Controlling the Levels of Estrogen Receptor α and β Activation in Postmenopausal Women During Hormone Replacement Therapy: A Novel Strategy for Achieving Optimal Health Outcomes

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Abstract. Recent studies from our laboratory have lead to the suggestion that the human ERα and ERβ signaling systems are differentially activated under different physiological conditions. In a non-pregnant young woman, the ERα system is preferentially activated over the ERβ system, mainly by estrone and its major oxidative metabolite, 2-hydroxyestrone. These two estrogens are among the quantitatively major estrogens present in a young woman, and they have approximately 4-fold preferential activity for ERα over ERβ. During pregnancy, however, ERβ is preferentially activated over ERα, which is caused by various pregnancy estrogens, mainly estriol and other *D*-ring derivatives of 17β-estradiol. These estrogens have preference for binding to ERβ over ERα, and some of them are produced in unusually large quantities. In light of this new finding, it is hypothesized that estrogens ideal for female hormone replacement therapy should be those that will produce a hormonal condition mirroring what is found in a non-pregnant young woman rather than in a pregnant woman. The endogenous estrogen derivatives, such as the sulfated conjugates of estrone, are among the ideal candidates for achieving this clinical purpose. In comparison, Premarin, the most commonly used hormone replacement therapy that contains a mixture of conjugated estrogens isolated from pregnant mare's urine, is less suitable because several of its estrogenic components can produce a strong, preferential over-stimulation of the human ERβ signaling system.

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Introduction

At present, the most commonly-used estrogen for HRT is Premarin, consisting of a mixture of mostly sulfated estrogens isolated from pregnant mare's urine. The hormonal activity of these conjugated estrogens *in vivo* results from their enzymatic hydrolysis to release biologically-active estrogens. For decades, the general scientific belief has always been that "an estrogen is an estrogen", *i.e.*, all estrogens were assumed to exert similar pharmacological actions in the body. However, this dogmatic view has been gradually changed in the past decade. A recent study from our laboratory compared the activity of a number of endogenous estrogen metabolites, including many of those contained in Premarin, for human ERα and ERβ [5]. It was found that while E_2 (perhaps the best-known endogenous estrogen) has nearly the highest and almost identical binding affinity for human ERα and ERβ, many of its metabolites have widely different preference for the activation of human ERα and ERβ [5]. In addition, the predominant estrogens that are present in a pregnant woman are very different from those present in a non-pregnant woman. Furthermore, these estrogens have different preference for activation of human ERα and ERβ. Based on these lines of information, it is proposed that differential activation of ERα over ERβ is an important factor for achieving optimal clinical outcomes in post-menopausal HRT. In this article, I also briefly discussed the types of estrogens that would be more ideal for human use as menopausal HRT.

Different endogenous estrogens are produced in pregnant and non-pregnant women

A large number of endogenous estrogen derivatives are known to be present in humans. Studies have been conducted in the past to determine human urinary excretion of various estrogens (mostly as conjugates) as a global indicator of the biosynthesis and metabolism of endogenous estrogens *in vivo* [9,10]. Based on recent data from our laboratory, it is estimated that the total daily amount of various urinary estrogens excreted from a late pregnant woman is 2 to 3 orders of magnitude higher than the amount excreted by a non-pregnant woman of the same age group. In addition, the composition of the urinary estrogens in pregnant and non-pregnant women is also widely different. Representative profiles of various endogenous estrogens found in the urine of pregnant and non-pregnant young women are summarized in Table 1.

In the urine samples obtained from non-pregnant young women, the conjugated forms of 2-OH-E_1, followed by 16α-OH-E_2 (E_3), 16α-OH-E_1 and E_1, are the predominant estrogens. The amount of E_2 and its major metabolites 2-OH-E_2 and 2-methoxy-E_2 was much less than that of E_1 and its corresponding metabolites. The relative composition of various estrogens in circulation is believed to be largely comparable to what is seen in the urine. The presence of higher levels of E_1 over E_2 in a non-pregnant woman is largely attributable to the high levels of the oxidative 17β-hydroxysteroid dehydrogenase (17β-HSD), which catalyzes the facile conversion of E_2 to E_1. The conversion of E_1 to 2-hydroxy-E_1 or E_2 to 2-OH-E_2 is catalyzed by various cytochrome P450 enzymes [11–14], and the subsequent *O*-methylation to form 2-methoxy-E_1/E_2 is catalyzed by catechol-*O*-methyltransferase (COMT) [7,15].

There is a drastic change in the endogenous estrogen composition during pregnancy. E_3 becomes the predominant estrogen and is produced in unusually large quantities. The daily amount of this estrogen (in its conjugated forms) released into the urine of a late pregnant woman is 200–1000 times higher than any of the quantitatively-major estrogens produced in a non-pregnant woman. Notably, several other *D*-ring estrogen derivatives, such as 17-epi-E_3, 16-epi-E_3, 16,17-epi-E_3 and estetrol (15α-OH-E_3), are also produced in readily detectable quantities at late stages of pregnancy. These *D*-ring derivatives are usually only present at low or undetectable levels in non-pregnant young women. Similar results were also reported in earlier studies [11,12].

In summary, although E_2, the best-known endogenous estrogen, is not the predominant estrogen produced in the body of a pregnant

TABLE 1. Comparison of daily urinary secretion (mean ± S.D.) of endogenous estrogen metabolites during the pre-ovulatory phase (days –6 to –10), ovulation (day 0), and post-ovulatory phase (days 6 to 10) of a normal non-pregnant woman with that of five pregnant women.

Estrogen	Non-pregnant woman (μg/24 hour urine)			Pregnant woman (μg/24 hour urine)
	Days –6 to –10 (pre-ovulatory phase)	Day 0 (ovulation)	Days 6 to 10 (post-ovulatory phase)	
Estrone (E$_1$)	6.2 ± 3.9	30.8 ± 15.6	16.2 ± 12.4	49.1 ± 37.8
17β-Estradiol (E$_2$)	0.9 ± 0.8	3.8 ± 1.9	1.6 ± 0.2	26.5 ± 11.3
2-Hydroxyestrone (2-OH-E$_1$)	5.7 ± 4.0	22.5 ± 12.0	8.8 ± 1.8	22.1 ± 11.4
4-Hydroxyestrone (4-OH-E$_1$)	0.7 ± 0.4	2.2 ± 0.2	1.1 ± 0.3	2.4 ± 0.8
16α-Hydroxyestrone (16α-OH-E$_1$)	2.4 ± 1.5	13.6 ± 6.4	5.0 ± 3.3	532.4 ± 948.8
2-Methoxyestrone (2-MeO-E$_1$)	2.4 ± 0.2	0.5 ± 0.8	0.8 ± 0.4	6.9 ± 3.4
2-Hydroxyestradiol (2-OH-E$_2$)	0.8 ± 0.4	1.8 ± 1.2	1.2 ± 0.3	3.4 ± 3.6
4-Hydroxyestradiol (4-OH-E$_2$)	ND	ND	ND	0.5 ± 0.1
2-Methoxyestradiol (2-MeO-E$_2$)	ND	ND	ND	11.5 ± 14.5
Estriol (E$_3$)	6.9 ± 2.3	28.8 ± 12.2	15.7 ± 5.8	11174.8 ± 9304.3
16-Epiestriol (16-EpiE$_3$)	ND	ND	ND	562.3 ± 626.3
17-Epiestriol (17-EpiE$_3$)	ND	ND	ND	ND
16,17-Epiestriol (16,17-EpiE$_3$)	ND	ND	ND	176.7 ± 72.2
2-Hydroxyestriol (2-OH-E$_3$)	ND	ND	ND	86.8 ± 73.7
Estetrol (15α-OH-E$_3$)	ND	ND	ND	302.0 ± 273.3

Note: **The collection of human urine samples was approved by the Institutional Review Board. The urinary estrogens were determined by using the GC/MS method as described below. An aliquot (1 mL) of the urine sample was transferred to a 1.5-mL microcentrifuge tube containing 200 μL 2 M Na$_2$AC buffer (pH 5.0), and the mixture was centrifuged at 14,000 *rpm* for 5 min. The supernatant (1 mL) was transferred to a small glass tube containing 20 μL of 0.5 ng/μL E$_2$-D2 (in pure ethanol) as the internal standard, and 75 μL of H-2 sulfatase as the enzyme for hydrolysis of estrogen conjugates. The reaction mixture was incubated at 37°C for 12 hours. After incubation, the tubes were centrifuged at 4,000 *rpm* for 10 min, and the supernatants were transferred to another set of test tubes and extracted with 5 mL of hexane/ethyl acetate (*v:v*, 3:2). The organic extracts were removed and dried under a stream of nitrogen gas. BSTFA (100 μL) was added for derivatization at 65°C for 1 hour. The TMS derivatives of estrogen metabolites were detected by using the GC/MS. The GC/MS apparatus consisted of an Agilent 6890N GC with 7683 auto-sampler, an Agilent 5973 MS Network, coupled with a HP-5MS capillary column. The front inlet temperature was 260°C, and the column flow rate was 1.0 mL/min. The oven temperature was as follows: The starting was set at 180°C, then increased at 4°C/min to 260°C, hold at this temperature for 5 min, then further increased at 5°C/min to 300°C, and hold for 5 min at 300°C (with the AUX temperature at 280°C).**
"ND" denotes that the estrogen metabolite of interest was not detected.

woman or of a non-pregnant woman. The major endogenous estrogens that are produced in a non-pregnant woman are vastly different in quantity and also in composition from those produced in a pregnant woman.

The biological activity of pregnancy and non-pregnancy estrogens is different

As discussed above, the endogenous estrogens formed in a non-pregnant woman are

vastly different in quantity and composition from those produced in a pregnant woman. There is also evidence showing that the endogenous estrogens produced in a non-pregnant young woman will exert very different physiological functions from the estrogens produced during pregnancy.

First, studies in recent years by us and also by others have shown that some of the E_2 derivatives can exert unique biological functions that are not shared by the parent hormone E_2 (reviewed in ref. [6–8,14–16]). For instance, an earlier study showed that 4-OH-E_2, a well-known hydroxylated metabolite of E_2, has a far stronger blood cholesterol-lowering effect in rats than E_2 [16], although its uterotropic activity [16,17] and ER-binding affinity are slightly lower than E_2 [5,17]. Also, it is well documented that the catechol estrogens are chemically-reactive and potentially genotoxic/mutagenic, which have been suggested to play an important role in mediating hormonal carcinogenesis [18–20]. In contrast, 2-methoxyestradiol, a nonpolar endogenous E_2 metabolite with little binding affinity for human ERα and ERβ, has a strong anti-proliferative, antiangiogenic and apoptotic actions [7,21]. It has been suggested that increased biosynthesis of this nonpolar estrogen metabolite is highly beneficial for the protection against estrogen-induced hormonal cancers [7,15].

Interestingly, an earlier study showed that the E_2 15α-hydroxylase activity which catalyzed the formation of 15α-OH-E_2 and 15α-OH-E_3 (estetrol) is selectively elevated by 50 to 70-fold in a localized area of the uterine endometrium where the imbedding of the fertilized ovum took place [22]. Although the exact biological functions of the 15α-hydroxylated estrogens are not clear at present, it is likely that the formation of 15α-OH-E_2 and 15α-OH-E_3 (estetrol) may serve important physiological functions, such as facilitating the imbedding process. Similarly, it is of note that the amount of 15α-hydroxylated estrogens present in the urine of a late-stage pregnant woman can be used as a reliable indicator of fetal well-being, in particular of fetal lung functions [23–27].

Second, in a recent study, our laboratory studied a large number of endogenous estrogen derivatives for their binding affinity for human ERα and ERβ [5]. We found that the major estrogens present in a non-pregnant young woman have clearly different preference for the activation of ERα vs ERβ compared to the estrogens present in a pregnant woman. Some of the relevant data are briefly discussed below.

It was observed that E_1 and 2-OH-E_1, two of the quantitatively-major estrogens present in a non-pregnant woman, have a modest but significant preference for binding to human ERα over ERβ [5]. E_1 had 3- to 4-fold higher preference for binding to human ERα than for ERβ. Similarly, 2-OH-E_1 (the 2-hydroxylated metabolite of E_1) also has approximately 4-fold preference for activation of ERα over ERβ. Notably, E_1 and 2-OH-E_1 have markedly lower binding affinity for human ERα and ERβ compared to E_2. I believe the relatively lower binding affinity of E_1 and 2-OH-E_1 actually is an advantage rather than a disadvantage, because they would pose a lower risk for causing over-stimulation of the ERα and ERβ signaling systems in $vivo$.

Very differently, E_3, the quantitatively-predominant estrogen produced during human pregnancy, has a significant preference for binding to ERβ over ERα [5]. Although E_3 had a rather low binding affinity for human ERα compared to E_2 (RBA 11% of E_2), it retained a relatively high binding affinity for ERβ (RBA 35% of E_2). Therefore, E_3 has an approximately 3 to 1 preference for binding to ERβ than for ERα. Similarly, 16α-OH-E_1, another well-known hydroxylated metabolite of E_1 that is also formed in very large quantity during pregnancy, has a higher binding preference for ERβ than ERα when compared to E_1.

16,17-Epiestriol had a very low binding affinity for human ERα, but it had a preferential affinity for ERβ; the difference of its binding affinity for ERβ over ERα is 18-fold. Notably, this unique endogenous estrogen metabolite is usually undetectable in a non-pregnant woman, but it is present at considerable levels during pregnancy (Table 1).

Taken together, it is clear that there is a distinct difference in the ratio and also intensity of ERα and ERβ activation in a non-pregnant young woman compared to a pregnant woman.
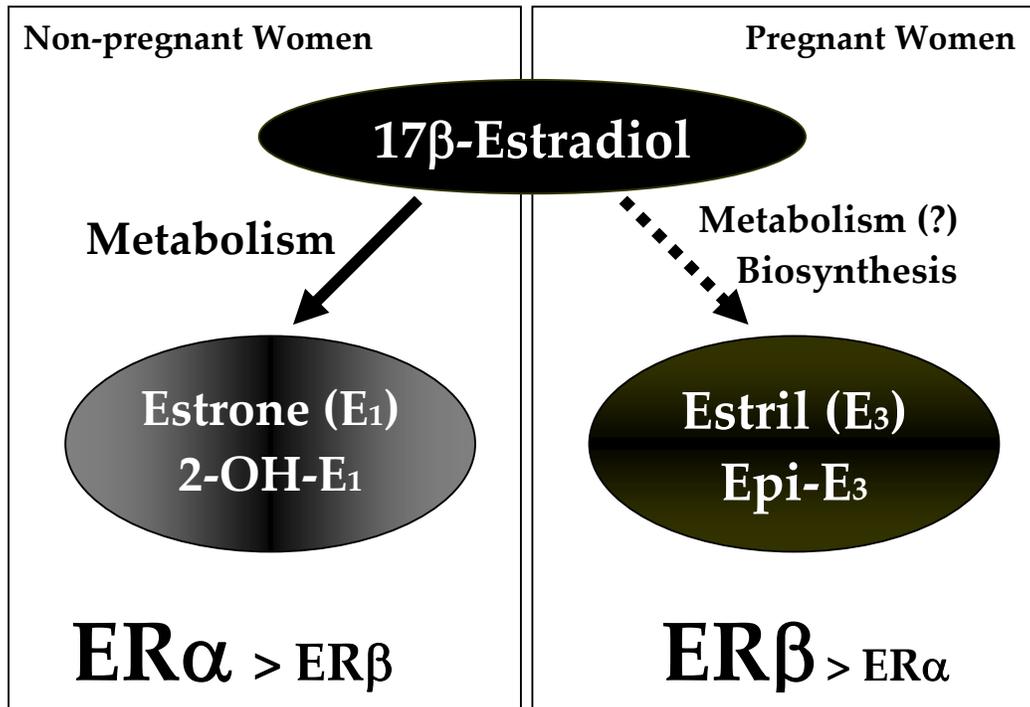
Differential Activation of the Human ERα and ERβ

Non-pregnant Women

Pregnant Women

17β-Estradiol

Metabolism

Metabolism (?)
Biosynthesis

Estrone (E₁)
2-OH-E₁

Estril (E₃)
Epi-E₃

ERα > ERβ

ERβ > ERα

Figure 1. Differential activation of the human ERα and ERβ in non-pregnant women *vs.* in pregnant women. In a non-pregnant woman, estrone (E_1) is believed to be the major circulating estrogen. 17β-Estradiol (E_2) biosynthesized in the body is quickly converted to E_1 by the 17β-hydroxysteroid dehydrogenase (17β-HSD) present in various non-gonadal tissues, such as the liver. E_1 has rather low binding affinity for ERα and ERβ compared to E_2, but it has a preferential binding affinity for ERα over ERβ. 2-OH-E_1 is the major metabolite of E_1 *in vivo*, and it is a very weak estrogen also with a preference for ERα over ERβ. Therefore, it is apparent that there is a preferential activation of the ERα system over the ERβ system in a non-pregnant woman. In comparison, during pregnancy, the quantitatively-predominant estrogens present in the circulation are estriol (E_3) or 16α-OH-E_1, which are either metabolically formed from 16α-hydroxylation of the endogenous estrogens (such as E_2 or E_1), or are formed via other biosynthetic pathways in the feto-placental unit. Note that both of these two estrogens, which are formed in relatively small quantities in a non-pregnant woman, have a preferential binding affinity for ERβ over ERα. Therefore, there is a clear difference in the ER activation profiles in a non-pregnant woman *vs.* that in a pregnant woman. For hormone replacement therapy (HRT), the ideal situation is to mimic the hormonal status in a non-pregnant healthy young woman instead of mimicking the hormonal condition in a pregnant woman. Notably, the current hormone replacement therapy (HRT) with the use of Premarin would largely mimic the hormonal conditions of a pregnant woman because our recent study (5) showed that many of the estrogens contained in Premarin (isolated from pregnant mare's urine) would also produce a preferential stimulation of the human ERα over ERβ.

The major estrogens produced in a non-pregnant woman would modestly favor the activation of ERα over ERβ. However, during pregnancy, there is a preponderance of activation of ERβ over ERα, which is exerted by various pregnancy estrogens, mainly E_3, which is produced in unusually large quantities. Such a preferential activation of ERβ is believed to play an indispensable role in mediating the various actions of endogenous estrogens that likely are required for the development of the fetus as well as for fulfilling other physiological functions related to pregnancy such as the suppression of autoimmune response against the fetus. These suggestions are in line with the observations that ERβ has a wide distribution in maternal reproductive and lymphatic organs in rats as well as in various tissues in the fetus [28–30].

Selection of estrogens for postmenopausal hormone replacement therapy (HRT)

Premarin, the most commonly-used HRT, contains a mixture of conjugated estrogens isolated from pregnant mare's urine. The major estrogens produced in a pregnant mare are quite different from those produced in a pregnant woman, and they do not contain E_3. However, they contain a number of unique equine estrogens, many of which are basically not produced in humans. Interestingly, our recent analysis showed that several of the equine estrogens contained in Premarin are functionally similar to the human pregnancy estrogen E_3 with respect to their preferential binding ability for human $ER\beta$ compared to $ER\alpha$ [5].

Let me give a few examples here. Our recent study showed that while 17β-dihydroequilenin has a low binding affinity for $ER\alpha$ (35% of E_2), it has a high binding affinity for $ER\beta$ (*RBA* 100% of E_2). Equilin (*i.e.*, 7-dehyro-E_1) has a decreased binding affinity for $ER\alpha$ compared to E_1 (its *RBA* 40% of E_1), but it has drastically increased binding affinity for $ER\beta$ (its *RBA* 631% of E_1). Similarly, *D*-equilenin has a much weaker binding affinity than E_1 for human $ER\alpha$ (*RBA* 20% of E_1), but its binding affinity for $ER\beta$ is >3 times higher than that of E_1.

Taken together, it is evident that many of the equine estrogens contained in Premarin have a strong, differential binding affinity for human $ER\beta$ over $ER\alpha$, which is very similar to the human pregnancy estrogen E_3.

Ideally, when an estrogen or a combination of estrogens is being evaluated for use in postmenopausal HRT, its ability to more closely mimic the hormonal environment found in a normal non-pregnant young woman but not one found in a pregnant woman is a crucial pharmacological property. Since very different types of estrogens are produced in pregnant *vs* non-pregnant women and they serve widely different physiological purposes, it is logical that the use of endogenous estrogens found in a non-pregnant young woman would be better for HRT than the use of estrogens predominantly produced during pregnancy. The former may include a combination of the sulfates of E_1 and 2-OH-E_1 and possibly other endogenous estrogens (such as the conjugates of 2-methoxyestrogens). The inclusion of methoxyestrogen sulfates as part of the HRT may be beneficial because of the strong antitumorigenic activity of 2-methoxyestradiol [7,15]). Given that many of the endogenous estrogens may have a rather rapid metabolic disposition in the body, some other naturally-occurring or synthetic estrogens that have longer half-lives and can also provide a similar preferential activation of the $ER\alpha$ system as E_1 may also be useful as alternatives. For instance, since 17α-E_2 has similar ER-binding preference as E_1 but it cannot be converted to E_2 by 17β-hydroxysteroid dehydrogenase (17β-HSD), the sulfate conjugates of 17α-E_2 may serve as alternatives to E_1-3-sulfate to achieve similar biological functions.

Here I also like to suggest that using sulfated estrogens for human HRT would be better than using the corresponding parent estrogens. The main reasons are: (*i*) The sulfated estrogens are inactive themselves (with little or no binding affinity for human $ER\alpha$ and $ER\beta$ [5]), but they can be enzymatically hydrolyzed to release bioactive estrogens in a variety of tissues in the body. Earlier studies showed that the estrogen target organs, such as the breast and uterus, contained much higher levels of estrogen sulfatase activity than other tissues [34–37]. As such, oral administration of estrogen sulfates would have the natural cushion effect which would avoid causing unwanted over-stimulation of the ER systems throughout the body. Instead, they usually would only activate those target tissues or cells that are most in need of estrogenic stimulation. Here it is also of note that several recent studies have shown that the estrogen target cells can actively transport E_1-3-sulfate into the cells [38,39]. Moreover, these cells may selectively adjust their ability to actively transport E_1-3-sulfate into the cells to release the biologically-active estrogens, depending on their hormonal needs. Theoretically, such a mechanism would offer certain degrees of target organ selectivity of estrogenic stimulation. (*ii*) Compared to estrogen glucuronides, estrogen sulfates are probably better because they usually have a lower clearance rate and a longer half-lives ($T_{1/2}$) in human, thereby

making them pharmacologically more useful [40, 41].

Based on the discussion given above, it is suggested that the use of estrogens to produce a modest level of stimulation of both ERα and ERβ systems with a slight preference for the ERα system would be better for postmenopausal HRT than the use of estrogens that confer a predominant activation of the ERβ system. It is apparent that Premarin, the most widely prescribed HRT, may not be the most suitable combination of estrogens for achieving this clinical purpose. Notably, while there is considerable amount of E_1-3-sulfate contained in Premarin, which presumably is good for its intended purpose as a HRT, the fact is that it also contains many other very potent equine pregnancy estrogens which would jointly produce a strong over-stimulation of the ERβ system. Similarly, genistein, a potent and preferential partial agonist of human ERβ, would be even less suitable than Premarin for use as postmenopausal HRT because it will essentially produce a near selective ERβ stimulation. This suggestion is in agreement with recent clinical observations showing that the singular use of genistein is mostly ineffective as a HRT in postmenopausal woman [42,43].

Conclusions

For decades, the general scientific belief had been that all estrogens would exert the same or highly similar pharmacological actions in a woman's body. In fact, when the oral tablet of Premarin was first approved by the U.S. Food and Drug Administration (FDA) for human use in 1942, its 0.625 mg dosage (still in use today) was actually assigned solely on the basis of its estrogenic potency in a rat bioassay that was found to be equivalent to 0.625 mg of sodium E_1-3-sulfate. This bioassay mostly measured the ERα-mediated uterotropic activity. Even to this day, little is known about the precise hormonal strength of Premarin and each of the bioactive components for human ERα and ERβ systems.

E_2 is among the most potent endogenous estrogens and has almost equal binding affinity for human ERα and ERβ, but it is not a major estrogen present in a woman. In fact, it is E_1 or E_3 that

is the quantitatively major estrogen present in a woman under different physiological conditions. Although their binding affinities for ERα and ERβ are lower than E_2, they provide a differential activation of the ERα or ERβ signaling system. Our recent study showed that the endogenous estrogens (such as E_1 and 2-OH-E_1) that are present in a non-pregnant woman would mainly activate the ERα system, whereas the estrogens (such as E_3 and epi-E_3) that are present in pregnant woman would provide a predominant activation of the ERβ system. Therefore, the facile metabolic conversion of E_2 to E_1 or of E_2 to E_3 in a woman provides an important means for achieving differential activation of the ERα or ERβ signaling system under different physiological conditions.

It is suggested that the more suitable estrogens for human HRT would be those that can mimic the physiological estrogenic stimulation in a premenopausal non-pregnant woman, but not that in a pregnant woman. Based on this new concept, it appears that the naturally-occurring estrogens like estrone (E_1) and estrone-3-sulfate (E_1-3-sulfate) would be more suitable for use as postmenopausal HRT than Premarin, which are essentially composed of pregnancy estrogens (with a strong ERβ preference). It is apparent that a balanced activation of the ERα and ERβ systems with a modest preference toward the ERα system would be better for HRT compared to estrogens that would predominantly activate the ERβ system. An optimally-adjusted activation of the ERα and ERβ signaling systems is believed to help maximize the beneficial effects of HRT, in addition to minimizing its untoward effects.

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