5α-H-Org 4060 was metabolized into 3α-OH,5α-H-Org 4060 (33.6%). Interestingly, in aorta there was also formation of an additional FM33 radioactive peak (4.3%), cochromatographing with 3β-OH,5α-H-Org 4060 (figure 7b).

Org OM38

Also Org OM38, the 7α-methyl derivative of norethisterone, was less metabolized than norethisterone itself. In uterus, vagina and aorta a single radioactive peak appeared (O21) that was not present in the control incubation (figure 5b). The O21 metabolite from all tissues had the same retention time in HPLC system I as the unlabeled 3α-OH-Org OM38 standard. After isolation, this metabolite also cochromatographed with the same standard on HPTLC (data not shown). Org OM38 was metabolized to 3α-OH-Org OM38 (O21) at 4.0%, 7.6% and 4.5% in uterus, vagina and aorta, respectively (see also table 2).

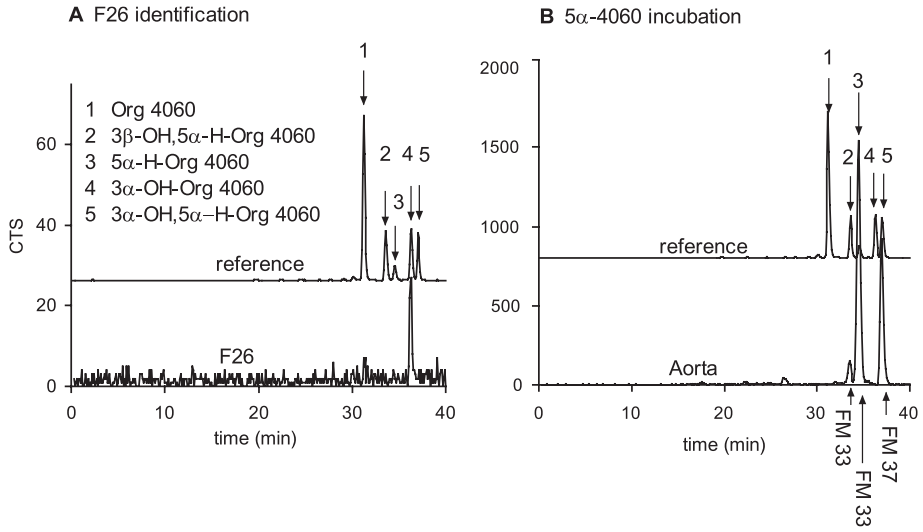

Figure 6: *Analysis ^{3}H-Org 4060 metabolites using HPLC system II. The upper trace represents a radiochromatogram of tritiated Org 4060 metabolites. Panel A shows the identification of the F26 Org 4060 metabolite from uterus as 3α-OH-Org-4060. Panel B shows the conversion in aortic tissue of ^{3}H-5α-H-Org 4060 into 3α-OH,5α-H-Org 4060 and 3β-OH,5α-H-Org 4060.*

Org 34694

Similar to Org 4060, also Org 34694 was poorly metabolized in uterus vagina and aorta (table 2). Three radioactive peaks (figure 5c) appear in the tissue incubations (T8, T9 and T26). The third radioactive peak (T26) had the same retention time as an unlabeled 3α-OH-Org 34694 reference. Because of the low amount of radioactivity, attempts to isolate T26 were unsuccessful.

TABLE 2

Metabolites of norethisterone, Org 4060, 5α-H-Org 4060, Org OM38 and Org 34694

parent compound	peak ID	metabolite	% of radioactivity		
			uterus	vagina	aorta
NET	N4	unknown	2.6 ± 0.7	9.3 ± 2.8	nd
	N17	norethisterone (NET)	61.1 ± 2.6	29.5 ± 6.7	94.1 ± 1.1
	N18	unknown	1.8 ± 0.4	0.5 ± 0.2	nd
	N19	3β-OH,5α-H-NET	0.5 ± 0.2	6.3 ± 1.9	nd
	N22	3α-OH,5α-H-NET	26.9 ± 2.9	37.1 ± 3.6	1.4 ± 0.8
	N23	5α-H-NET	3.8 ± 1.2	14.1 ± 1.9	0.2 ± 0.05
Org 4060	F8	unknown	2.5 ± 0.2	2.4	nd
	F23	Org 4060	93.3 ± 1.6	90.2	95.6
	F26	3αOH-4060	1.3 ± 0.2	2.5	1.0
5α-H-4060	FM18	unknown	14.5	15.2	nd
	FM33	3β-OH-,5α-H-Org 4060	nd	nd	4.3
	FM34	5α-H-Org 4060	45.2	37.9	59.6
	FM37	3α-OH,5α-H-Org 4060	35.8	40.4	33.6
Org OM38	O19	Org OM38	93.3 ± 0.5	88.3	92.4
	O21	3α-OH-OM38	4.0 ± 0.7	7.6	4.5
Org 34694	T8	unknown	0.9 ± 0.1	1.2	0.9
	T9	unknown	1.2 ± 0.1	3.2	0.4
	T24	Org 34694	93.0 ± 0.1	93.9	92.9
	T26	3α-OH-34694	0.6 ± 0.3	0.6	0.7

nd = not detected, values are mean ± SEM

Discussion

The incubations with progesterone in uterus tissue confirmed the presence of 5α-reductase, 3α-HSD and 20α-HSD, as was described earlier (Lisboa and Holtermann, 1976). The same progesterone metabolizing enzymes also appeared to be present in vagina and, to a lesser extent, in aorta.

Next it was investigated whether these enzymes also acted upon the 19-nor-progestagen norethisterone and norethisterone derivatives. In the three tissues, 5α-reduction followed by 3α-reduction was the major pathway of norethisterone metabolism. Norethisterone was metabolized into mainly 3α-OH,5α-H-

norethisterone in uterus, vagina and aorta of proestrus- and ovariectomized rats. Conversion of norethisterone was higher in vagina than in uterus, while aorta showed only limited conversion. In uterus and vagina, also the formation of 3β-OH,5α-H-norethisterone was demonstrated. The conversion of all norethisterone derivatives (Org OM38, Org 4060 and Org 34694) was much lower than of norethisterone itself (table 2). Furthermore, the formation of 5α-reduced forms of Org OM38, Org 4060 or Org 34694 was not found. Only the formation of 3α-hydroxylated forms of Org OM38, Org 4060 and Org 34694 in uterus, vagina and aorta could be demonstrated.

There is limited information as to the physiological function of the progesterone metabolizing enzymes in the female reproductive tract and the cardiovascular system. In mice uterus, there is a combined action of 5α-reductase, 3α-HSD and 20α-HSD to catabolize progesterone in uterus, and the impaired progesterone metabolism in the cervix of 5α-reductase type 1 isoenzyme knockout mice leads to a parturition defect in these mice (Mahendroo et al., 1999) .

The reduction of the 3-keto, Δ4 moiety of norethisterone and norethisterone derivatives may be important for their pharmacology in HRT target tissues, since this reduction changes its progestagenic character. While norethisterone acts as a progestagen in prepubertal rabbits, where it induces the uteroglobin content of uterine flushing, neither 5α-H-norethisterone or 3β-OH,5α-H-norethisterone shows this effect (Cerbon et al., 1990). Moreover, 5α-H-norethisterone and 3β-OH,5α-H-norethisterone exhibited strong *in vivo* estrogenic effects by inducing progesterone receptor (PR) mRNA in rabbit uterus (Pasapera et al., 1995) and both PR and c-fos mRNA in rat uterus (MendozaRodriguez et al., 1999). It was also shown that 3α-OH,5α-H-gestodene and 3β-OH,5α-H-gestodene posses a weak intrinsic estrogenic activity (Lemus et al., 2000). The mixed hormonal profile of norethisterone with both progestagenic and estrogenic properties may thus be mediated by metabolism of norethisterone. Since Org 4060, Org OM38 and Org 34694 were not 5α-reduced in uterus, vagina or aorta, estrogenic activity through formation of tetrahydro-reduced metabolites seems unlikely in these tissues. The pharmacological significance of the formation of minor amounts of 3α(OH)-Org 4060, Org OM38 and Org 34694 needs further investigation.

It was also shown that, although Org 4060 is not 5α-reduced in uterus, vagina or aorta, 5α-H-Org 4060 itself is readily metabolized in all three tissues into mainly 3α-OH,5α-H-Org 4060 (40.4% , 35.8% and 33.6%, for uterus, vagina and aorta, respectively), and into 3β-OH,5α-H-Org 4060 (4.3%) in aorta. This, together with the fact that 3α-OH-5α-H-norethisterone and not 5α-H-norethisterone is the main metabolite of norethisterone, demonstrates that 5α-reduction is the rate limiting step in the metabolism of norethisterone and norethisterone derivatives in the three HRT target tissues. Apparently, both the 11β-ethyl substituent that is the only structural difference between norethisterone and Org 4060, and the 7α-methyl substituent of Org OM38 render both Org 4060 and Org OM38 poor substrates for 5α-reduction, compared to norethisterone. The steric hindrance of the 7α-methyl

substituent for steroid 5α-reduction is also known from 7α-methyl-19-nortestosterone. In contrast to 19-nortestosterone, 7α-methyl-19-nortestosterone is not 5α-reduced (Sundaram et al., 1995). Substitutions at the 11β-position of the steroid skeleton have not been reported before to interfere with 5α-reductase activity. Interestingly, two 19-nor-progestagen steroids with a 11- methylene substituent, desogestrel and Org 30569, were extensively metabolized in rat liver microsomes into mainly 3α-(OH),5α-H-reduced derivatives (Verhoeven et al., 1998b). Apparently, the rigidity that is imposed on the steroid C ring by the double bond of the 11-methylene group in desogestrel and Org 30569 does not interfere unfavorably with the activity of 5α-reductase (Verhoeven et al., 1998a). It is difficult to speculate on the nature of the interference of the 11β-ethyl group of Org 4060 with the 5α-reductase enzyme. The extreme insolubility of 5α-reductase, a reflection of the membrane-bound nature of the enzyme, has made its purification and structural characterization very difficult. Still, photolabeling and mutagenesis studies have indicated a N-terminal steroid D-ring binding domain, a NADPH cofactor binding domain and a putative C-terminal catalytic steroid A-ring region (Thigpen and Russell, 1992; Wang et al., 1999; Bhattacharyya et al., 1999). There seems to be considerable tolerance of 5α-reductase for substitutions to the steroid D-ring. All known 5α-reductases accept both testosterone and progesterone as a substrate. Also norethisterone, a 17α-ethinylated-19-nortestosterone, is readily 5α-reduced. Moreover, human 5α-reductase type 1 or 2 can 5α-reduce campestrol, a C-27 plant steroid, reduction in the plants (Li et al., 1997). Maybe the 11β-ethyl substitution of Org 4060 does not interfere with the steroid D-ring binding domain of 5α-reductase, but rather with the NADPH binding domain.

The fact that 3α-OH,5α-H-Org 4060 was formed from 5α-H-Org 4060 in comparable amounts in uterus, vagina and aorta shows the presence of 3α-HSD in all three tissues. The 3α-HSD enzyme is known to work in concert with 5α-reductases in target tissues to regulate levels of active steroid (Penning, 1997). Only one isoform of 3α-HSD has been described in rat, with high level expression in the liver. It seems probable that this enzyme is also involved in the conversion of Org 4060 and Org OM38 in 3α-OH-Org 4060 and 3α-OH-Org OM38 in uterus and vagina.

In the rat, four isoforms of 3β-HSD have been described, each with its own enzymatic characteristics and tissue expression pattern. Which of these isoforms is responsible for the conversion of 5α-H-norethisterone into 3β-OH,5α-H-norethisterone remains unclear. Apart from their oxidative 3β-hydroxysteroid dehydrogenase / Δ^5-Δ^4 isomerase activity, homogenates of HeLa cells expressing rat 3β-HSD type 1 and type 2 are also able to reduce dihydrotestosterone into 5α-androstan-3β,17β-diol (Zhao et al., 1991). Ribonuclease protection analysis revealed the presence of rat 3β-HSD type 1 and type 2 mRNA in rat uterus (Simard et al., 1993). On the other hand, the rat 3β-HSD type 3 displays no isomerase activity and is considered a pure 3-ketosteroid reductase. Its expression however has only been

demonstrated in male rat liver (Labrie et al., 1992). So, either the 3β-reduction of 5α-norethisterone in vagina and the uterus is catalyzed by type 1 and 2 3β-HSD or there may be another 3β-HSD isoform present in these tissues with ketosteroid reductase activity.

Overall it was shown in this paper that norethisterone is metabolized to its estrogenic 5α-reduced metabolites in uterus, vagina and aorta. Substitutions at the 7α- and 11- position as in Org 4060, Org OM38 and Org 34694 prevented the metabolism to 5α-reduced forms. However, limited conversion of Org 4060, Org OM38 and Org 34694 into their 3α-OH-reduced form does occur in uterus, vagina and aorta. Furthermore, the conversion of 5α-H-Org 4060 into 3αOH,5α-H-Org 4060 in all three tissues demonstrates that 5α-reduction is the rate limiting step in the formation of 3α-OH,5α-H-reduced metabolites of norethisterone and norethisterone derivatives in HRT target tissues. It could thus be possible that circulating 5α-reduced norethisterone derivatives are a source for generating 3α-OH-,5α-H-reduced metabolites in uterus, vagina and aorta. It also indicates that *in vivo* effects of 5α-reduced norethisterone and 5α-reduced norethisterone derivatives in HRT target tissues should be interpreted with prudence, because of their conversion to 3α-OH,5α-H-reduced forms, which display estrogenic activity.

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CHAPTER 4

Metabolism of norethisterone and norethisterone derivatives in uterus and vagina of postmenopausal women; the role of 5-alpha reductase

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in preparation

Abstract

Treatment of postmenopausal bone loss with Hormone Replacement Therapy (HRT) usually consists of a combination of estrogen and a progestagen such as norethisterone (17α-ethynyl-19-nor-testosterone). The metabolism of radiolabeled norethisterone and several norethisterone derivatives was studied in uterus and vagina, two target tissues of HRT. Metabolism of norethisterone in these target tissues may play a role in the final biological response to norethisterone and its derivatives. The norethisterone derivatives are Org OM38 (7α-methyl-norethisterone), Org 4060 (11β-ethyl-norethisterone) and Org 34694 (7α-methyl,11-ethylidene-norethisterone). Uterus and vagina biopsies were obtained from postmenopausal women following hysterectomy and incubated with the ^{3}H labeled steroids (50 nM). The radioactive metabolites were analyzed by HPLC and HPTLC. Norethisterone was extensively converted in vaginal tissue (51.1 ±1.2%) into mainly 5α-reduced metabolites. Equal amounts of 3β-OH, 5α-H-norethisterone (15.9 ± 2.0%), 3α-(OH), 5α-H-norethisterone (15.3 ± 1.7%) and 5α-H-norethisterone (15.3 ± 1.7%) were found. Surprisingly, in human uterus, norethisterone was not metabolized. However, the 3α-hydroxysteroid dehydrogenase and 3β-hydroxysteroid dehydrogenase activities in uterus and vagina were similar, as was revealed by the incubations with 5α-H-Org 4060. Therefore, 5α-reductase activity maybe specifically decreased in the postmenopausal uterus. HEK293 cells expressing human 5α-reductase type 1 and type 2 were both able to metabolize norethisterone to 5α-H-norethisterone. Org OM38, Org 4060 and Org 34694 were not metabolized in vagina, uterus or cells expressing human 5α reductase type 1 or 2. Apparently the 7α-methyl group of Org OM38 and the 11β-ethyl group of Org 4060 render them poor substrates for 5α-reduction.

Overall it was demonstrated that tetrahydro metabolites of norethisterone could be formed in vagina tissue of postmenopausal women, but not in uterus. The 5α-reduction of norethisterone is the first step the formation of these metabolites. Furthermore, the 7α-methyl group of Org OM38 and the 11β-ethyl group of Org 4060 prevent their 5α-reduction by both human 5α-reductase isoenzymes.

Introduction

Hormone Replacement Therapy (HRT) for postmenopausal women often consists of a combined estrogen and progestagen treatment, given either sequentially or continuously. The treatment should ideally have beneficial estrogenic effects on bone, the cardiovascular system, climacteric complaints and vagina. An estrogenic, proliferative response should be absent in the uterus and the breast. Norethisterone is a synthetic steroid that is used as a progestagen in combined HRT for postmenopausal women. However, both progestagenic and anti-progestagenic effects of norethisterone have been described on uteroglobin and progesterone receptor expression in the uterus of progesterone treated prepubertal

rabbits (Pasapera et al., 1995). Moreover, estrogenic activity of norethisterone in rat uterus is mediated through 5α-reduced metabolites of norethisterone (Mendoza Rodriguez et al., 1999). Metabolism of norethisterone in HRT target tissues may thus contribute to its mixed hormonal profile.

The metabolism of norethisterone was studied in uterus and vagina, two HRT target tissues, of postmenopausal women. Also the effect of substitutions at the 7 and 11 position on the norethisterone skeleton was studied. The 7α and 11β position are known to maintain good estrogen receptor binding when they are substituted with small lipophilic groups such as a methyl- or ethyl group (Zeelen and Bergink, 1980). The norethisterone derivatives studied are: Org OM38, Org 4060 and Org 34694. All three steroids are similar to norethisterone, but have a 7α-methyl- (Org OM38), an 11β-ethyl- (Org 4060) or both a 7α-methyl- and an 11-ethylidene substituent (Org 34694).

Biopsies of uterus and vagina were obtained from post-menopausal women following hysterectomy. To investigate which steroid metabolizing enzymes are present, tissue fragments of uterus and vagina were incubated with ^{3}H-labeled progesterone. Subsequently, fragments of uterine and vaginal tissue were incubated with ^{3}H-labeled norethisterone and the ^{3}H-labeled norethisterone derivatives (Org OM3, Org 4060 and Org 34694). Furthermore, HEK293 cells transiently expressing the human 5α-reductase type 1 or the human 5α-reductase type 2 isoenzyme were incubated with the ^{3}H-labeled steroids. The metabolites were separated using HPLC and HPTLC. Identification of metabolites was performed by cochromatography with unlabeled steroids in the different chromatographic systems.

Materials and methods

Chemicals

[1,2,6,7-^{3}H]-progesterone (3589 GBq/mmol) was purchased from NEN Life Science products (Hoofddorp, The Netherlands). The radiolabeled steroids [16-^{3}H]-Norethisterone (1010 GBq/mmol), [16-^{3}H]-Org OM38 (1400 GBq/mmol), [11-^{3}H]-Org (560 GBq/mmol) and [16α-^{3}H]-Org 34694 (925 GBq/mmol) were a gift from Organon (Oss, The Netherlands). The radiolabeled, 5α-reduced metabolite of Org 4060 and the 3α-hydroxylated metabolite of Org 4060 were synthesized from [11-^{3}H]-Org 4060 by Organon and yielded [11-^{3}H]- 5α-H-Org 4060 and [11-^{3}H]-3α-OH-Org 4060 with specific activities of ca. 500 GBq/mmol. The radiolabeled, 5α,3α-reduced metabolite of Org 4060 and the 5α,3β-reduced metabolite were produced from [11-^{3}H]- 5α-Org 4060. The unlabeled norethisterone, Org 4060, Org OM38 and Org 34694 and their metabolites, used as reference steroids were a gift from Organon.

All other chemicals were obtained from local commercial sources and were of analytical grade.

Patients

Uterus and vagina biopsies were obtained from four postmenopausal women (age 55-88 years) following vaginal hysterectomy. The indication for surgery in all women was a uterine prolapse. The participants gave their written, informed consent and the study was approved by the local ethics committees of the participating study centers.

Tissue collection and incubation

The uterine and vaginal tissue was collected in ice-cold Leibowitz medium (L-15), buffered with HEPES at pH 7.4 and supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin. The uterus was opened longitudinally and sections were cut from the middle part of the uterus in such a way that each section included the luminal epithelial layer and an underlying endometrial layer of approximately 10 mm thickness. The vaginal tissue and the uterine fragments were minced and 50 mg of tissue minces were transferred to vials containing 2 ml of Leibowitz medium (L-15) and ^{3}H-labeled steroid dissolved in 50 µl propyleneglycol. The final concentration of ^{3}H-labeled steroids was 50 nmol/l. The fragments were incubated at 37°C for 24 hours in a shaking water bath. For each labeled steroid, a 24 hour control incubation without tissue fragments was done. 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 ethanol. The ethanol extract was dried under a stream of nitrogen, redissolved in 300 µl methanol and combined with the medium. To this mixture, 2.5 µg of appropriate unlabeled carrier steroids was added. 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 and HPTLC analysis. The recovery of radioactivity in the methanol eluates was 90.0 ± 3.0%, 89.2 ± 6.8%, 89.3 ± 6.8% and 89.6 ± 5.9% for norethisterone, Org 4060, Org OM38 and Org 34694, respectively.

HPLC analysis of metabolite profiles

Separation of the steroids and their metabolites was performed using a Waters spherisorb S5 ODS-2 column (4.6 x 250 mm) (Phase Separations B.V, Emmen, The Netherlands) with a guard column (4.6 x 10 mm) and a gradient of Water (solvent A) and acetonitrile (solvent B). The analytes were eluted with 40% of solvent B for 5 minutes, followed by a linear gradient of 40-75% solvent B in 35 minutes. After 5 minutes of elution with 75% solvent B, initial values were re-established in 5 minutes. The flow rate was 1.0 ml/ min. Alternatively, a second elution system with methanol as solvent B was used. In this system (system II), the analytes were

eluted with 50% of solvent B for 5 minutes, followed by a linear gradient of 50-90% solvent B in 35 minutes. After 5 minutes of elution with 90% solvent B, initial values were re-established in 5 minutes.

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 215 nm (system I) or 254 nm (system II), and a Flo-one model A200 on-line radioactivity detector (Canberra Packard, Groningen, The Netherlands).

Immediately before analysis, the methanol Sep-Pak eluates were dried under a stream of nitrogen and redissolved in 500 µl 40% (v/v) acetonitrile / water. An aliquot of 100 µl was injected on the column.

HPTLC analysis

A 200 µl aliquot of the methanol Sep-Pak eluate was dried under a stream of nitrogen and redissolved in 20µl propanol. Together with appropriate standard steroids, the sample was applied to the sample concentration zone of a HPTLC plate (10x10 cm, Merck kieselgel 60 F254). The plates were first run in toluene-cyclohexane (50:50) for 10 minutes to concentrate the sample at the border of the concentration zone. After drying, the plates were developed in dichloromethane-diethylether (80:20) for 15 minutes at 4°C. the reference standards were visualized under UV light. Steroids with a Δ4-3-keto group were visible with exposure to 254 nm UV light, while the other steroids were detected at 360 nm UV light, after spraying with primulin (Wright, 1971). For detection of the radioactive compounds on the HPTLC silica plate, the plates were developed on X-ray film after spraying with a scintillation reagent (EN^{3}HANCE, Life Science products). To identify progesterone and norethisterone metabolites, the radioactive spots were scraped from the HPTLC plate and the steroids were eluted from the silica with 2 times 500 µl of dichloromethane. The solvent was evaporated under a gentle stream of nitrogen and an aliquot was run on HPTLC using diisopropylether-chloroform-hexane (7:2:1) as the mobile phase to verify the cochromatography with the unlabeled steroid. Another aliquot was run on HPLC to establish the retention time of the metabolite in this system.

Cell culture and transfection

The human 5α-reductase isoenzymes type 1 and type 2 cDNAs subcloned into the pCMV7 expression vector were a kind gift from D.W. Russell. HEK293 cells were cultured as described previously (Blomenrohr et al., 1997). In a 24-well plate, 20.000 cells were plated and transiently transfected 24 hours later with 1 µg human 5α-reductase isoenzyme DNA using the SuperFect transfection method (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

The cells were incubated with the tritiated steroids (50 nM) in 1 ml serum free medium 48 hours after transfection. Aliquots of medium (100 µl) were collected after 0.5, 2, 3, 4 and 24 hours of incubation and analyzed on HPLC system I. The

transfections were tested by measuring their ability to convert testosterone into dihydrotestosterone. Mock-transfected cells did not convert testosterone into dihydrotestosterone (data not shown). Each steroid was tested with cells from three separate transfections.

Results

Progesterone metabolism

Progesterone was readily metabolized in vaginal tissue and less in uterine tissue. Figure 1 shows representative HPLC and HPTLC radiochromatograms of a uterine and a vaginal tissue incubation with progesterone.

Cochromatography with unlabeled reference steroids on HPTLC in two systems indicated the identity of the formed metabolites as is shown in table 1. The most abundant metabolites in the vagina were 5α-Pregnan-3,20-dione, 5α-Pregnan-32β-ol-20-one, 5α-Pregnan,20α-ol-3-one and 5α-Pregnan-3α-ol-20-one and an unknown metabolites P8 and P10. These metabolites indicate the presence of 5α-reductase, 3α-hydroxysteroid dehydrogenase, 3β-hydroxysteroid dehydrogenase and 20α-hydroxysteroid dehydrogenase activity in the tissue.

TABLE 1

Progesterone metabolites (percentage of total radioactivity) in uterus and vagina

peak ID	**metabolite**	**% of radioactivity**	
		Uterus	Vagina
P8	Unknown	nd	10
P10	Unknown	1	19
P26	4-pregnen-20α-ol-3-one	5	3
P30	5α-pregnan-3β,20α-diol	<1	1
P31	progesterone	81	24
	5α-pregnan-3α,20α-diol	nd	<1
P34	5α-pregnan, 20α-ol-3-one	<1	7
P36	5α-pregnan-3β-ol-20-one	2	8
P37	5α-pregnan-3α-ol-20-one	<1	4
P39	5α-pregnan-3,20-dione	3	17

nd = not detected

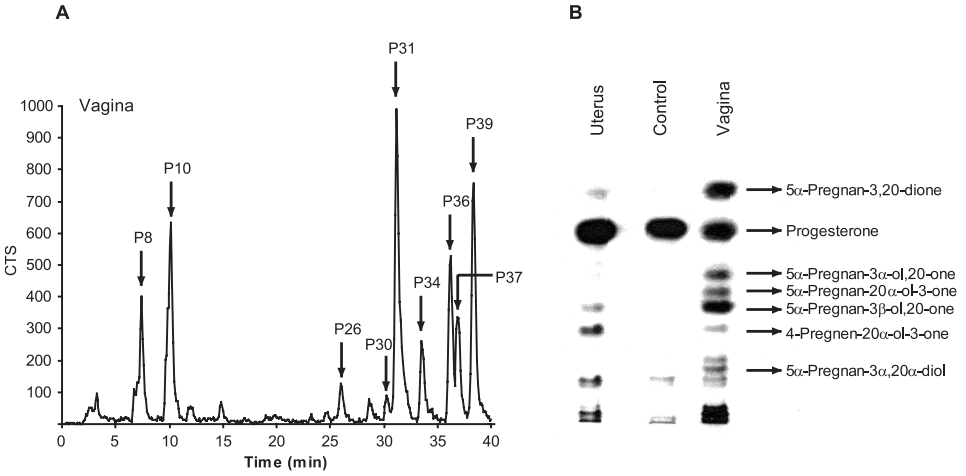

Figure 1: *Analysis of ^{3}H-progesterone metabolites in incubations with tissue fragments from vagina and uterus of postmenopausal women. A representative HPLC radiochromatogram of a vagina incubation is shown in panel A. The arrows indicate the formation of two unknown metabolites (P8 and P10) and identified metabolites (P26, P30, P34, P36, P37 and P39). Panel B shows the cochromatography on HPTLC with unlabeled steroid standards for an incubation of uterine and vaginal tissue with ^{3}H-progesterone. An incubation without tissue was used as a control. The 5α, 3α, 3β and 20α reduced metabolites were identified as indicated by the arrows.*

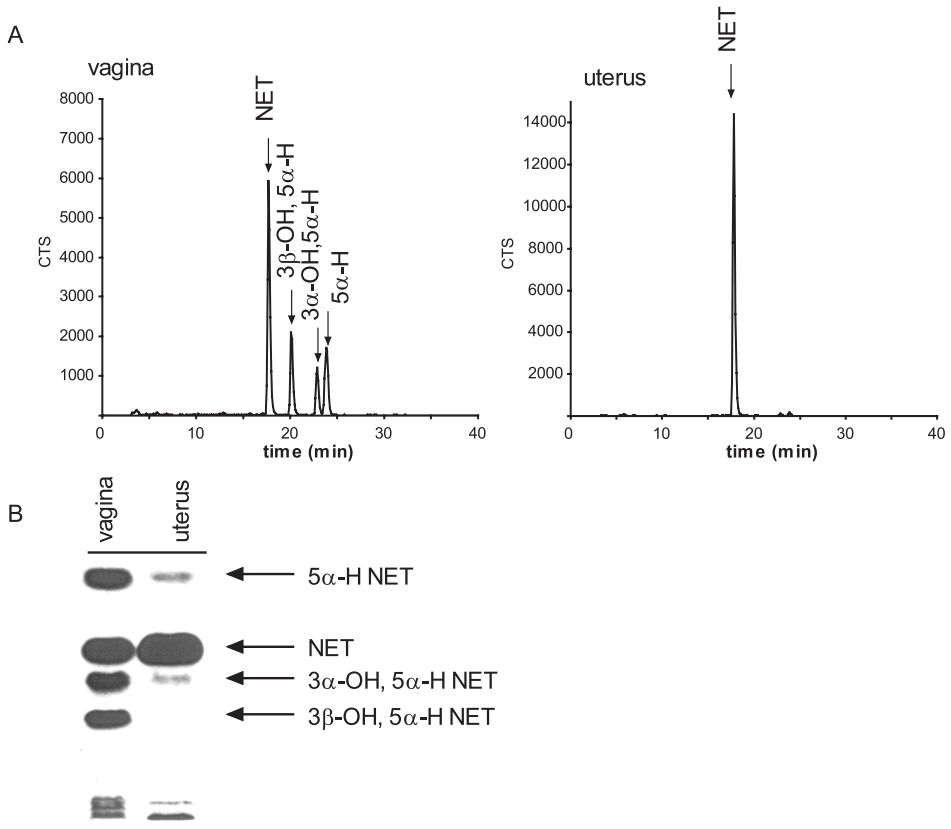

Figure 2: *Analysis of ^{3}H-norethisterone (NET) metabolites in incubations with tissue fragments from vagina and uterus of postmenopausal women. Panel A shows representative HPLC (system- I) radiochromatograms of vagina and uterus incubations. Panel B shows a representative HPTLC autoradiogram of vagina and uterus incubations. In both chromatographic systems, the formation of 3β-OH, 5α-H-NET, 3α-OH, 5α-H-NET and 5α-H-NET could be demonstrated.*

Norethisterone, Org OM38, Org 4060 and Org 34964 metabolism

Norethisterone was metabolized in vaginal tissue incubations to 3β-OH, 5α-H-norethisterone (15.9 ± 2.0%), 3α-OH, 5α-H-norethisterone (15.3 ±1.7%) and 5α-H-norethisterone (17.7 ± 1.0%). The identity of the metabolites was determined by cochromatography with unlabeled standards in HPLC and HPTLC analysis, as is illustrated in figure 2. Hardly any metabolism of norethisterone was detected in the uterus fragment incubations. Less than 1% of the norethisterone was metabolized into 3β-OH,5α-H-norethisterone 3α-OH,5α-H-norethisterone or 5α-H-norethisterone, as is illustrated in figure 3.

The norethisterone derivatives Org OM38, Org 4060 or Org 34694 were not metabolized in the tissue incubations with uterine or vaginal tissue. In contrast, 5α-H-Org 4060 was metabolized in uterus to 3β-OH, 5α-H-Org 4060 (25.9 ± 1.9%) and 3α-OH, 5α-H-Org 4060 (25.9 ± 1.8%). Also in vagina tissue incubations, 5α-H-Org 4060 was metabolized in uterus to 3β-OH, 5α-H-Org 4060 (38.3 ± 4.0%) and 3α-OH, 5α-H-Org 4060 (20.7 ± 2.4%), as is illustrated in figure 4.

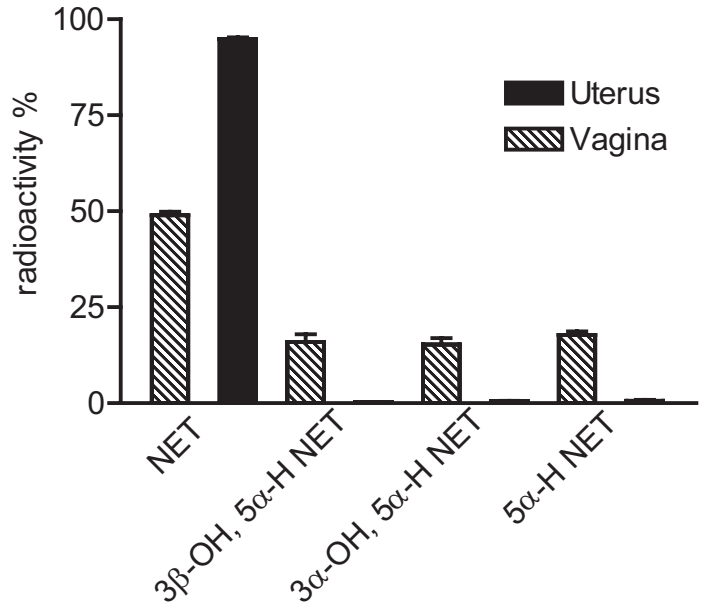

Figure 3: *Metabolism of ^{3}H-norethisterone (NET) in uterine and vaginal tissue of postmenopausal women. ^{3}H-norethisterone and its metabolites after a 24-hour incubation of uterus and vagina tissue from postmenopausal women were analyzed using HPLC. Bars indicate average ± SEM.*

Incubations of 5α-reductase transfected HEK293 cells.

Testosterone was readily 5α-reduced into 5α-dihydrotestosterone in HEK293 cells after transfection with the human 5α-reductases, as is shown in figure 5. As in the tissue fragment incubations, there was no metabolism of Org OM38, Org 4060 or Org 34694 in incubations with HEK293 cells transfected with human 5α-reductase type 1 or 5α-reductase type 2. However, norethisterone was 5α-reduced by HEK293 transfected with human 5α-reductase 1 and 5α-reductase type 2. After 24 hours of incubation, 18.8 ± 1.5% of norethisterone was metabolized into 5α-H-norethisterone by HEK293 cells transfected with human 5α-reductase type 1 and into 33.2 ± 3.7% 5α-H-norethisterone by HEK293 cells transfected with human 5α-reductase type 1, as shown in figure 6.

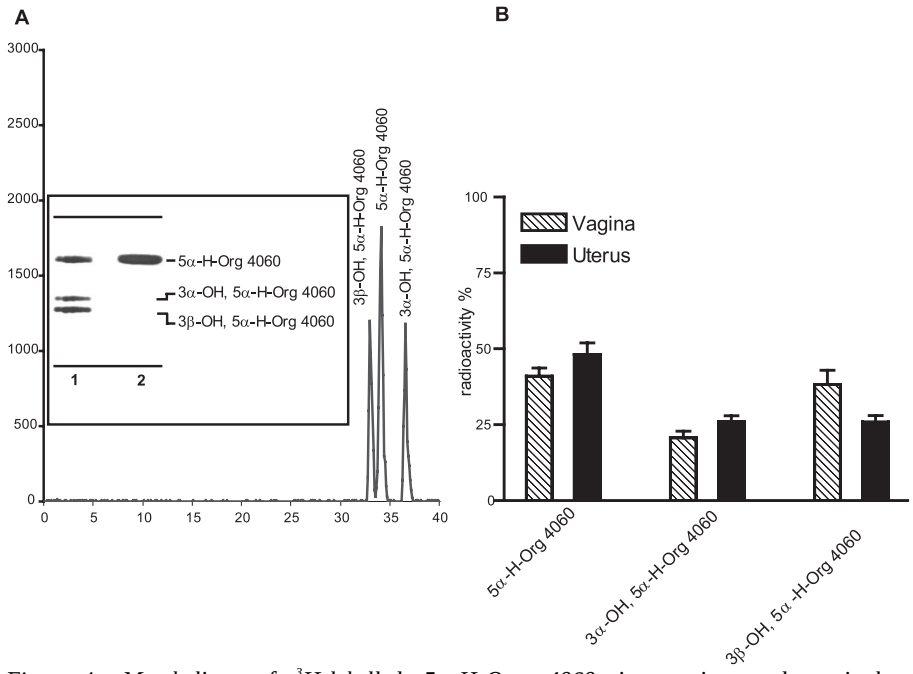

Figure 4: *Metabolism of ^{3}H-labelled 5α-H-Org 4060 in uterine and vaginal tissue of postmenopausal women. ^{3}H-5α-H-Org 4060 and its metabolites after a 24-hour incubation of uterine and vaginal tissue from postmenopausal women were analyzed using HPLC and HPTLC. Panel A shows a representative HPLC (system II) radiochoromatogram of ^{3}H-5α-H-Org 4060 and its metabolites from a uterus incubation. A HPTLC autoradiogram of the same sample is shown in the insert (lane 1), together with a control incubation without tissue (lane 2). Panel B summarizes the metabolism of ^{3}H-labelled 5α-H-Org 4060 in uterus and vagina tissue. Bars indicate average ± SEM.*

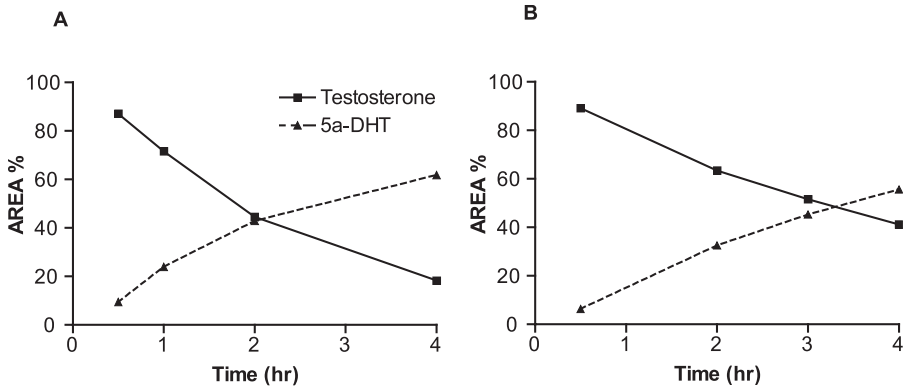

Figure 5: *Metabolism of testosterone in HEK293 cells transfected with human 5α-reductase type 1 (panel A) and type 2 (panel B). Aliquots of medium were analyzed by HPLC (system I) after 0.5, 1.0, 2.0 and 4.0 hours of incubation. HEK293 cells transfected with both human 5α-reductase isoforms were able to metabolize testosterone into dihydrotestosterone (5α-DHT). Mock-transfected cells did not convert testosterone into 5α-DHT (data not shown).*

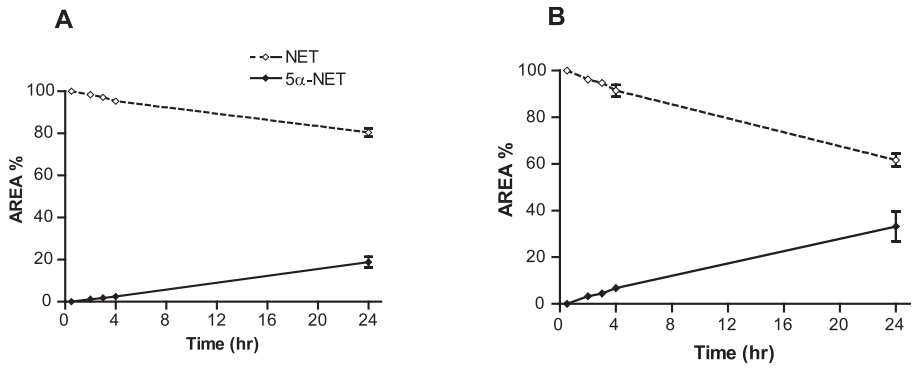

Figure 6*: Metabolism of norethisterone (NET) in HEK293 cells transfected with human 5α-reductase type 1 (panel A) and type 2 (panel 2). The cells were transfected and 48 hours later, they were incubated with ^{3}H-labelled norethisterone (50 nM) in serum free medium. Aliquots of medium were analyzed by HPLC (system I) after 0.5, 2.0, 4.0 and 24 hours of incubation. HEK293 cells transfected with both human 5α-reductase isoforms were able to metabolize norethisterone into 5α-H-norethisterone (5α-NET). Org OM38, Org 4060 and Org 34694 were not metabolized in HEK293 cell transfected with human 5α-reductase type 1 or type 2 (data not shown).*

Discussion

The present study shows that 5α-reduction is the major metabolic pathway of norethisterone in vaginal tissue of postmenopausal women. All metabolites were 5α-reduced, followed by 3α- and 3β-reduction of the 3-keto group. Also progesterone was metabolized by the same reductive activities. Approximately equal amounts 5α-H-norethisterone (17.7 ± 1.0%), 3β-OH, 5α-H-norethisterone (15.9 ± 2.0%) and of 3α-OH, 5α-H-norethisterone (15.3 ±1.7%) were formed. This demonstrates the presence of 5α-reductase, 3α-hydroxysteroid dehydrogenase and 3β-hydroxysteroid dehydrogenase activity in vaginal tissue of postmenopausal women. Tetrahydro metabolites have also been described as major metabolites in plasma after oral administration of norethisterone (for a review see (Stanczyk and Roy, 1990). The major plasma metabolites were 3α-OH, 5β-H-norethisterone and 3α-OH, 5α-H-norethisterone (Braselton et al., 1977; Pommier et al., 1995). However, norethisterone shows good adsorption and bioavailability (Orme et al., 1983) and circulating norethisterone may well be a source for local metabolism in vaginal tissue. Especially, the formation of 3β-OH, 5α-H-norethisterone that is reported here is of interest, since this metabolite is known to have estrogenic properties (MendozaRodriguez et al., 1999). It remains to be elucidated to what extent this local metabolism plays a role in combined HRT using norethisterone and an estrogen.

In the uterus of postmenopausal women, there was no metabolism of norethisterone and the metabolism of progesterone in the uterus was markedly reduced compared to the vagina. However, the presence in the human uterus of

both type 1 and type 2 isoforms of 5α-reductase has been demonstrated previously, using immunocytochemistry (Aumuller et al., 1996) (Eicheler et al., 1994). The uterine surface epithelium and stromal cells stained strongly with the human 5α-reductase type 1 isoform, whereas there was a less strong staining with the 5α-reductase type 2 isoform. It seems therefore likely that there is a decreased expression of 5α-reductase in the uterus of postmenopausal women. The finding that uterine tissue of postmenopausal women was almost as active as vaginal tissue in metabolizing 5α-H-Org 4060 to 3β-OH, 5α-H-Org 4060 and 3α-OH, 5α-H-Org 4060 demonstrates that this could not be due to an overall decline of steroid metabolism in the uterus. It also demonstrates that 5α-reduction of norethisterone is required for subsequent 3α- or 3β-reduction of the 3-keto group.

HEK293 cells expressing type 1 and type 2 human 5α-reductase were both capable of metabolizing norethisterone into 5α-H-norethisterone. In contrast, none of the norethisterone derivatives Org OM38, Org 4060 or Org 34694 was 5α-reduced by any of the two 5α-reductase isoforms expressed in HEK293 cells. Similarly, neither Org OM38, Org 4060 nor Org 34694 was metabolized in uterus or vagina tissue incubations. It is concluded that the hindrance of substituents at the 7α- and the 11-position of the norethisterone steroid skeleton render Org OM38, Org 4060 and Org 34694 poor substrates for human 5α-reductase isoenzymes. The effect of a 7α-methyl substituent was documented previously with 7α-methyl-19-nortestosterone. Whereas 19-nortestosterone is 5α-reduced by rat liver, prostrate and epididymis homogenates, 7α-methyl-19-nortestosterone is not (Agarwal and Monder, 1988). The finding that Org 4060, with an 11β-ethyl group, also resisted 5α-reduction is rather surprising. The related steroids Org 30659 [(17α)-17-hydroxy-11-methylene-19-norpregna-4,15-dien-20-yn-3-one] and desogestrel, both with an 11-methylene substituent, are readily 5α-reduced in rat microsome incubations (Verhoeven et al., 1998a; Verhoeven et al., 1998b). It is not clear whether the resistance of Org 34694 to 5α-reduction is due to its 11-ethylidene or to its 7α-methyl substituent, since the 7α-methyl group alone (Org OM 38) is already sufficient to prevent 5α-reduction in our incubations.

Overall, It was demonstrated that norethisterone is metabolized into estrogenic, tetrahydro metabolites in vaginal tissue of postmenopausal women, but not in the uterus. This seems to be caused by the lack of 5α-reductase activity in the uterus. Norethisterone was 5α-reduced by both type 1 and type 2 human 5α-reductase, but a 7α-methyl (Org OM38) or a 11β-ethyl substituent (Org 4060) render norethisterone resistant to 5α-reduction in vagina or uterus tissue or in incubations with HEK293 cells transiently expressing human 5α-reductase isoenzymes. It remains to be elucidated what role the metabolism of norethisterone in vagina tissue of postmenopausal women plays in the effects on this organ in combined HRT with norethisterone and an estrogen.

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CHAPTER 5

The metabolism of Org OD14 and its derivatives in rat and human target tissues for Hormone Replacement Therapy.

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in preparation

Abstract

Uterine and vaginal tissue from postmenopausal women and uterine, vaginal and aortic tissue from ovariectomized rats were incubated with radiolabeled Org OD14. The radioactive metabolites were analyzed by HPLC and HPTLC. Org OD14 (50 nM) was mainly 3α-reduced to Org 4094, in rat uterus (75 ± 3%) and vagina (67 ± 1%). In rat aorta, Org OD14 was metabolized to mainly Org OM38, its Δ4 isomer (27 ± 1%), Org 4094 (24 ± 2%) and an unidentified compound (27 ± 1%). In contrast, Org OM38 and Org 30126 (3β-reduced Org OD14) were the major metabolites of Org OD14 in uterine and vaginal tissue of postmenopausal women. In incubations at higher Org OD14 concentrations, the conversion of Org OD14 to Org OM38 was predominant over the conversion of Org OD14 to Org 30126. This was explained by a 6 fold higher apparent Km value and a 10 fold higher Vmax value for the conversion of Org OD14 to Org OM38 as compared to the conversion of Org OD14 to Org 30126. The 3β-HSD inhibitor epostane had no effect on Org OD14 metabolism in human uterine and vaginal tissue microsomes. Furthermore, HEK293 cells expressing the human 3β-HSD type 1 and 2 isoforms did not metabolize Org OD14. Moreover, the 3β-reduction of Org OD14 to Org 30126 was shown to NADPH dependent, while the isomerization of Org OD14 to Org OM38 did not require a cofactor. It therefore was concluded that the human 3β-HSD isoforms are not involved in the metabolism of Org OD14, and that the 3β-reduction and the Δ5-10 to Δ4 isomerization may be catalyzed by different enzymes.

Also Org 30126 was metabolized differently in rat and human tissues. Org 4094 was the major metabolite in rat uterus (50 ± 1%), vagina (50 ± 3%) and aorta (18 ± 9.1%). In both uterine and vaginal tissue from postmenopausal women, no metabolism of Org 30126 could be detected.

We propose different metabolic pathways for Org OD14 in HRT target tissues of rat and human. The rat is therefore a poor model to study the uterine and vaginal metabolism and pharmacology of Org OD14.

Introduction

Since estrogen receptors are widely distributed throughout the body, the effect of postmenopausal estrogen deficiency is pleiotropic. Increased bone resorption may lead to osteoporosis, there are profound vasomotor symptoms, unfavorable cardiovascular changes and vaginal atrophy. Estrogen replacement can effectively prevent osteoporosis and other symptoms (The Writing Group for the PEPI, 1996), that are related to the marked decline in plasma estradiol levels after menopause. Organs that are also positively affected by estrogen replacement are the vagina and the cardiovascular system (Bush, 2000). Estrogen replacement, however may lead to cell proliferation in the uterus and the breast. In particular in the uterus, unopposed estrogen replacement treatment leads to an increased risk of developing uterine cancers. Therefore, an ideal therapy would be one that acts as an estrogen on the

bone, the cardiovascular system and the urogenital system, but not on the uterus or the breast.

Org OD14, (7α,17α)-17-hydroxy-7-methyl-19-norpregn-5(10)-en-20-yn-3-one), is a synthetic steroid used in Hormone Replacement Therapy (HRT) and has estrogenic, progestagenic and androgenic properties in several bioassays (Vies van der, 1987; Markiewicz and Gurpide, 1990). In postmenopausal women, it causes an increase in bone mineral density, reduces vaginal atrophy but it has no estrogenic effect on the endometrium (for a review see (Moore, 1999)). Metabolism of Org OD14 to its Δ4-isomer Org OM38 (7α,17α)-17-hydroxy-7-methyl-19-norpregn-4-en-20-yn-3-one) by 3β-hydroxysteroid dehydrogenase / isomerase (3β-HSD) occurs in the human endometrium and is implicated to suppress estrogenic action in the endometrium through the progestagenic effect of Org OM38 (Tang et al., 1993). Therefore the mixed hormonal profile of Org OD14 is due to its metabolites. The *in vitro* binding of Org OD14 and its known metabolites to sex steroid receptors is listed in table 1.

TABLE 1

Relative binding affinity to the ER and PR for Org OD14 and its derivatives.

Steroid	**Relative Binding Affinity (%)**	
	ER (E2=100%)	**PR** (Org 2058= 100%)
Org OD14	<1	1
Org OM38 (Δ4-Org OD14)	<1	10
Org 30126 (3β-OH-Org OD14)	2	nb
Org 4094 (3α-OH-Org OD14)	2	nb

nb = no binding. Adapted from Markiewicz and Gurpide, 1990

These metabolites are Org OM38 with moderate PR binding and no ER binding, while 30126, (3β,7α,17α)-7-methyl-19-norpregn-5(10)-en-20-yn-3,17-diol, and Org 4094, (3α,7α,17α)-7-methyl-19-norpregn-5(10)-en-20-yn-3,17-diol, show only weak ER binding but no PR binding.

Org OD14 also acts as an estrogen agonist on bone in ovariectomized (OVX) rats (Ederveen and Kloosterboer, 1999). The mature OVX rat is considered a good model for studying early postmenopausal bone loss, and is a valuable tool to evaluate the effect on bone of agents for HRT. It is not clear whether the rat is also a good model to study the effect of Org OD14 on target tissues other than bone. Since at least the protective effect of Org OD14 on the human endometrium seems to be dependent on its metabolism to Org OM38, it is of importance to compare the metabolism of Org OD14 in target tissues of both rat and human origin.

Therefore the metabolism of Org OD14 was studied in rat uterus, vagina and aorta as well as in human uterus and vagina. Uterus and vagina biopsies were obtained from postmenopausal women following hysterectomy. Tissue fragments from the biopsies and from rat uterus, vagina and aorta were incubated with ^{3}H-

labeled Org OD14, Org 30126 and Org 4094 and their metabolites were analyzed using HPLC and HPTLC. To further elucidate the role of 3β-HSD in the metabolism of Org OD14 in the uterus and vagina of postmenopausal women, tissue homogenates, microsome fractions and HEK293 cells transiently expressing human 3β-HSD type 1 and type 2 were also studied.

Materials and methods

Chemicals

[6,7-^{3}H]-dehydroepiandrosterone (2200 GBq/mmol) was purchased from NEN Life Science products (Hoofddorp, The Netherlands). The radiolabeled steroids [16-$^{3}H_2$]-Org OD14 (1600 GBq/mmol), [16-$^{3}H_2$]-Org OM38 (1400 GBq/mmol), [16-$^{3}H_2$]-Org 30126 (1480 GBq/mmol) and [16-$^{3}H_2$]-Org 4094 (1400 GBq/mmol) and the unlabeled Org OD14, Org OM38, Org 30126, Org 4094 and epostane were a gift from Organon (Oss, The Netherlands). All other chemicals were obtained from local commercial sources and were of analytical grade.

Patients

Uterus and vagina biopsies were obtained from four postmenopausal women (age 55-88 years) following vaginal hysterectomy. The indication for surgery in all women was a uterine prolapse. The participants gave their written, informed consent and the study was approved by the local ethics committees of the participating study center.

Human tissue

The uterus and vaginal tissue was collected in ice-cold Leibowitz medium (L-15), buffered with HEPES at pH 7.4 and supplemented with 100 U/ml penicillin and 100μg/ml streptomycin. The uterus was opened longitudinally and sections were cut from the middle part of the uterus in such a way that each section included the luminal epithelial layer and an underlying endometrial layer of approximately 10 mm thickness. The vaginal tissue and the uterine fragments were minced and 50 mg of tissue minces were transferred to vials containing 2 ml of Leibowitz medium (L-15) and used for tissue fragment incubations. The remainder of the tissue was frozen in liquid nitrogen and stored at -80°C until homogenization or microsome preparation.

Rat tissue

Four mature female Wistar strain Hsd/Cpd:Wu rats (Harlan, The Netherlands), with a weight between 225- and 250 g were fed with standard pelleted diet (RMH-B; Hope Farms BV, Woerden, The Netherlands), and tap water *ad libitum*. The animals were subjected to a 14h-light/10h-dark daily cycle. The rats were ovariectomized two weeks before sacrifice. All animal procedures were approved by the Animal Ethics Committee.

The uterus, vagina and aorta were dissected immediately after sacrifice. The organs were minced and 50 mg was used for tissue fragment incubations.

Tissue fragment incubation

The tissue fragments were transferred to vials containing 2 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 the appropriate ^{3}H-labeled steroid dissolved in 50 µl propyleneglycol. The final concentration of ^{3}H-labeled steroids was 50 nmol/l. The uterus and vagina tissue of two patients was also incubated with increasing Org OD14 concentrations in the range of 50 nM to 50 µM. The fragments were incubated at 37°C in a shaking water bath. For rat tissue fragments, 200 µl of medium was removed from the incubation vial after 45, 90 and 180 minutes of incubation. For human tissue fragments, 200 µl of medium was removed from the incubation vial after 45, 90, 180 and 360 minutes of incubation. A control incubation in L-15 medium without tissue was performed for each radiolabeled steroid. After incubation, medium aliquots were immediately acidified by adding 10 µl 1M HCl and 100 µl was injected on the HPLC column after centrifugation (10000g, 5 min). The radioactivity in an aliquot of the medium was counted by LSC and the remainder was removed from the tissue fragments. The tissue was extracted three times with 1 ml ethanol and the combined ethanolic extracts were dried under a stream of nitrogen and reconstituted in 250 µl methanol / 1mM HCl (50/50). At the end of the incubation period, the medium contained 64 ± 4.5% , 58 ± 8.4% and 62 ± 8.0% of the total radioactivity for Org OD14, Org 30126 and Org 4094, respectively.

Preparation of tissue homogenates and microsomes

For the preparation of tissue homogenates, uterine or vaginal tissue was homogenized (250 mg tissue/ml) in 50 mM phosphate buffer (pH 7.4) / 0.1 mM EDTA containing 20 % glycerol using an Ultra Turrax. After pelleting of cell debris (800g, 5 min), the supernatant was frozen in liquid nitrogen and stored at -80°C until use. For the preparation of microsomes, human uterine and vaginal tissue was homogenized (250 mg tissue / ml) in KCl 154 mM / EDTA 0.1 mM. After pelleting of cell debris (800g, 5 min), the homogenate was centrifuged at 10.000g for 10 minutes. The supernatant was removed and centrifuged at 100.000g for one hour, and the pellet was resuspended in 1 ml 50 mM phosphate buffer (pH 7.4) / 0.1 mM EDTA containing 20% glycerol, frozen in liquid nitrogen and stored at -80°C.

HEK293 cell culture and transfection with human 3β-HSD type 1 and type 2

The human 3β-HSD isoenzymes type 1 and type 2 cDNAs subcloned into the pCMV (3β-HSD type 1) and pcDNA3.1 (3β-HSD type 2) expression vector were a kind gift from J.I. Mason. HEK293 cells were cultured as described previously (Blomenrohr et al., 1997). In a 10 cm dish, 10^{6} cells were plated and transiently

transfected 24 hours later with 10µg human 3β-HSD isoenzyme plasmid using the SuperFect transfection method (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The cells were washed with PBS, scraped from the dish and homogenized in 2 ml buffer (50 nM phosphate buffer, pH 7.4, 0.1 mM EDTA in 20% glycerol) using a Potter homogenizer. The cell homogenate was centrifuged (800g, 5 min) and the supernatant was frozen in liquid nitrogen and stored at –80°C until use.

Incubations of cell- and tissue homogenates and microsomes

Appropriate amounts of homogenate or microsome fraction were incubated with tritiated steroids in 50 mM phosphate buffer (pH 7.4) / 0.1 mM EDTA containing 20% glycerol at 37°C for 60 minutes at a final volume of 250 µl. The different steroid concentrations were generated using unlabeled steroid added to 100 pmol of ^{3}H-steroid. The reaction was started by adding the appropriate cofactor (final concentration 1 mM) and stopped with 10 µl 40% trichloroacetic acid. A control incubation in buffer without tissue was performed for each radiolabeled steroid. After addition of 200 µl of methanol, the incubation mixture was centrifuged at 10.000g (5 min) and 100 µl of the supernatant was injected on the HPLC column.

HPLC analysis of metabolite profiles

Separation of the steroids and their metabolites was performed using a Waters spherisorb S5 ODS-2 column (4.6 x 250 mm) (Phase Separations B.V, Emmen, The Netherlands) with a guard column (4.6 x 10 mm) and a gradient of water (solvent A) and methanol (solvent B). The analytes were eluted with 60% of solvent B for 5 minutes, followed by a linear gradient of 60-85% solvent B in 35 minutes. After 5 minutes of elution with 85% solvent B, initial values were re-established in 5 minutes. 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 254 nm , and a Flo-one model A200 on-line radioactivity detector (Canberra Packard, Groningen, The Netherlands).

HPTLC analysis:

A 200 µl aliquot of the incubation medium was extracted three times with dichloromethane. The combined extracts were dried under a stream of nitrogen and redissolved in 20 µl propanol. Together with appropriate standard steroids, the sample was applied to the sample concentration zone of a HPTLC plate (10x10 cm, Merck kieselgel 60 F254). The plates were first run in toluene-cyclohexane (50:50) for 10 minutes to concentrate the sample at the border of the concentration zone. After drying, the plates were developed in dichloromethane-diethylether (80:20) for 15 minutes at 4°C. The reference standards were visualized under UV light. Steroids with a Δ4-3-keto group were visible by exposure to 254 nm UV light, while

the other steroids were detected at 360 nm UV light, after derivatization with primulin (Wright, 1971). For detection of the radioactive compounds on the HPTLC silica plate, the plates were developed on X-ray film after spraying with a scintillation reagent (EN^{3}HANCE, Life Science products).

Results

Metabolism of Org OD14, Org 30126 and Org 4094 in rat uterus, vagina and aorta.

Org OD14

Org OD14 was rapidly metabolized in rat uterine and vaginal tissue fragments into mainly Org 4094, its 3α-reduced derivative. After 180 minutes of incubation, Org OD14 was almost completely metabolized, with Org 4094 representing 75.3 ± 2.6% and 67.1 ± 1.0% of the total radioactivity in uterus and vagina, respectively. Also minor amounts of Org OM38, Org 30126 and an unknown metabolite with a retention time of 11 minutes (U11) were formed. In aortic tissue fragments, Org OD14 was also extensively metabolized, and after 180 minutes of incubation Org OM38, Org 4094, U11 and Org 30126 represented 27.0 ± 0.8%, 23.5 ± 2.3%, 27.4 ± 1.2% and 7.8 ± 0.7% of the total radioactivity, respectively. Figure 1 shows representative HPLC chromatograms of rat uterine and rat aortic tissue incubations and figure 2 shows the time course for the conversion of Org OD14 in rat uterine, vaginal and aortic tissue incubations. Also in homogenates of rat uterus there was extensive metabolism of Org OD14 into Org 4094. This conversion was NADPH dependent but could not be inhibited by indomethacin up to 25 μM , a powerful 3α-HSD inhibitor (Figure 3a).

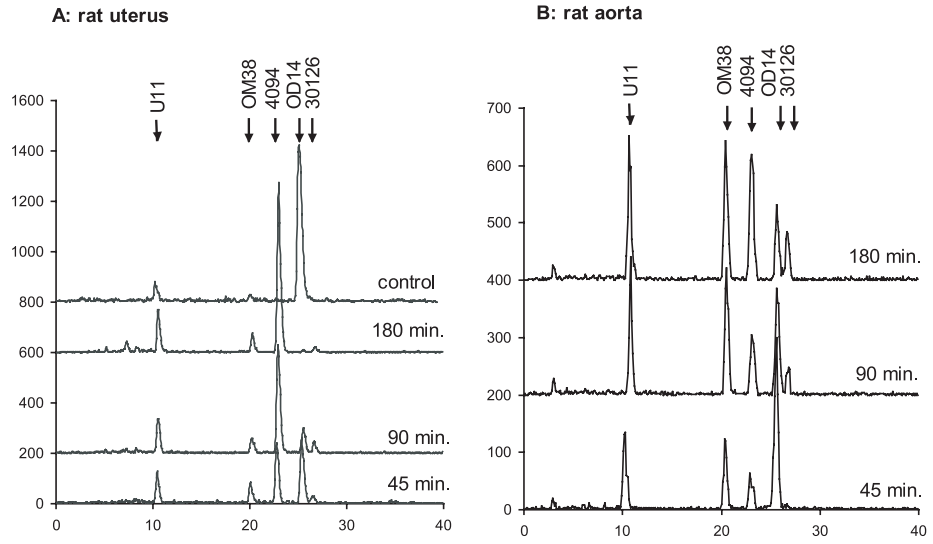

Figure 1: *Representative HPLC chromatograms from incubations of rat uterine (a) and rat aortic (b) tissue fragments. The tissue fragments were incubated with radiolabeled Org OD14 (50 nM) in L-15 medium. Aliquots of the incubation medium were analyzed after 45, 90 and 180 minutes of incubation. An incubation (180 min.) of Org OD14 in L-15 medium without tissue was used as a control. In uterine tissue, Org 4094 was the major metabolite of Org OD14 after 180 minutes, while in aortic tissue similar amount of U11, Org OM38 and Org 4094 could be identified.*

Org 30126

Org 30126 was extensively converted in rat uterus and vagina into Org 4094. After 180 minutes of incubation, Org 30126 and Org 4094 represented 49.8 ± 0.5% and 49.4 ± 3.4% of total radioactivity, respectively. In rat aorta, Org 30126 was also converted into Org 4094, although to a lesser extent (18.0 ± 9.1% of total radioactivity after 180 minutes of incubation). Figure 4 shows the time course for the conversion of Org 30126 in rat vagina and aorta incubations. In homogenates of rat uterus, the metabolism of Org 30126 into Org 4094 was shown to be a two step reaction. With NADP as cofactor, Org 30126 was metabolized to Org OD14. In the presence of both NADP and NADPH, there was further metabolism of Org 30126 to Org 4094. The 3β-HSD inhibitor epostane had no effect on the conversion of Org 30126 up to 25 μM, whereas indomethacin (10 μM) inhibited completely the metabolism of Org 30126 to Org OD14 or Org 4094 in rat uterus homogenates, as is shown in figure 3b.

Org 4094

Metabolism of Org 4094 in incubations of tissue fragments from rat uterus, vagina or aorta could not be detected.

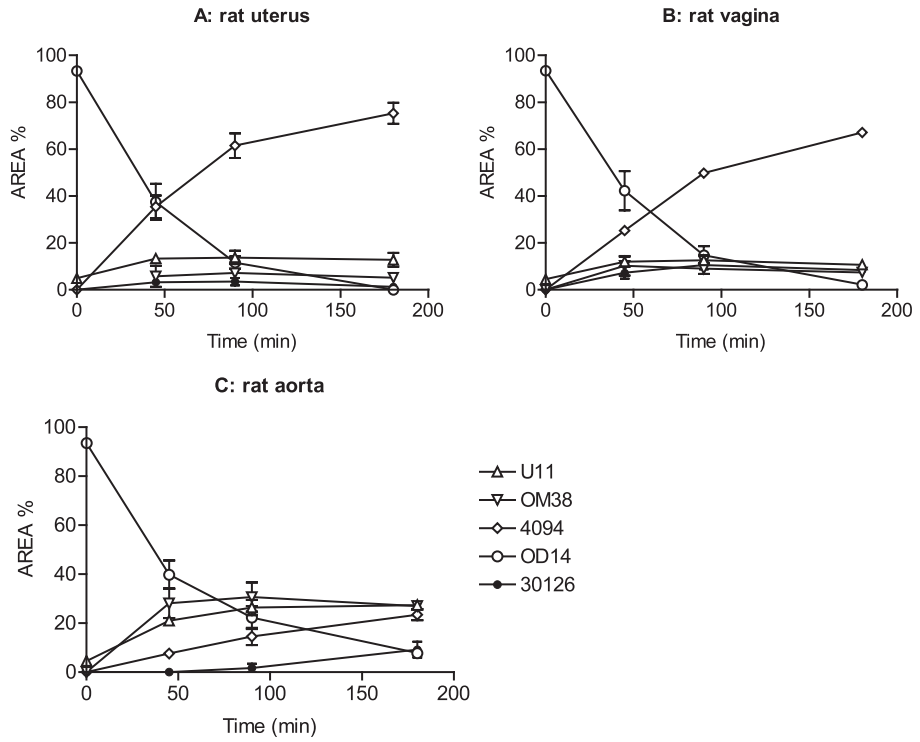

Figure 2: *Rat (n=4) uterine (a), vaginal (b) and aortic (c) tissue fragments were incubated with radiolabeled Org OD14 (50 nM) in L-15 medium. The metabolites were analyzed using HPLC after 45, 90 and 180 minutes of incubation. Mean area percentages ± SEM are presented of the radioactive peaks of U11, Org OM38, Org 4094, Org OD14 and Org 30126 at the respective time points.*

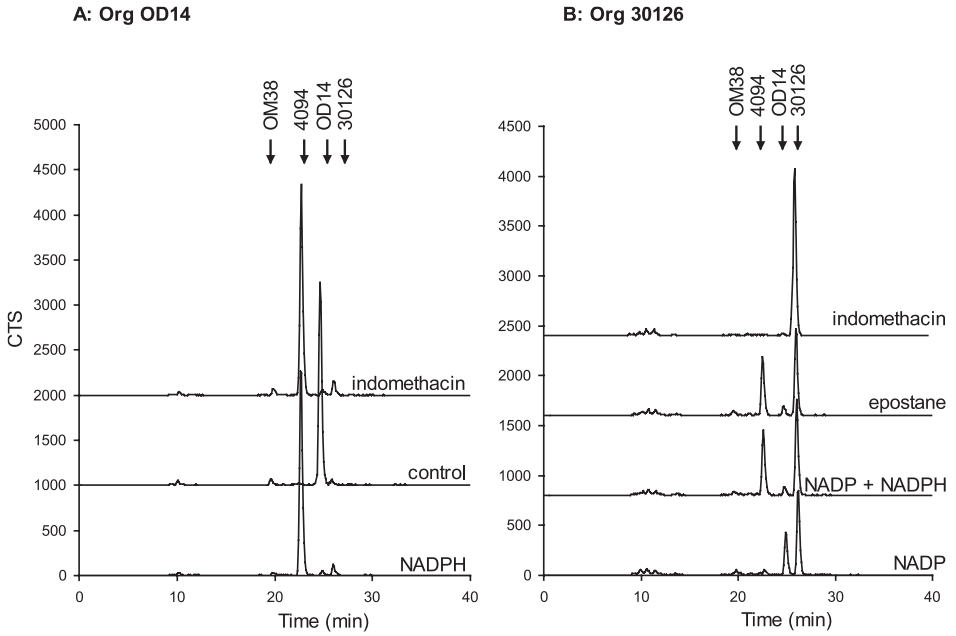

Figure 3: *Homogenates of rat uterus were incubated with radiolabeled Org OD14 or Org 30126 (50 nM) for 60 minutes in 50 mM phosphate buffer (pH7.4) / 0.1 mM EDTA containing 20% glycerol. HPLC chromatograms are shown for incubations of homogenates without cofactor (control) or with NADP and / or NADPH (1 mM) as indicated in the graphs. Furthermore, chromatograms are shown from incubations of Org OD14 or Org 30126 with the required cofactor(s) plus the typical 3β-HSD inhibitor epostane (25 μM) or indomethacin (10 μM), a typical 3α-HSD inhibitor.*

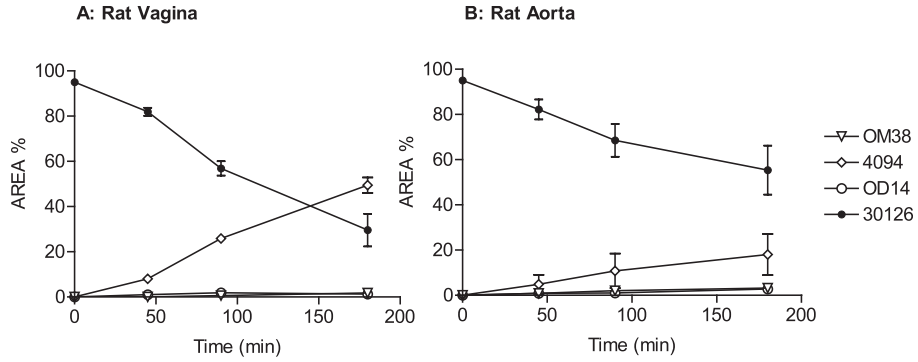

Figure 4: *Rat (n=4) vaginal (a) and aortic (b) tissue fragments were incubated with radiolabeled Org 30126 (50 nM) in L-15 medium. The metabolites were analyzed using HPLC after 45, 90 and 180 minutes of incubation. Mean area percentages ± SEM are presented of the radioactive peaks of Org OM38, Org 4094, Org OD14 and Org 30126 at the respective time points.*

Metabolism of Org OD14, Org 30126 and Org 4094 in uterus and vagina of postmenopausal women.

Org OD14

Org OD14 was extensively metabolized in uterine and vaginal tissue from postmenopausal women. The major metabolites of Org OD14 in both tissues were Org 30126, Org OM38 and an unknown metabolite with a retention time of 11 minutes (U11).

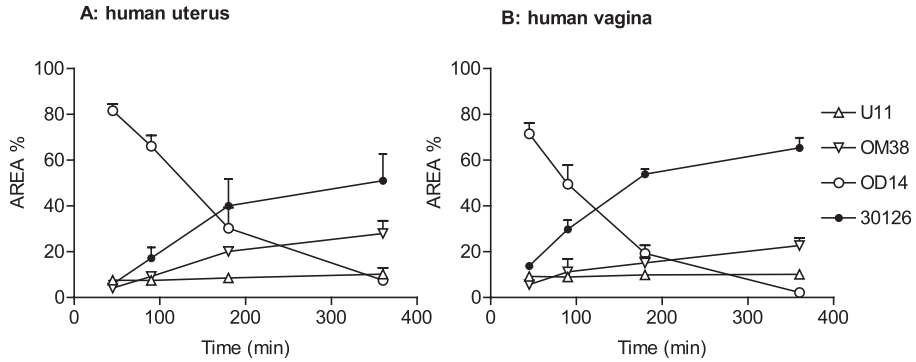

Figure 5: *Uterine (a) and vaginal (b) tissue fragments from postmenopausal women were incubated with radiolabeled Org OD14 (50 nM) in L-15 medium. The metabolites were analyzed using HPLC after 45, 90, 180 and 360 minutes of incubation. Mean area percentages ± SEM are presented of the radioactive peaks of Org OM38, Org 4094, Org OD14 and Org 30126 at the respective time points.*

Figure 5 shows the time course conversion of Org OD14 (50 nM) in uterine and vaginal tissue fragments of four postmenopausal women. With increasing substrate concentration of Org OD14 (50 nM - 50 µM) there was a shift in the ratio of the metabolites Org OM38 and Org 30126 in both tissues. At lower concentrations of Org OD14, Org 30126 was the predominant metabolite, whereas at higher concentrations of Org OD14, Org OM38 formation was predominant over Org 30126 formation (figure 6). Using homogenates of vaginal tissue, apparent Km values were estimated to be 179 ± 20.3 µM for the conversion of Org OD14 to Org OM38, and 28 ± 7.8 µM for the conversion of Org OD14 to Org 30126 (Figure 6). Both activities were also present in the microsome preparations of both tissues. NADPH, rather then NADH, was the preferred cofactor for the conversion of Org OD14 to Org 30126. The conversion of Org OD14 to Org OM38 appeared to be cofactor independent. The conversion of Org OD14 to either Org OM38 or Org 30126 could not be inhibited by epostane up to 25 µM (Figure 7).

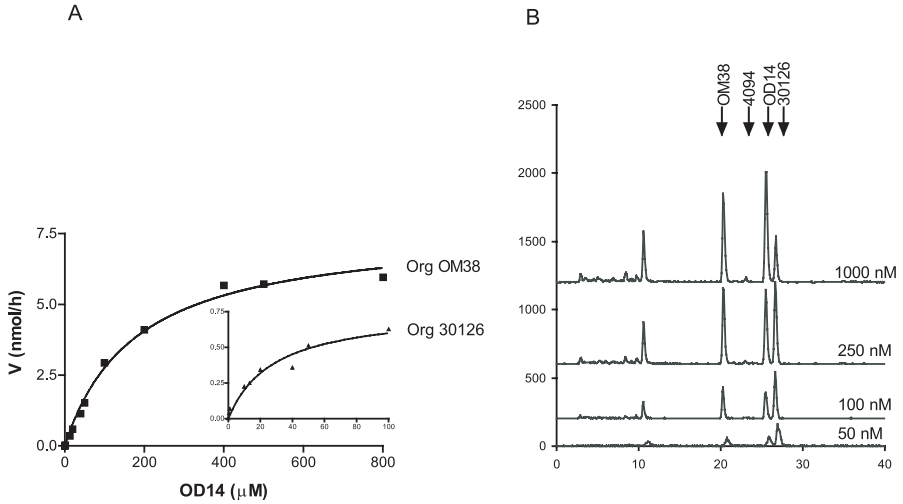

Figure 6: *homogenates of vaginal tissue from postmenopausal women (A) were prepared as described in the materials and methods section, and incubated with increasing concentrations of radiolabeled Org OD14 (0.1μM-100μM) for 60 minutes in 50 mM phosphate buffer (pH7.4) / 0.1 mM EDTA containing 20% glycerol. The initial velocity of the formation of Org OD14 and Org 30126 is plotted against the Org OD14 concentration and the data are fitted to the Michealis-Menten equation using a least-square analysis. The apparent Km values were estimated to be 179 ± 20.3 μM and 28 ± 7.8 μM for the formation of Org OM38 and Org 30126, respectively. Panel B shows representative HPLC chromatograms from incubations of vaginal tissue with increasing Org OD14 concentrations (50 – 1000 nM) as indicated in the graph. Note that relatively more Org OM38 compared to Org 830126 was formed with increasing substrate concentrations.*

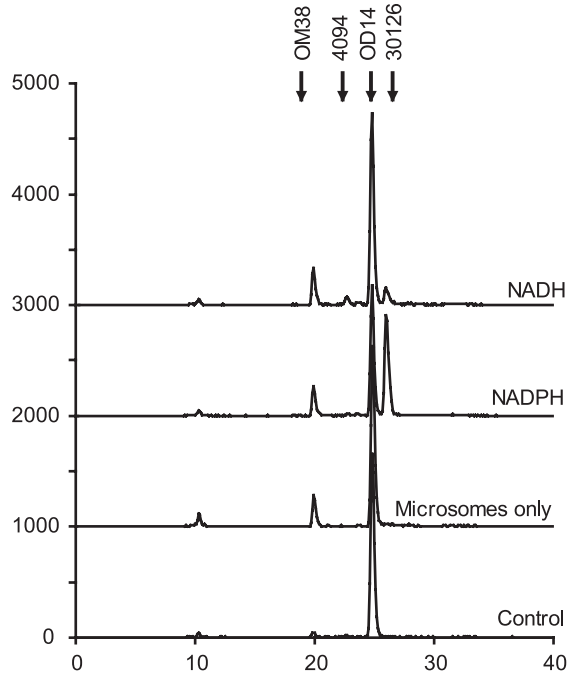

Figure 7:
Microsomes were prepared from vaginal tissue of postmenopausal women as described in the materials and methods section and were incubated with radiolabeled Org OD14 (50 nM) for 60 minutes in 50 mM phosphate buffer (pH7.4) / 0.1 mM EDTA containing 20% glycerol. HPLC chromatograms are shown for incubations of microsomes without cofactor (microsomes only) or with NADH or NADPH (1 mM) as indicated in the graphs. An incubation of Org OD14 in buffer without tissue was used as a control. The 3β-HSD inhibitor epostane (25 μM) showed no effect on microsomal 3β-HSD metabolism (not shown).

Org 30126 and Org 4094

In contrast to the rat, no metabolism of Org 30126 in fragments of vaginal and uterine tissue of postmenopausal women could be detected. In homogenates of both tissues, there was some conversion (< 2%) of Org 30126 to Org OD14 with NADP as the preferred cofactor. Org 4094 was metabolized to Org 30126, amounting to 2.9 ± 0.9% of total radioactivity in uterine tissue and to 6.9 ± 1.5% in vaginal tissue (Figure 8).

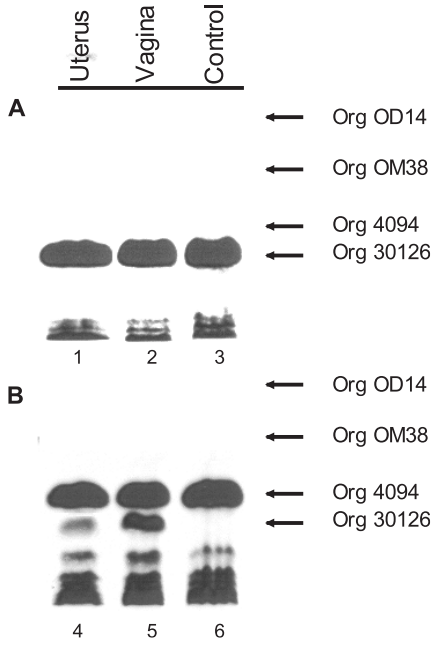

Figure 8:
Representative HPTLC chromatograms from incubations of uterine and vaginal tissue fragments of postmenopausal women. The tissue fragments were incubated with 50 nM radiolabeled Org 30126 (A) or Org 4094 (B) in L-15 medium. Aliquots of the incubation medium were analyzed after 180 minutes of incubation as described in the materials and methods section. An incubation of the radiolabeled steroids in L-15 medium without tissue was used as a control. No metabolites of Org 30126 could be detected, while there was minor conversion of Org 4094 to Org 30126 in uterus and vagina.

Metabolism of Org OD14, Org 30126 and Org 4094 in HEK293 cells transiently transfected with human 3β-HSD type 1 and type 2, respectively

HEK 293 cells were successfully transfected with human 3β-HSD type 1 and type 2, respectively. Homogenates of cells transfected with either human 3β-HSD types catalyzed the NAD dependent conversion of DHEA (1 μM) to androstenedione, whereas mock-transfected cells did not. The 3β-HSD reaction with DHEA in the homogenates could be blocked completely by epostane (500 nM). However, these homogenates did not metabolize Org OD14, Org 30126 or Org 4094. There was only a minor conversion of Org OD14 to Org 30126 using NADPH as a cofactor, but this activity was also present in mock-transfected cells.

Discussion

In this study we compared the conversion of Org OD14 in rat and human uterine and vaginal tissue, respectively. The metabolism of Org OD14 in rat uterus and vagina was shown to be very different from its metabolism in uterus and vagina of postmenopausal women. Although Org OD14 was extensively metabolized in uterus and vagina of both species, the conversion of Org OD14 was demonstrated to follow rather different metabolic routes (figure 9). In rat uterus and vagina, Org OD14 was converted to Org 4094, the 3α-reduced metabolite of Org OD14. In uterus and vagina of postmenopausal women, Org OD14 was converted to mainly Org OM38, the Δ4 metabolite of Org OD14, and to Org 30126, the 3β-reduced metabolite of Org OD14. Furthermore, an unidentified metabolite (U11) was formed. Since it is assumed that the unique protective effect of Org OD14 on the human endometrium is mediated by its local conversion to the progestagen Org OM38 (Tang et al., 1993; Moore, 1999), the finding that Org OD14 metabolism in rat uterus generates Org 4094 instead of Org OM38 has major implications for the use of the rat as a model to test the uterine pharmacology of Org OD14. It can be anticipated from the results presented in this paper that the effect of Org OD14 on the rat uterus will be largely estrogenic because of its rapid conversion to Org 4094. The metabolism of Org OD14 to Org OM38 has been demonstrated before in human uterus by Tang *et al*. (1993). The conversion of Org OD14 to Org 30126 by uterus and vagina of postmenopausal women, however, has never been observed before. In the present study two results may explain why Tang *et al.* did not report the conversion of Org OD14 to Org 30126. Firstly, we showed that at higher concentrations of Org OD14, such as were used by Tang *et al*., the conversion of Org OD14 into Org OM38 is dominating the conversion into Org 30126. The 6 fold higher apparent Km value and the 10 fold higher apparent Vmax value for the conversion of Org OD14 to Org OM38 in tissue homogenates reflected this. Secondly, the conversion of Org OD14 to Org 30126 was shown to be NADPH dependent, while no cofactors were used in the report of Tang *et al*.

The species specific preference for the 3α- or 3β-reduction of Org OD14 has also been demonstrated in hepatocyte incubations of rat and human origin (Sandker et al., 1994). The major metabolites in rat hepatocytes were all 3α-reduced forms of Org OD14, whereas in human hepatocytes there was also formation of 3β-reduced metabolites of Org OD14.

There was also a striking difference between uterine and vaginal tissue from rats and postmenopausal women, respectively, regarding the metabolism of Org 30126. While Org 30126 was extensively converted to Org 4094 by rat uterine and vaginal tissue, no conversion of Org 30126 could be detected in these tissues of postmenopausal women.

Using homogenates of rat uterus, it was demonstrated that there is a two step conversion of Org 30126 to Org 4094. With NADP as cofactor, Org 30126 was converted to Org OD14. Adding both NADP and NADPH to rat uterus homogenates resulted in the conversion of Org 30126 to Org 4094.

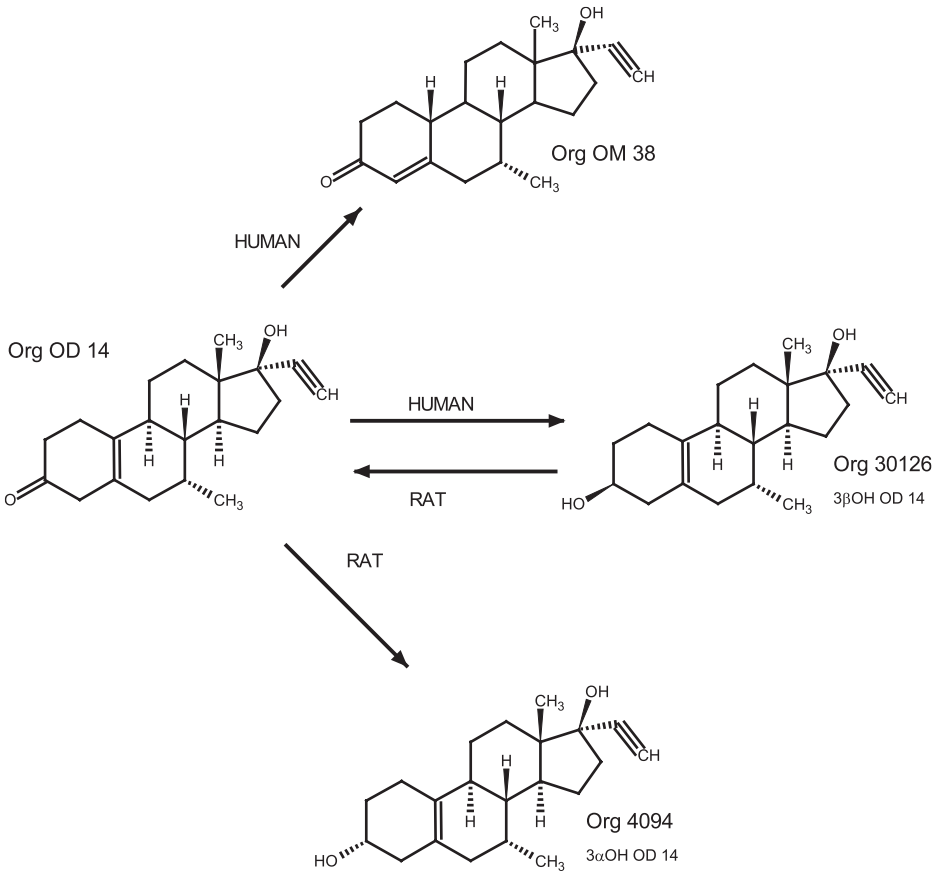

Figure 9: *Preferred metabolic pathways of Org OD14 and its derivatives in rat and human.*

The Org 30126 to Org OD14 conversion could not be inhibited by epostane, a powerful 3β-HSD inhibitor (Lopez, 1989; Takahashi et al., 1990). Likewise, indomethacin, a classical 3α-HSD inhibitor, could not inhibit the Org OD14 to Org 4094 conversion. Surprisingly, indomethacin was able to completely block the conversion of Org 30126 in homogenates of rat uterus. These data suggest that in the rat uterus and vagina the conversion of Org 30126 to Org 4094 proceed with Org OD14 as an intermediate. The reaction seems however not to be catalyzed by sequential oxidative and reductive of 3β-HSD and 3α-HSD enzymes, since typical inhibitors had no effect on these reactions. Rather there may be a (3β→3α)-hydroxysteroid epimerase present in rat uterus and vagina, as was suggested for the epimerization of 3β-OH-norethynodrel to 3α-OH-norethynodrel in rat liver (Freudenthal et al., 1971b). This reaction was localized in the 100.000g supernatant of rat liver homogenates and was found to be species specific (Freudenthal et al.,

1971a). Maybe a similar enzyme is also present in rat uterus and vagina and acts upon Org OD14, the 7α-methyl derivative of norethynodrel. We speculate that this putative enzyme might be a member of the aldo-keto reductase (AKR) family, in view of its inhibition by indomethacin.

No metabolism of Org 4094 could be demonstrated in rat uterus, vagina or aorta. In uterus and vagina of postmenopausal women, there was minor (2.9% and 6.9%, respectively) metabolism of Org 4094 to Org 30126. Maybe this conversion is catalyzed by recently characterized human (3α→3β) hydroxysteroid epimerase which was shown to be expressed in the human uterus (Huang and Luu-The, 2000).

The incubations with rat aorta tissue demonstrated that Org OD14 is also extensively metabolized in a non-reproductive tissue. Org OD14 was metabolized in rat aorta into approximately equal amounts of Org OM38, Org 4094 and the unidentified metabolite U11. Also Org 30126 could be identified as a metabolite of Org OD14 in this tissue.

The nature of the enzyme(s) involved in the metabolism of Org OD14 to Org OM38 and Org 30126 in uterus and vagina of postmenopausal women was investigated using tissue homogenates and microsomes. To our surprise, the conversion of Org OD14 to Org OM38 was independent of any β-nicotinamide adenine cofactor. There are several indications that this conversion is however an enzyme catalyzed reaction. Firstly, there was a substrate saturable activity for the conversion of Org OD14 to Org OM38, which displayed Michealis-Menten kinetics. Secondly, the activity was also present in microsome preparations from uterine and vaginal tissue. We could however not support the finding of Tang *et al.* that the conversion of Org OD14 to Org OM38 in human uterus is catalyzed by a 3β-hydroxysteroid dehydrogenase / isomerase. HEK293 cells transfected with human 3β-HSD type 1 or type 2 were unable to convert Org OD14 to OM38, although these cells showed a considerable 3β-HSD activity with DHEA as substrate.

The reductive 3-ketosteroid oxidoreductase activity that converts Org OD14 to Org 30126 was shown to be NADPH rather than NADH dependent and could not be inhibited by epostane. Furthermore, the HEK293 cells transfected with human 3β-HSD type 1 and type 2 did not convert Org OD14 to Org 30126. Moreover, no conversion of DHEA to androstenedione was detected in homogenates of uterus or vagina from postmenopausal women (data not shown). Summarizing these data, it indicates that the 3-keto reduction of Org OD14 is not catalyzed by human 3β-HSD type 1 or 2. There may thus be an as of yet uncharacterized human 3β-HSD isoform present in uterus and vagina similar to *e.g.* rat 3β-HSD type 3 (Launoit de et al., 1992) or the murine 3β-HSD type 4 (Clarke et al., 1993) , which act primarily as 3-ketosteroid reductases with NADPH as their cofactor.

In summary, important differences between OVX rat and postmenopausal women in the metabolism of Org OD14 and its derivatives in uterus and vagina are presented in this paper. Org OD14 was rapidly metabolized to the weak estrogen Org 4094 in rat uterus, while in the uterus of postmenopausal women Org OD14

was metabolized to both the weak estrogen and the progestagen Org OM38. This discrepancy renders the rat a poor model to study uterine effects of Org OD14. The finding that the formation of Org OM38 in the postmenopausal uterus dominates over the formation of Org 30126 at higher Org OD14 concentrations indicates that a better absorption of Org OD14 may result in a more significant progestagenic effect on the uterus. The estrogen / progestagen balance in the uterus in HRT with Org OD14 will however ultimately be determined by the tissue concentrations of Org OM38, Org 30126 and Org 4094. Whether the accumulation of these metabolites is a result of local metabolism or results from disposition of plasma metabolites of Org OD14 remains to be elucidated.

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CHAPTER 6

Summarizing discussion and perspectives

Overview

The response of different tissues to steroid treatment is determined by a series of factors that include the availability for the tissue of active steroid, the composition of the steroid receptors in the tissue (type and concentration of receptor) and the presence of steroid receptor coregulatory proteins. In this thesis we concentrated on an important factor that determines the availability of active steroid: the steroid metabolism in the tissue. Such steroid metabolism may generate a metabolite in the tissue that is less active than the mother-compound and thereby change the level of active steroid in the tissue. Alternatively, a metabolite may be generated that binds more efficiently to a different steroid receptor, thus changing the nature of the effect of a steroid in the tissue.

This phenomenon is of particular interest in Hormone Replacement Therapy (HRT). A key feature of HRT for postmenopausal women is the tissue specific action of the applied hormonal active compounds. The primary target of HRT is the skeleton, where an estrogen agonistic activity is required to prevent bone loss. On the other hand, the endometrium and the breast need to be protected from this estrogen agonistic activity. Therefore, progestagens are included in HRT to suppress the unfavorable effects of the estrogen on the endometrium and the breast. The metabolism of both estrogens and progestagens in such target tissues may influence the desired estrogen / progestagen balance in those tissues.

The ovariectomized (ovx) rat is frequently used as a model to study the pathogenesis and treatment of postmenopausal bone loss and to evaluate the effectiveness of new compounds for HRT. It is less clear whether the ovx rat is also a good model to investigate target tissues other than the bone. Species differences in metabolism in target tissues of compounds for HRT may well play a role herein.

To evaluate these differences, we compared the metabolism of several estrogens and progestagens in rat and human target tissues of HRT. These steroids are the estrogens estradiol, ethynylestradiol and moxestrol, and selected 19-norprogestagens derived from norethisterone (NET). Furthermore the metabolism of Org OD14, a steroid with a mixed estrogenic, androgenic and progestagenic profile was studied (for structures of the steroids, see chapter 1). First, we investigated whether these steroids can be metabolized in the uterus, vagina and aorta of the rat. Next, we compared the steroid metabolism of the rat with that in the uterus and vagina of postmenopausal women.

The estrogens were selected as derivatives of estradiol with substitutions at the 17 position (ethynylestradiol; 17α-ethynyl-estradiol) or both the 11 and 17 position (moxestrol; 11β-methoxy,17α-ethynyl-estradiol) in the steroid skeleton. Similarly, the norethisterone derivatives were selected with substitutions at the 7 (Org OM38; 7α-methyl-NET), 11 (Org 4060; 11β-ethyl-NET) or both the 7 and 11 position (Org 34694; 7α-methyl, 11-ethylydene-NET). In this way the effect on the estradiol and norethisterone metabolism in HRT target tissues of these substitutions of could be established.

Estrogen metabolism in HRT target tissues: comparing the rat and the human

In Chapter 2, the metabolism of estradiol, ethynylestradiol and moxestrol in the uterus, vagina and the aorta of the ovariectomized rat was investigated. These data were compared to uterine metabolism of estradiol in the human as presented in the literature.

Estradiol metabolism

An overview of the uterine and vaginal metabolism of estradiol in both species and its regulation by ovarian hormones is presented in figure 1.

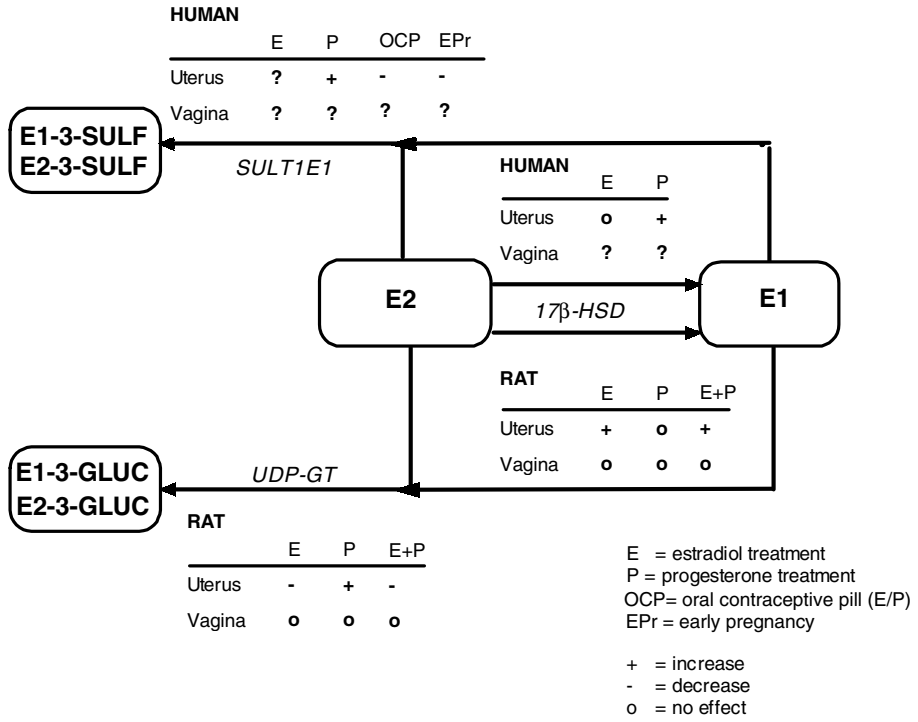

Figure 1: *an overview of uterine and vaginal estradiol (E2) metabolism in the rat and the human. The hormonal regulation of the different steps in the estradiol metabolism is indicated in the tables.*

In the uterus and vagina of the rat, estradiol was oxidized to estrone by 17β-hydroxysteroid dehydrogenase (17β-HSD). Furthermore, estradiol and estrone were glucuronidated at the 3 position. No formation of estradiol or estrone sulfates was detected. This is in contrast to estradiol metabolism in the human uterus, where 3-sulfation is the preferred conjugation of estradiol and estrone (Clarke et al., 1982). Another striking difference between the uterine estradiol metabolism of the rat and the human is its regulation by ovarian hormones. In the human uterus,

estradiol is metabolized by 17β-HSD and estrogen sulfotransferase (Liu and Tseng, 1979b). 17β-HSDs are NAD(H)- and / or NADP(H)-dependent enzymes that catalyze the oxidation and reduction of 17β-hydroxy and 17-ketosteroids, respectively. The oxidative type 2 17β-HSD is the predominant isoform in the human uterus (Peltoketo et al., 1999). A single human estrogen sulfotransferase enzyme is known (SULT1E1), shown to have a high affinity for estradiol (Falany et al., 1995). Both, the human 17β-HSD and the sulfotransferase activity are induced by progestagens (Tseng and Liu, 1981; Tseng, 1978; Clarke et al., 1982), and their activity is maximal during the secretory phase of the menstrual cycle (Tseng and Gurpide, 1975). The progestagen-induced inactivation of estradiol by 17β-HSD and estrogen sulfotransferase was suggested to facilitate the transition from the proliferative to the secretory endometrium after ovulation. In the rat, 17β-HSD activity is not induced by progesterone. However, as we demonstrated in ovx rats, the uterine metabolism of estradiol is induced by estradiol treatment. Furthermore, the glucuronidation of estradiol is progesterone-induced and estradiol-suppressed (Chapter 2 of this thesis). Moreover, in the same study we showed that estradiol and progesterone control the estradiol metabolism in a tissue specific manner. The activity of 17β-HSD and UDP-GT in the rat uterus, but not in the vagina, is affected by *in vivo* hormonal treatment.

Ethynylestradiol and moxestrol metabolism

An overview of the uterine and vaginal metabolism of ethynylestradiol in both species and its regulation by ovarian hormones is presented in figure 2.

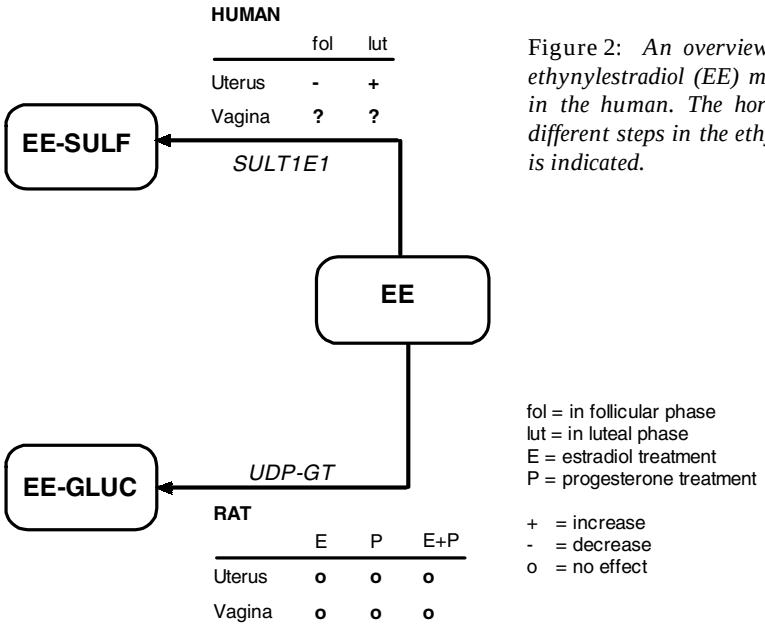

Figure 2: *An overview of uterine and vaginal ethynylestradiol (EE) metabolism in the rat and in the human. The hormonal regulation of the different steps in the ethynylestradiol metabolism is indicated.*

Similar to estradiol, ethynylestradiol was preferably glucuronidated rather than sulfated in the rat uterus and vagina. In contrast to the glucuronidation of estradiol, there was no effect of estradiol or progesterone treatment on the uterine glucuronidation of ethynylestradiol, suggesting that different UDP-GT isoenzymes are involved. In contrast, in the human, a single SULT1E1 enzyme mediates the conjugation of ethynylestradiol and estradiol in the human uterus and the sulfation activity for both substrates varies in parallel during the menstrual cycle (Rubin et al., 1999). Moreover, estradiol and ethynylestradiol were sulfated by expressed human estrogen sulfotransferase with similar maximal velocities at substrate concentrations of around 20 nM (Falany et al., 1995)

There was no metabolism of moxestrol in any of the rat tissues that were investigated. This indicates that the 11β-methoxy substituent renders 17α-ethynylated estrogens into poor substrates for glucuronidation in the rat.

Estrogen inactivation in target tissues: oxidation or conjugation?

Both, oxidation by 17β-HSD and conjugation of estradiol constitute a local inactivation of estradiol. The product of oxidative 17β-HSD activity, estrone, is considered to be a weaker estrogen, with an affinity for the estrogen receptor α of 60% compared to estradiol, and 37% for the estrogen receptor β (Kuiper et al., 1997). Estradiol-3-glucuronide and estradiol-3-sulfate do not show a significant estrogen receptor binding. Recently, it was shown that there is a coordinated action of estrogen sulfotransferase, glutathion and an organic anion transporter (multi drug resistant protein 1; MRP1) to generate an efflux of estrone- and estradiol-3-sulfates from intact cells (Qian et al., 2001). In view of the ubiquitous tissue distribution of human MRP1 (van Aubel et al., 2000), this mechanism may provide estrogen sensitive cells with the means of active transport of the inactivated estrogen. But which of the two metabolic pathways is the more important for estrogen inactivation?

In isolated human endometrial glands it was established that 17β-HSD activity is dominant over sulfation at higher (> 0.1 μM) estradiol concentrations (Liu and Tseng, 1979a). A similar effect was shown in the human *Ishikawa* endometrial cancer cell that possesses both 17β-HSD and estrogen sulfotransferase activity. In these cells, estradiol-3-sulfate was the major metabolite at lower estradiol concentrations (<10nM), while at higher estradiol concentrations (>0.1μM), estrone is the major metabolite (Hata et al., 1987). This phenomenon is a reflection of the Km values, which were reported to be 0.4 μM and 5 nM for the conversion of estradiol by 17β-HSD and the human estrogen sulfotransferase, respectively. The conversion of estradiol to estrone by 17β-HSD also shows a higher Vmax value than the conjugation by estrogen sulfotransferase. It appears therefore that estrogen-3-sulfation is dominant at estradiol concentrations that correspond to the normal physiological range and the range for combined HRT. At higher estradiol concentrations, the inactivation to estrone by 17β-HSD is more important.

The effectiveness of human estrogen sulfotransferase to decrease the response to estradiol has been demonstrated *in vitro.* Human *Ishikawa* endometrial adenocarcinoma cells were stably transfected with SULT1E1. This resulted in an 8-fold induced estrogen sulfotransferase activity compared to mock-transfected cells. However, the SULT1E1 transfected cells were 200-fold less sensitive for the induction of alkaline phosphatase by estradiol or ethynylestradiol than mock-transfected *Ishikawa* cells. Moreover, no such difference was observed for the diethylstilbestrol (DES) induced alkaline phosphatase activity in these cells (Kotov et al., 1999). Similarly, human breast cancer MCF-7 cells, transfected with estrogen sulfotransferase, were less sensitive to the growth-stimulating properties of estradiol than control cells that do not express estrogen sulfotransferase (Falany and Falany, 1997).

The most significant difference between rat and human may therefore be the difference in regulation of ethynylestradiol conjugation by progesterone. Sulfoconjugation of ethynylestradiol in the human uterus is induced by progesterone, whereas there is no induction of ethynylestradiol glucuronidation in the uterus of the rat. Combined treatment with ethynylestradiol and a progestagen will result in increased estrogen inactivation in the human uterus, but not in the rat uterus.

Metabolism of the progestagenic substance norethisterone and three norethisterone derivatives in HRT target tissues: comparing the rat and the human.

The metabolism of the progestagenic compound norethisterone and three norethisterone derivatives in HRT target tissues is described in the chapters 3 and 4. In order to investigate which progestagen metabolizing activities are present in the target tissues, the metabolism of progesterone was studied. Next, the metabolism of norethisterone and its derivatives was studied. The norethisterone metabolites have a 7α-methyl (Org OM38), an 11β-ethyl (Org 4060) or both, a 7α-methyl and an 11-etlylydene substituent (Org 34694).

Progesterone metabolism

Our strategy was to study the metabolism of progesterone first, to have an indication as to which progesterone-metabolizing enzymes are present in the tissues. Table 1 presents an overview of the progesterone metabolism in uterus and vagina tissue from the rat and postmenopausal women. The progesterone metabolites that were identified as described in Chapter 3 and 4, demonstrate the presence of steroid 5α-reductase (5αR), 20α-hydroxysteroid dehydrogenase (20α-HSD), 3α-hydroxysteroid dehydrogenase (3α-HSD) and 3β-hydroxysteroid dehydrogenase (3β-HSD) activity. They also demonstrate the central role of 5α-reductase. In the uterus and vagina of both species, almost all metabolites were 5α-reduced. It is also apparent from these data that there is a strong preference for 3α-

reduction in the rat, while in the human both 3α- and 3β-reduced metabolites are formed. It is also clear that in the uterus of postmenopausal women progesterone is metabolized to a lesser extent than in the vagina.

TABLE 1

Progesterone metabolites (percentage of total radioactivity) in uterus and vagina from rat and human

metabolite	**Human**		**Rat**	
	Uterus	Vagina	Uterus	Vagina
Unknown (sum)	1	29	4	13
Progesterone	81	24	#	#
4-pregnen-20α-ol-3-one	5	3	5	1
5α-pregnan, 20α-ol-3-one	<1	7	nd	<1
5α-pregnan-3α,20α-diol	nd	<1	37	27
5α-pregnan-3β,20α-diol	<1	1	nd	nd
5α-pregnan-3,20-dione	3	17	11	10
5α-pregnan-3α-ol-20-one	<1	4	38	40
5α-pregnan-3β-ol-20-one	2	8	nd	nd

nd= not detected # = not enough resolution on HPLC to quantify

Data adapted from chapter 3 and 4.

Norethisterone metabolism

The enzyme activities demonstrated with progesterone could give a prediction for the metabolism of norethisterone and its derivatives in rat and human target tissues, and the formation of 5α-, 5α,3α-, and 5α,3β-reduced metabolites of these steroids was likely. For the metabolism of norethisterone, this prediction proved to be a reliable one. Figure 3 schematically presents the metabolism of norethisterone in human and rat vagina as is described in Chapter 3 and 4. In summary, norethisterone was extensively 5α-reduced in uterus and vagina of the rat and in the postmenopausal vagina, but much less so in the postmenopausal uterus. In rat uterus and vagina, 3α-OH, 5α-H-NET (27 and 37%) and 5α-H-NET (4 and 14%) were the most abundant metabolites, whereas similar amounts of 5α-H-NET (18%), 3α-OH,5α-H-NET (15%) and 3β-OH,5α-H-NET (16%) were formed in the postmenopausal vagina. There was hardly any norethisterone metabolism in the postmenopausal uterus.

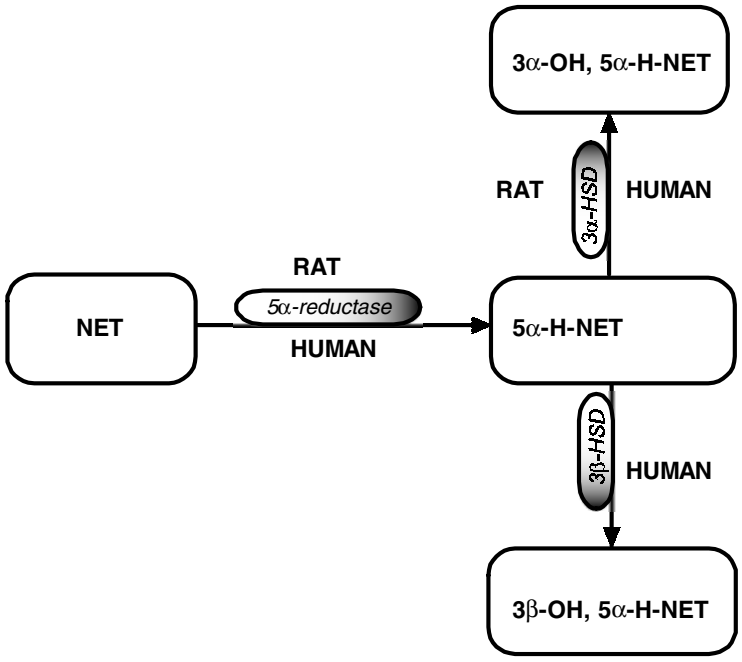

Figure 3: *An overview of the metabolism of norethisterone (NET) in target tissues of the rat and postmenopausal women*

Org OM38, Org 4060 and Org 34694

Substitutions on the 7-position as in OM38, or the 11-position as in Org 4060 ,or at both the 7- and the 11-position as in Org 34694 dramatically reduced the conversion of these compounds in the rat and metabolism was even not detectable in the uterus and vagina of postmenopausal women. Using HPTLC to identify the radioactive metabolites that were collected after HPLC separation, the formation of 3α-reduced metabolites of Org OM38, Org 4060 and Org 34694 in rat uterus, vagina and aorta could be demonstrated. The 3α-reduced metabolites, however, constituted not more than 4-8% (Org OM38), 1-3% (Org 4060) and less than 1% (Org 34694) of the total radioactivity. Since it is known that 7α-methyl-19-nor-testosterone can not be 5α-reduced (Sundaram et al., 1995), we tried whether it was possible to pinpoint the reduced metabolism to the 5α-reductase enzyme. To this end the metabolism of 5α-H-Org 4060 was studied in the rat and postmenopausal women. In uterus, vagina and aorta of the rat, 5α-H-Org 4060 was readily metabolized to 3α-OH, 5α-H-Org 4060, and in both the vagina and the uterus of postmenopausal women to 3α-OH, 5α-H-Org 4060 and to 3β-OH, 5α-H-Org 4060 (figure 4).

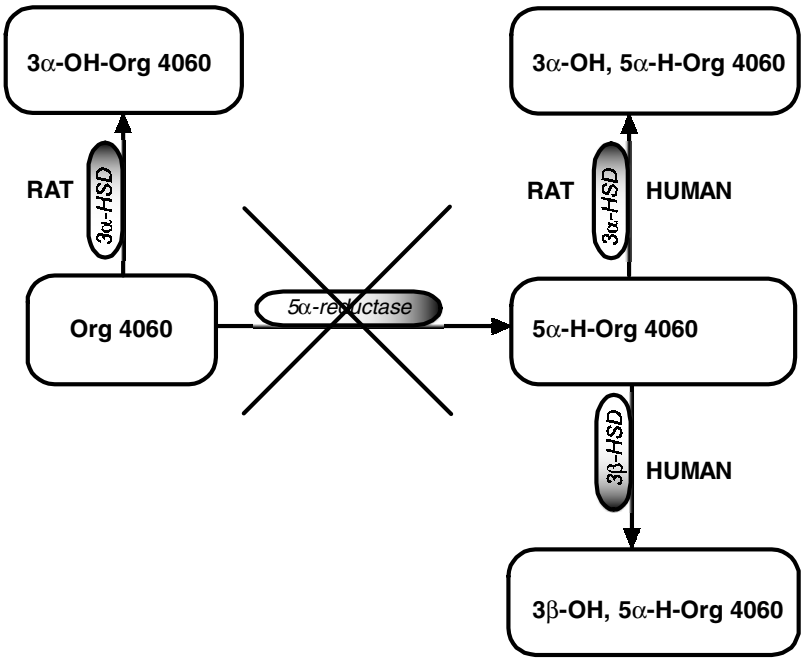

Figure 4: An overview of the metabolism of Org 4060 and 5α-H-Org-4060 in target tissues of the rat and postmenopausal women.

Several conclusions can be drawn from the above experiments:

- The 7α-methyl and the 11β-ethyl substitution to norethisterone generate steroids which are poor substrates for 5α-reduction in rat or postmenopausal target tissues.
- After 5α-reduction of norethisterone, the 11β-ethyl substitution does not interfere with rat 3α-HSD or human 3α- or 3β-HSD activity.
- The uterus of postmenopausal women is 5α-reductase deficient since neither progesterone or norethisterone were 5α-reduced in these tissues.

In order to confirm the inhibitory effect of the 7- and 11-substitutions on the 5α-reduction of norethisterone, we transiently expressed human 5α-reductase type 1 and 2 in HEK293 cells. Upon transfection with the expression vectors for both enzymes, the HEK293 cell efficiently metabolized testosterone (50 nM) to 5α-DHT. Similarly, norethisterone (50 nM) was metabolized to 5α-H-norethisterone in HEK293 cell expressing human 5α-reductase type 1 or type 2. However, Org OM38, Org 4060 and Org 34694 were not metabolized in these cells. This confirms that the lack of metabolism in the vagina of postmenopausal women of these norethisterone derivatives is attributable to the fact that they are poor substrates for both human

5α-reductase enzymes. Thus, Org OM38, Org4060 and Org 43694 are not metabolized by uterine tissue of post-menopausal women.

7α-methyl and 11β-ethyl substitution: a balance between metabolism and receptor binding.

Besides their progestagenic action, several progestagens that are derived from 19-nortestoterone have been reported to display some estrogenicity. Norethisterone, gestodene and levonorgestrel are all 19-nortestosterone derivatives and are all able to induce the proliferation of MCF-7 cells at concentrations of 10^{-7} M. At the same or higher concentrations, progesterone and medroxyprogesterone acetate (MPA) did not show this stimulatory effect on MCF-7 cell proliferation. Moreover, the cell proliferation that was induced by the 19-nortestosterone-derived progestagens could be antagonized by the anti-estrogens 4OH-Tamoxifen and ICI 164,384, indicating that the effect of the 19-norprogestins is mediated by the estrogen receptor (Schoonen et al., 1995). A similar estrogenic effect was reported in *Ishikawa* endometrial cancer cells. In these cells, 19-nortestosterone-derived progestagens were able to induce the Alkaline Phosphatase (AP) activity. Again, there was no estrogenic response of MPA. By comparing the ability of several progestagen analogues to induce AP activity in *Ishikawa* cells, two structural elements were identified on the progestagen molecule that seem to confer the estrogenicity. The presence of a 17β-OH group was mandatory, while removal of the 19-methyl group enhanced the estrogenicity (Botella et al., 1995). However, none of the 19-nortestosterone-derived progestagens that are estrogenic in the *in vitro* assays show appreciable affinity for the estrogen receptor.

It was therefore concluded that the estrogenicity of the 19-nortestosterone-derived progestagens is due to their metabolism to more estrogenic metabolites. Indeed, it was shown for norethisterone that several 5α-reduced metabolites show significant estrogen activity in the rat uterus *in vivo* (Mendoza Rodriguez et al., 1999). Similarly, 5α-reduced metabolites of gestodene show estrogenic activity in a hERα-mediated transactivation assay (Lemus et al., 2000). The most potent estrogenic metabolite of both 19-norprogestagens was their 3β,5α-tetrahydro reduced derivative.

The finding that 7α-methyl and 11β-ethyl substitution of norethisterone generates poor substrates for 5α-reductase implies that these 19-nortestosterone derived progestagens will not show estrogenic effects due to their metabolism to more estrogenic metabolites. However, Table 2 presents what happens to the estrogen receptor binding of 3β-OH, 5α-H-norethisterone upon substitution of a 7α-methyl , an 11-ethyl or a 7α-methyl- and an 11-ethylidene. Substituents at both the 7- and the 11-position enhance the RBA for the ER.

TABLE 2

The influence of 7- and 11-substitutions on the RBA for the estrogen receptor (ER) of 3β-OH, 5α-H-NET.

substituent	RBA for ER (%) (E2 = 100%)
none [1]	8
7α-methyl [1]	21
11β-ethyl [2]	142
7α-methyl, 11-ethylidene [2]	100

[1] *adapted from Schoonen et al., 2000*
[2] *unpublished data from Organon*

The most potent estrogens are thus the most difficult to be formed, but it is conceivable that minor amounts of the norethisterone derivatives are 5α-reduced by the liver, the organ with the highest 5α-reductase capacity. Since 5α-H-Org 4060 was readily 3β- and 3α-reduced in the postmenopausal uterus and vagina, it may be possible that small amounts of the 5α-reduced 19-norprogestagens are further 3β- and 3α-reduced in target tissue, generating potent estrogens.

Metabolism of Org OD14 and its derivatives in HRT target tissues: comparing the rat and the human.

In Chapter 5 the metabolism of Org OD14 (7α-methyl-norethynodrel), its 3β-reduced derivative Org 30126 and 3α-reduced derivative Org 4094 was studied. Org OD14 is a synthetic steroid used in Hormone Replacement Therapy and has estrogenic, progestagenic and androgenic properties in several bioassays (van der Vies, 1987; Markiewicz and Gurpide, 1990). In postmenopausal women, it causes an increase in bone mineral density, reduces vaginal atrophy but it has no estrogenic effect on the endometrium (Moore, 1999). Metabolism of Org OD14 to its Δ4-isomer Org OM38 (7α,17α)-17-hydroxy-7-methyl-19-norpregn-4-en-20-yn-3-one) by 3β-hydroxysteroid dehydrogenase/ isomerase (3β-HSD) occurs in the human endometrium and is implicated to suppress estrogenic action in the endometrium through the progestagenic effect of Org OM38 (Tang et al., 1993). Therefore the mixed hormonal profile of Org OD14 is due to its metabolites.

Org OD14 metabolism

Org OD14 was very rapidly metabolized in all tissues that were investigated. At a concentration of Org OD14 of 50 nM, virtually all of the substrate was metabolized after 180 and 360 minutes in rat as well as in human tissues. The metabolites, however, were different in rat and human tissues, respectively. Org OD14 was metabolized to the estrogen Org 4094 (3α-OH-Org OD14) in the rat uterus and vagina, while the estrogen Org 30126 (3β-OH-Org OD14) and the progestagen Org OM38 (Δ4 Org OD14) were the major metabolites in the uterus

and vagina of postmenopausal women. In rat aortic tissue, Org Om38, Org 4094 and an unidentified metabolite were the major metabolites

The conversion of Org OD14 to its Δ4 isomer Org OM38 in human uterus has been reported previously. It was thought to be catalyzed by 3β-HSD/isomerase (Tang et al., 1993). Two isoforms of human 3β-HSD/isomerase have been described. They both act as 3β-HSD/isomerases in catalyzing the conversion of pregnenolone (P5) and dehydro-epiandrosterone (DHEA) to progesterone and androstenedione, respectively (Simard et al., 1995). Human 3β-HSD/isomerases first catalyse the NAD dependent oxidation of the 3β-OH group of P5 or DHEA. The NADH that is being produced induces a conformational change that activates the isomerase activity (Mason et al., 1998)

In Chapter 5 several data are presented suggesting that the conversion of Org OD14 to Org OM38 (Δ4 Org OD14) and Org 30126 (3β-OH Org OD14) in the uterus and vagina of postmenopausal woman is not catalyzed by a human 3β-HSD/ isomerase type 1 or type 2.

Firstly, the conversion of Org OD14 to Org 30126 was shown to be NADPH- rather than NADH dependent, and the conversion could not be inhibited by the classical 3β-HSD inhibitor epostane. Secondly, the conversion of Org OD14 to Org OM38 did not require a cofactor. Moreover, HEK293 cells transfected with the human 3β-HSD type 1 or 2 did not metabolize Org OD14. Thus, an as of yet uncharacterized human 3β-HSD isoform similar to rat 3β-HSD type 3, which acts as a NADPH-dependent 3-ketosteroid reductase may be responsible for the conversion of OD14 by the human uterus.

Another interesting result of these studies was the observation that for the isomerization of Org OD14 to its Δ4-isomer Org OM38 there was a 6 fold higher apparent Km value and a 10 fold higher Vmax value compared to the conversion of Or OD14 to Org 30126. This means that at a higher plasma concentration of Org OD14, relatively more Org OM38 than Org 30126 may be formed in the uterus. This may predispose good absorbers of Org OD14 to experience a more pronounced progestagenic effect in the uterus.

Org 30126 and Org 4094 metabolism

Org 30126 was not metabolized in the human tissues, while it was converted to Org 4094 in the rat uterus and vagina. This conversion was shown to be a two step NADP(H) dependent reaction with Org OD14 as an intermediate. Org 30126 was also metabolized to Org 4094 by aortic tissue of the rat, although to a lesser extent. No appreciable metabolism of Org 4094 was detected in any of the tissues from both species. An overview of the metabolic routes of Org OD14 in rat and human is presented in figure 5.

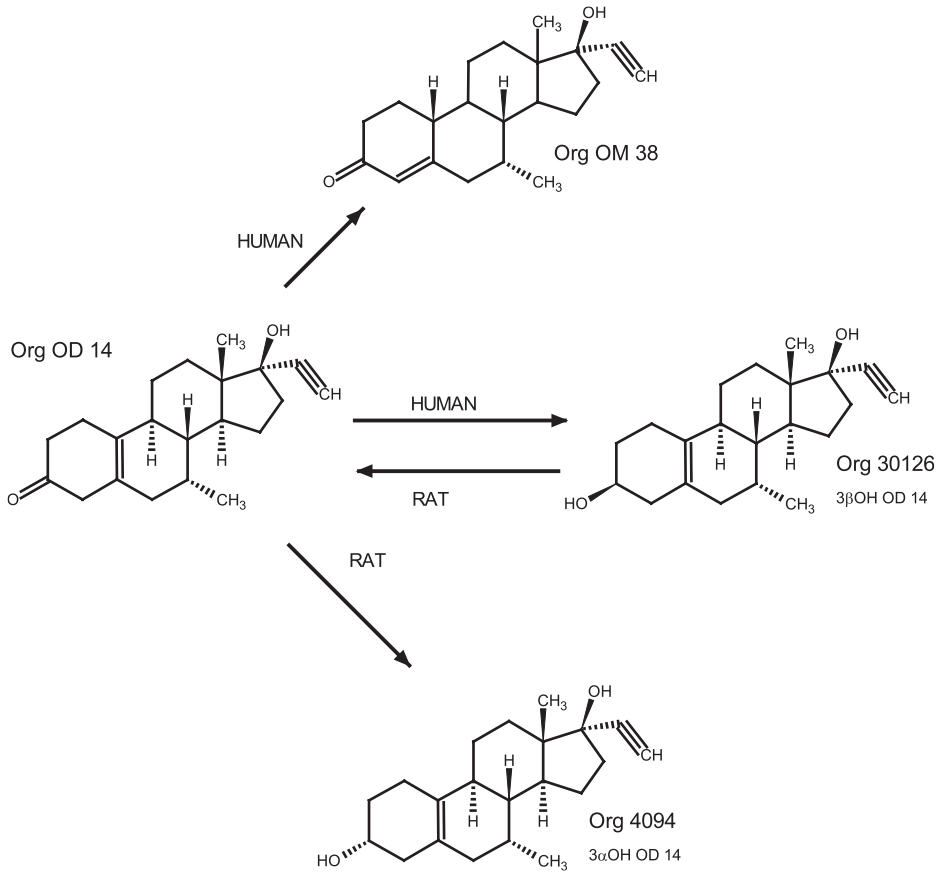

Figure 5: *Preferred metabolic pathways of Org OD14 and its derivatives in rat and human.*

We have to conclude that, in view of the important differences in uterine Org OD14 metabolism between the rat and the human, the rat is not suited to be a model for the study of uterine effects of Org OD14. The progestagen Org OM38 is not formed in the rat uterus as it is in the human, but rather the weakly estrogenic substance Org 4094, leading to an unopposed estrogenic effect in the rat uterus.

Concluding remarks and perspectives

The role of systemic metabolism versus target tissue metabolism.

In this thesis many questions are answered concerning the target tissue specific metabolism of norethisterone derivatives and Org OD14. It is now quite clear what the metabolic routes are in the selected tissues of both rat and human. Also the limitations of the rat as a model for steroid metabolism studies in human target tissues have been identified. Up to this stage we have concentrated on the characteristics of the steroid metabolism in the tissue. We have established the steroid-metabolizing capacity of especially uterine and vaginal tissue. The next step would be to learn how this steroid metabolizing capacity actually affects the steroid concentrations in the tissue. A complicating factor for such research is the fact that this can only be studied in an *in vivo* experiment. The systemic metabolism of the studied compound will also have its effect on the intra-tissue levels of the steroid. However, by comparing the "tissue metabolite profile" with the "plasma metabolite profile" it will be possible to evaluate the *in vivo* relevance of the target tissue metabolism for the metabolite levels in the tissue.

An indication that target tissue metabolism is an important factor in the regulation of steroid levels in the tissue may be obtained by considering the parturition defect in 5α-reductase type 1 knockout mice. In both wildtype and mutant mice the plasma progesterone levels drop dramatically 2 days before term and the progesterone levels in the uterus and cervix of the wildtype mice drop accordingly. However, in knockout mice, impaired progesterone metabolism leads to sustained high levels of the hormone in the cervix and in the uterus, giving rise to an impaired cervical ripening and low delivery rates as a consequence. (Mahendroo et al., 1999)

Comparing norethisterone derivatives and Org OD14

Norethisterone is usually used as the progestagen in a estrogen / progestagen combined HRT. However, the two types of 19-norprogestagens, the norethisterone derivatives and Org OD14, may represent two different strategies to generate a single HRT compound that acquires both estrogenic and progestagenic properties through metabolism. In the human, Org OD14 is rapidly converted to its estrogenic metabolites and to a progestagen, that may counteract unfavorable estrogen action. Norethisterone and also its derivatives Org 4060, Org OM38 and Org 34694 on the other hand are all strong progestagens. The estrogenic properties could be generated from these components through the action of 5α-reduction and 3β-reduction. This latter concept may still harbor some possibilities to generate analogues that are more readily 5α-reduced and still retain good estrogen receptor affinity of its 3β, 5α-reduced metabolites. It may be interesting to consider norethisterone analogues that have small lipophilic substitutions at the 12 or 9 position. Substitutions to the steroid skeleton of estradiol at those positions are known to maintain good ER binding. Substituents to the steroid skeleton of

norethisterone at the 12 and 9 positions may not interfere with 5α-reductase. *In vitro* systems stably expressing the 3α, 3β and 5α reducing enzymes may prove useful tools to evaluate the effect of such substitutions on their metabolism. In order establish such tools, the expression of these enzymes in the tissues of interest should be studied, allowing for a proper choice of the *in vitro* setting.

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Samenvatting

Bij vrouwen na de menopause is er een sterke daling van de oestradiol concentraties in het bloed. Hoewel oestradiol oorspronkelijk alleen beschouwd werd als een geslachtshormoon, heeft dit hormoon ook invloed op een groot aantal andere organen, waaronder het skelet, het cardiovasculaire systeem en het centrale zenuwstelsel. Een van de meest dramatische gevolgen van de daling van de bloedspiegel van dit hormoon is een toename van de botresorptie die kan leiden tot osteoporose. Een effectieve behandeling van deze conditie is mogelijk door middel van een Hormoon Vervangings Therapie (HVT) waarin het deficiente oestradiol wordt gesupplementeerd door toediening van een oestrogeen. Een nadeel van deze oestrogenen is dat de incidentie van borst- en endometrium kanker toeneemt. Dit verschijnsel lijkt gerelateerd te zijn aan de stimulatie door oestrogenen van de proliferatie in de borst en het endometrium. Deze twee organen zijn zeer gevoelig voor oestrogenen. Om de oestrogeen gestimuleerde proliferatie in de borst en het endometrium tegen te gaan, wordt daarom vaak een progestageen aan HVT toegevoegd. Door deze combinatie wil men bereiken dat het gewenste oestrogene effect op het bot blijft bestaan, terwijl de ongewenste proliferatie in de borst en het endometrium wordt onderdrukt door het progestageen.

Het effect van de de steroiden die gebruikt worden in HVT op de organen die door deze therapie beinvloed worden (doelweefsels) is afhankelijk van een groot aantal factoren. De belangrijkste hiervan zijn achtereenvolgens: de beschikbaarheid van de steroiden in het weefsel, de soort en concentratie van steroid receptoren die aanwezig zijn in het doelweefsel en de aanwezigheid van eiwitten die betroken zijn bij de co-activatie/co-repressie van steroid receptoren. In dit proefschrift concentreerden we ons op de eerste stap in deze cascade. De beschikbaarheid van een circulerend steroid hormoon in een doelweefsel wordt bepaald door de opname uit de bloedbaan en het metabolisme in dat weefsel. Het steroid metabolisme in het weefsel kan leiden tot een activatie of een inactivatie van het steroid hormoon. Een voorbeeld van steroid hormoon activatie door metabolisme in het doelweefsel is de omzetting van testosterone naar het sterkere androgeen 5α-dihydrotestosterone in de prostaat. Een andere mogelijkheid is dat door metabolisme een steroid wordt gevormd dat affiniteit heeft voor een andere steroid receptor. De aromatisering van C19 androgenen tot C18 oestrogen door het enzym aromatase in b.v. vetweefsel is hier een goed voorbeeld van.

Het is goed denkbaar dat de steroiden die gebruikt worden bij HVT op een vergelijkbare manier worden gemataboliseerd in de verschillende doelweefsels. In dit proefschrift werd daarom een studie gedaan naar het vermogen van een aantal geselecteerde doelweefsels voor HVT (uterus, vagina en aorta) om een aantal oestrogenen en progestagenen te metaboliseren. De oestrogen die werden bestudeerd zijn oestradiol, ethynylestradiol (17α-ethynyl-estadiol) en moxestrol (11β-methoxy-17α-ethynyl-estradiol). De progestagenen die werden bestudeerd zijn norethisteron (NET) en een aantal derivaten van NET. De derivaten van NET zijn Org OM38 (7α-methyl-NET), Org 4060 (11β-ethyl-NET) en Org 34694 (7α-

methyl,11-ethylidene-NET). Op deze wijze kon de invloed van een 17α-ethynyl en 11β-methoxy substituent op het metabolisme in HVT doelweefsels van oestradiol bepaald worden. Evenzo kon de invloed op het metabolisme worden bepaald van een kleine lipofiele substitutie op de 7α en 11-positie van norethisterone. Bovendien werd ook het metabolisme bestudeerd van Org OD14 (ook bekend als livial of tibolone), een steroid met zowel oestrogene, progestagene en androgene eigenschapen. Van Org OD14 is bekend dat het effectief is als een oestrogeen agonist op bot, maar geen stimulatie van het endometrium veroorzaakt vanwege de vorming in het endometrium van een metaboliet met progestagene eigenschappen.

De geovariectomeerde (ovx) rat wordt beschouwd als een goed model om de pathogenese en behandeling van osteoporose bij postmenopausale vrouwen te bestuderen. Het is echter minder duidelijk of de ovx rat ook vergelijkbaar is met de mens als het gaat om effecten van HVT op andere weefsels dan het bot. Omdat metabolisme in doelweefsel een belangrijke invloed heeft op de werking van steroiden werd onderzocht of het metabolisme van de geselecteerd steroiden in de rat vergelijkbaar is met dat in postmenopausale vrouwen

hoewel er in zowel de uterus als de vagina van de ovx rat sprake is van oxidatie van de 17β-OH groep door 17β-hydroxysteroid dehydrogenase (17β-HSD) en conjugatie van de 3-OH groep bleek het metabolisme van oestradiol in de uterus en vagina van de ovx rat sterk te verschillen van dat in de mens. De regulatie door estradiol en progesteron van de 17β-HSD activiteit en de conjugatie van estradiol in de ovx rat uterus was tegenovergesteld aan dat in de humane uterus. Bovendien bleek dat er in de rat uterus sprake is van een conjugatie van estradiol tot een glucuronide, terwijl in de humane uterus 3-sulfaten worden gevormd. De regulatie door estradiol en progesteron van het estradiol metabolisme was alleen duidelijk in de uterus; in de vagina had behandeling met deze hormonen geen invloed. De 17α-ethynyl groep verhinderde zoals te verwachten de oxidatie van ethynylestradiol door 17β-HSD, terwijl de 11β-methoxy substitutie van moxestrol de glucuronidering bleek te verhinderen. Er werd geen metabolisme van oestrogenen waargenomen in de rat aorta (hoofdstuk 1).

Ook het metabolisme van NET in de uterus en vagina van de rat en de mens bleek verschillend te zijn. In beide soorten was 5α-reductie de bepalende stap voor het metabolisme van norethisteron. De 3-keto groep van het gevormde 5α-H-NET werd in de vagina van beide soorten verder gereduceerd. Echter, in de vagina van postmenopausale vrouwen werd er zowel 3α-OH, 5α-H-NET als 3β-OH, 5α-H-NET gevormd, terwijl in de rat er voornamelijk 3α-OH, 5α-H-NET werd gevormd. Ook in de rat uterus en aorta werd voornamelijk 3α-OH, 5α-H-NET gevormd terwijl er nauwelijks NET metabolisme was in de uterus van postmenopausale vrouwen. De verschillen tussen rat en mens in vorming van 5α-gereduceerde en 3α,5α / 3β,5α-gereduceerde metaboliten van NET is van belang omdat 5α-reductie van NET de affiniteit van NET voor de progesteron receptor teniet doet. Bovendien vertonen 3α,5α en 3β,5α gereduceerde metabolieten van NET affiniteit voor de oestrogeen

receptor en worden zij ook geassocieerd met *in vivo* en *in vitro* oestrogene activiteit. Dit laatste geldt in sterkere mate voor 3β,5α-NET dan voor 3α,5α-NET. Zowel de 7α-methyl als de 11β-ethyl stubstitutie van Org OM38 en Org 4060, respectivelijk, verhinderde de 5α-reductie in beide soorten alsook in HEK293 cellen die humaan 5α-reductase type 1 of 2 tot expressie brengen (hoofdstuk 3 en 4).

De voorkeur voor 3α-reductie in de rat werd ook duidelijk in de studies met Org OD14. In uterus, vagina en aorta van de rat werd de 3-keto groep van Org OD14 omgezet tot 3α-OH Org OD14 (Org 4094). In de uterus en vagina van postmenopauale vrouwen daarentegen werd Org OD14 omgezet tot 3β-OH-Org OD14 (Org 30126) en tot de Δ4 isomeer van Org OD14 (Org OM38). Hieruit volgt dat het beschermende effect van Org OD14 op het endometrium door de vorming van het progestagene Org OM38 niet zal optreden in de rat. In de rat uterus, vagina en aorta werd Org 30126 ook omgezet naar Org 4094 met Org OD14 als tussenproduct. In de humane weefsels werd Org 30126 niet omgezet (hoofdstuk 5).

In dit proefschrift worden belanrijke verschillen aangetoond tussen de rat en de mens voor wat betreft het metabolisme van oestradiol, norethisteron en Org OD14 in enkele doelweefsels van HVT. Deze verschillen maken de rat minder geschikt voor het bestuderen van effecten op deze doelweefsels van de genoemde steroiden. Bovendien werd gevonden dat substitutie met een 7α-methyl of een 11β-ethyl groep de 5α-reductie van norethisteron verhinderd. Gezien de verschillen tussen de rat en de mens is het wellicht beter om *in vitro* systemen te ontwikkelen om het metabolisme van vergelikbare steroiden in HVT doelweefsel te testen. Hierbij zal verdere kennis van de expressie van de enzymen die betrokken zijn bij dit metabolisme onontbeerlijk zijn (hoofdstuk 6).

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Curriculum Vitae

Maarten Jan Blom werd op 13 oktober 1966 geboren te Capelle aan den IJssel. In 1985 haalde hij het eindexamen VWO te Eindhoven. In 1987 vervolgde hij zijn studie aan de Hogere Laboratorium Opleiding in Utrecht en in 1991 werd hier het diploma HLO chemie behaald. Van 1991 tot 1995 was hij als analist verbonden aan de vakgroep Veterinaire Farmacologie, Farmacie en Toxicologie te Utrecht waar onder leiding van Prof. Dr. J. Fink-Gremmels werd gewerkt aan het Ochratoxine A metabolisme. Vervolgens werkte hij als analist bij het Laboratorium Genetische en Metabole Ziekten onder leiding van Dr. Abelink en Dr. van Kuilenburg aan het metabolisme van biogene aminen in liquor cerebrospinalis en aan de activiteit van het enzym DPD in bloedcellen. Eind 1996 werd hij aangesteld als AIO bij de onderzoeksgroep Vergelijkende Endocrinologie van de faculteit biologie te Utrecht. Hier werd het in dit proefschrift beschreven onderzoek verricht onder de begeleiding van Prof. Dr. H.J.Th. Goos en Dr. J.G.D. Lambert. Vanaf april 2001 is hij werkzaam als senior scientist bij Xendo laboratories te Groningen.

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