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Estrogen receptor-beta agonist diarylpropionitrile counteracts the estrogenic activity of estrogen receptor-alpha agonist propylpyrazole-triol in the mammary gland of ovariectomized Sprague Dawley rats

Xiaozheng Song, Zhong-Zong Pan*

Department of Animal Science, Vermont Cancer Center, University of Vermont, Burlington, VT 05405, USA

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ABSTRACT

Although estrogen can bind both types of estrogen receptors, estrogen receptor-alpha (ERα) is dominant in mediating estrogenic activity in the mammary gland and uterus. Excessive estrogenic activity such as estrogen-based postmenopausal hormone replacement therapy increases the risk for breast and endometrial cancers. The adverse effect of estrogen on uterine endometrium can be opposed by progestins; however, estrogen-plus-progestin regimen imposes substantially greater risk for breast cancer than estrogen alone. In this study, we used ERα-selective agonist propylpyrazole-triol (PPT) and ERβ-selective agonist diarylpropionitrile (DPN) to activate ERα and estrogen receptor-beta (ERβ) separately in an ovariectomized rat model and determined whether PPT-activated ERα function in the mammary gland can be suppressed by DPN activated ERβ. Ovariectomized rats were randomly divided into six groups and treated with DMSO (control), DPN, PPT, PPT/DPN, PPT/Progesterone, and PPT/Progesterone/DPN, respectively. In the mammary gland, PPT but not DPN increased cell proliferation and amphiregulin gene expression; importantly, the stimulatory effect of PPT on mammary cell proliferation and amphiregulin gene expression can be suppressed by DPN. In the uterus, the effect of PPT on uterine weight and endometrial cell proliferation was not inhibited by DPN but can be inhibited by progesterone. These data provide *in vivo* evidence that PPT activated ERα activity in the mammary gland can be opposed by ERβ-selective agonist DPN, which may be explored for the development of better hormone replacement therapy regimen with less risk for breast cancer.

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1. Introduction

Estrogen has profound effects on a broad range of tissues and organs involved in many physiological processes. Drop in estrogen production after menopause is responsible for many postmenopausal symptoms, thus estrogen or estrogen-plus-progestin can be used for hormone replacement therapy (HRT) to ameliorate postmenopausal symptoms. A major adverse effect associated with estrogen-based HRT is the increased risk for breast cancer and uterine endometrial hyperplasia and malignancy [1,2]. The adverse effect of estrogen on uterine endometrium can be opposed by progestins; however, estrogen-plus-progestin HRT regimen imposes substantially greater risk for breast cancer than estrogen alone [1,3–6]. While the two types of estrogen receptors, ERα and ERβ, bind to natural estrogen with similar affinity, ERα is the dominant receptor that mediates the estrogenic responses in most estrogen regulated tissues including the mammary gland and uterus [7–12]. Deregulation of ERα expression and activity accounts for the majority of breast and endometrial cancers. Approximately 70% of breast tumors and 60% of endometrial tumors are ERα-positive tumors [13,14]. In many breast tumors, the percentage of ERα-positive cells is much higher than that in the normal mammary gland [14–17]. Furthermore, ERα may mediate cell proliferation differently in breast tumors. In the normal mammary gland, $ERα^+/Ki67^+$ cells are very rare and it is believed that ERα acts in a paracrine manner to promote neighboring ERα-negative cell to proliferate [11,15,16,18,19]. In ERα-positive breast tumors or cancer cell lines, the percentage of $ERα^+/Ki67^+$ cells are much higher than that in the normal mammary gland and that ERα may directly stimulate ERα-positive cancer cells to proliferate [15,19,20]. Deregulated expression of ERα in transgenic mice leads to mammary tumorigenesis and makes the uterus more susceptible to estrogen induced uterine tumorigenesis [21–23]. Unlike ERα, ERβ is not required for mammary gland and uterus development

Abbreviations: BrdU, 5-bromo-2′-deoxyuridine; BW, body weight; DPN, diarylpropionitrile; ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; HRT, hormone replacement therapy; IF, immunofluorescent; IHC, immunohistochemical; OVX, ovariectomy or ovariectomized; P4, progesterone; PPT, propylpyrazole triol; UWW, uterine wet weight.

* Corresponding author. Tel.: +1 802 656 0134; fax: +1 802 656 8196.
E-mail address: zpan@uvm.edu (Z.Z. Pan).

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[8,9,11,24,25]. Epidemiological studies indicate that ERβ expression is lost or decreased in many breast and endometrial tumors, indicating that ERβ may function as a tumor suppressor [26–28].

The precise mechanism(s) by which estrogen promotes tumorigenesis in the mammary gland and uterine endometrium is not fully understood. A major effect of estrogen on the mammary gland and uterus is to stimulate cell proliferation [29–32]. It has been found that estrogen-based HRT significantly increases breast epithelial cell proliferation in postmenopausal women [33]. Deregulation of cell proliferation by oncogenes and tumor suppressors is one of the hallmarks of cancer cells [34,35]. Consistent with its role in breast and endometrial malignancy, ERα is essential and sufficient to mediate estrogen induced cell proliferation [8,11,30,36,37]. In contrast to the positive role of ERα in cell proliferation, ERβ may function as a negative regulator of cell proliferation. Loss of ERβ could lead to increased cell proliferation, whereas overexpression of ERβ has been found to inhibit cell proliferation and xenograft tumor formation in several breast and endometrial cell lines [8,24,38–46]. The molecular mechanism of ERβ action is not fully understood [9,47,48]. Studies using *in vitro* cell lines have demonstrated that ERβ can antagonize ERα in gene expression, cell cycle progression, and cell proliferation [42–45,49–51]. ERα and ERβ may form a subtle balance to regulate estrogen signaling in mammary and endometrial cell proliferation, loss of the balance may lead to tumor initiation and progression [52].

In addition to genetic modification of estrogen receptor expression, ER-selective agonists have been developed to determine the biological functions of ERα and ERβ [9,47,53–55]. These ER-selective agonists may also be used for pharmacological interventions of estrogenic activity [9,53,55]. Despite the significance of estrogenic activity in mammary cell proliferation and tumorigenesis and that ERβ may function as a tumor suppressor, *in vivo* studies of the ERβ-selective agonists in the mammary gland are very limited [30,56,57]. It remains unknown whether endogenous ERβ can be activated to function as a tumor suppressor in the mammary gland *in vivo*. In this study, we used ER-selective agonists propylpyrazole triol (PPT) and diarylpropionitrile (DPN) to separately activate ERα and ERβ in an ovariectomized (OVX) rat model and determined whether ERα-mediated estrogenic activity in the mammary gland can be inhibited by DPN activated ERβ. In receptor competition binding assay for binding affinity relative to estradiol, PPT is an ERα-selective agonist that has a 410 fold higher relative binding affinity to ERα than to ERβ; DPN has a 70 fold higher relative binding affinity to ERβ than to ERα [58,59]. We demonstrated that ERα-mediated estrogenic activity in the mammary gland can be opposed by the ERβ-selective agonist DPN *in vivo*, suggesting that ERβ-selective agonists such as DPN may be explored for the development of better HRT regimens to reduce or eradiate the risk for breast cancer.

2. Methods

2.1. Animals

All animal experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Vermont. Ovariectomized (OVX) virgin female Sprague Dawley rats (Charles River - Canada) were housed with a 12-h light and dark cycle and ad libitum access to food and water. Rats were ovariectomized at 5–6 weeks old and rested for two weeks before treatment. Five to six rats were randomly assigned to each group and totally there are six groups, the control group, the DPN group, the PPT group, the PPT/DPN group, the PPT-plus-progesterone (PPT/P4) group, and the PPT/P4/DPN group. PPT, DPN, and progesterone were obtained from Tocris Bioscience and were dissolved in DMSO for stock solution. The drugs were administered by i.p. injection once a day for three consecutive days; the control group rats received the vehicle DMSO only. The dosage of the different drugs used in this study was as follows: PPT at 500 μg/kg BW (body weight), DPN at 1000 μg/kg BW, P4 at 20 mg/kg BW. BrdU (5-bromo-2′-deoxyuridine, from Sigma) solution in PBS was injected (i.p., 20 mg/rat/d) at the same time when the drug(s) was administered. Rats were sacrificed 16 h after the last injection for biopsy sample collection. The timing of treatment and biopsy after the last treatment was chosen based on other studies. In the literature, various lengths of treatment ranging from a couple of hours to several weeks were used for the evaluation of different endpoint parameters [30,31,37,56,57]. The primary endpoint of evaluation in this study was cell proliferation rate, the three day treatment period was chosen as it has been shown in several studies that two to three day treatment significantly increased mammary cell proliferation rate [30,31]. Another reason that we did not choose shorter than three days is the concern that the percentage of proliferating cells in OVX rats induced by shorter treatment period would be too low to allow the detection of any inhibitory effect. The drugs were administered in the afternoon for all three treatments; for the last treatment, drug injections for different animals (with ear tag numbers) were administered at 15 min intervals so that each individual animal was killed at 16 h post the last treatment for biopsy sample collection. Time course studies have shown that estrogen treatment for as short as 4 h significantly increased the percentage of cyclin D-staining cells in the mammary gland; in our previous studies using ERα-positive MCF-7 cell line treated with estrogen, we noticed that the percentage of cells with the Ki-67 proliferation marker started to increase around 12 h [20,30]. Based on these time course studies, we expected that the effect from the last treatment can be detected 16 h later. For mammary gland biopsy, the fourth pair of mammary glands was harvested from each rat and weighed. The right-side was fixed in neutral formalin for 48 h before being processed for paraffin embedding. The left-side was snap-frozen and stored in liquid nitrogen for RNA isolation. The uterus from each rat was first measured for uterine wet weight (UWW) and then fixed in neutral formalin for 24–48 h before being processed for paraffin embedding.

The dosage selection for this study was based on the dosages used by other studies, the relative binding affinity, and the relative transcriptional activity via ERE (estrogen response element) [12,31,36,37,43,56,58–63]. PPT from 50 μg/d/rat to 1000 μg/d/rat was shown with very good response in the uterine endometrium [36]. The body weight of the rats in this study was approximately 200 g, therefore the dose per rat was about 100 μg/d/rat for PPT, 200 μg/d/rat for DPN, and 4 mg/d/rat for progesterone. In the transcriptional activity assay using the U2OS cell system, it was shown that the maximal activity stimulated by PPT was comparable to that by estradiol, and that the EC50 for estradiol via ERα was 8 pM and the EC50 for PPT via ERα was 140 pM [43]. The ratio of 8 pM estradiol/140 pM PPT can be converted as 20 μg/kg BW estradiol/500 μg/kg BW PPT, a dosage that were expected to be functional in the mammary gland as well [30,31,56,64]. The binding affinity of PPT to ERα is approximately 49% of that of estradiol to ERα, or the conversion of 500 μg/kg BW PPT to 176 μg/kg BW estradiol [59]. DPN at 1000 μg/kg BW was within the range used by other studies for its effect on uterus, hot flush, osteoporosis, and cardioprotection [60–62,65–67]. The binding affinity of DPN to ERβ is approximately 18% of that of estradiol to ERβ, or the conversion of 1000 μg/kg BW DPN to 205 μg/kg BW estradiol [58]. Based on these calculations, the theoretically converted PPT and DPN (to estradiol) would have DPN binding to ERβ and PPT binding to ERα at a comparable level. Considering that estradiol may have a two to ten-fold higher binding affinity for ERα than for ERβ, the ratio of DPN-ERβ/PPT-ERα could be lower than the 1:1 ratio [12,43,63]. The binding affinity of PPT to ERβ is 0.12% of that of estradiol to ERβ, or

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