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Effect of Estradiol and Selected Antiestrogens on Pro- and Antioxidant Pathways in Mammalian Uterus

Sudhir Jain,* Deeksha Saxena,* Pradeep G. Kumar,* Samuel S. Koide,† and Malini Laloraya*

In this study, we examined the effect of 17β-estradiol and selected antiestrogens on uterine NADPH-oxidase activity, superoxide dismutase (SOD) activity, hydride ($H^{\cdot -}$), dienyl radical and $O_2^{\cdot -}$-radical generation, and membrane fluidity. NADPH oxidase activity was positively modulated in estradiol-treated animals and negatively regulated in animals that received injections of AF-45, RU-39411, tamoxifen, or ICI-182780. The SOD activity was markedly reduced in estradiol-treated animals when compared with the control animals. A positive modulation of SOD activity was observed upon treatment with AF45, RU39411, tamoxifen, and ICI 182780, though the potency varied among the individual test compounds. We observed detectable $H^{\cdot -}$-radical generation as evidenced from MNP-$H^{\cdot}$ adduct formation in the uterine cell preparations from untreated control animals. Estradiol produced a tremendous augmentation in the superoxide radical profiles in uterine cell preparations compared to the control levels. All the other compounds that were tested significantly lowered the superoxide levels in the test set-up. AF-45, RU-39411, tamoxifen, and ICI-182780 induced varying orders of suppression of $H^{\cdot -}$-radical generation in the test subjects. There was a significant enhancement in membrane fluidity, hydride radical levels, and dienyl radical generation in the estradiol-treated group. All the antiestrogens did not exhibit a similar action on these parameters. RU-39411 exhibited antiestrogen-like activity in modulating hydride levels and membrane fluidity, whereas it stimulated dienyl radical generation. Thus our tests showed that the selected antiestrogens failed to show estrogen-like activity in these assays. It appears that estradiol exerts feedback control over pro- and antioxidant pathways and that markers of oxidative status could be used as a measure to evaluate the antiestrogenic activity of estradiol agonists/antagonists. CONTRACEPTION 1999;60: 111–118 © *1999 Elsevier Science Inc. All rights reserved.*

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Introduction

Estrogens regulate growth and cell division in target tissues such as the uterus. The process of regulation is thought to involve the initial binding to high-affinity nuclear receptors, which, in turn, bind to specific DNA sequences that regulate gene transcription.[1] The actual series of events that mediate the mitogenic effect of estrogen in the uterus remains largely unknown.[2] One theory suggests that estrogens act directly as a mitogen on target cells. Another hypothesis is that estrogens promote uterine growth indirectly via a local (paracrine, autocrine) or systemic (endocrine) regulation of levels of certain growth factors, their receptors, or both.[2] The rodent uterus has been widely used as a model system to study interactions between estrogens and their intracellular receptors and the influence of estrogens on target organ growth.[3,4] In some estrogen target organs, 17β-estradiol induces the *de novo* synthesis of specific proteins that can be used as specific molecular markers of hormone action.[5–7] Estrogens have been observed to increase the overall rate of protein synthesis in the uterus,[8] and many laboratories have searched for molecular markers of estrogen action in the uterus. Several uterine proteins have been suggested as markers of estrogen action: peroxidase,[9] hydrolase,[10] plasminogen activator,[11] glucose-6-phosphate dehydrogenase,[12] estrogen receptor,[13] progesterone receptor,[14] and induced protein (IP).[15–17]

There are important practical consequences to further investigations of estrogen action related to the role of these hormones, because blockade of estrogen action appears to be a key to the control of certain malignancies and to the control of fertility. Directly related to this is the controversy surrounding the use of antiestrogens like tamoxifen.[18,19] Tamoxifen is representative of the most studied class of antiestrogens; typically, such agents are nonsteroidal in structure and manifest complex pharmacology encompas-

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sing variable tissue-, cell-, and gene-specific effects from estrogen-like actions to complete blockade of estrogen action characteristic of pure antagonism.[20,21] The advent of molecules that bind ER with a high affinity without activating receptor signaling provided for the first time the opportunity to study in an unequivocal manner the consequences of a full blockade of estrogen action.[22] ICI 182780 is a pure antiestrogen that blocks estrogen action in vitro and in vivo in a concentration (dose)-dependent and complete manner, whereas in the absence of endogenous estrogens this agent alone produces no estrogen-like effects.[23,24] Similarly, the hormonal properties of anordrin, an antiestrogen that has been found to have a postcoital antifertility effect in women and some laboratory animals,[25–27] or its 1000-times more potent metabolite anordiol (AF 45) in terms of antiestrogenic activity are not yet fully understood. RU39411, an antiestrogen that had long been thought to possess only antagonistic properties, was recently shown to possess some weak, estrogen agonist effects on the oviduct.[28]

In some of our recent reports, we demonstrated early pregnancy-associated elevation in free radical generation and the loss of superoxide dismutase (SOD) activity in mouse uteri,[29] the resulting lipid-kinking,[30] and the increase in membrane fluidity[31] as important events in mediating blastocyst implantation. Because preimplantation embryo development and the embryo implantation in the uterus are largely controlled by ovarian steroids, an examination of the effects of selected estrogenic/antiestrogenic drugs on the above biophysical and biochemical aspects of mammalian uterus during early pregnancy could yield more molecular insight into the mechanism of action of estrogens and could define the estrogenic or antiestrogenic nature of the drugs in question using novel parameters. This article reports our observations from some of the experiments designed in this line.

Materials and Methods

Reagents

Trizma HCl, Trizma base, diethyl dithiocarbamic acid (DDC), diethylene triamine penta acetic acid (DTPA), and NADPH and N-t-butyl-α-phenyl nitrone (PBN) were purchased from Sigma Chemical (St. Louis, MO). 16-Doxyl stearate (16-NS) and 2-methyl-2-nitrosopropane (MNP) were obtained from Aldrich Chemical (Milwaukee, WI). The following drugs were used: anordiol (AF-45, 2α,17α-diethynyl-A-nor-5α-androstane-2β,17β-diol) (Shanghai Institute of Materia Medica, China); tamoxifen ([Z]-2-[4-{1,2-diphenyl-1-butenyl}-phenoxyl]-N,N-dimethylethaneamine, Astra-Zeneca Pharmaceuticals, Chesire, UK); ICI 182780 {7α-(9-4,4,5,5,5-pentafluoropenyl sulphenyl)nonyl-estra-1,3,4(10-triene-3,17β-diol)} from Astra-Zeneca Pharmaceuticals, Chesire, England; RU39411 (3,17ψ-dihydroxy-11β-(4-dimethylaminoethoxyphenyl)estra-1,3,5-triene) Roussel-Uclaf, France, and 17β-estradiol (1,3,5[10]-estratriene-3,17β-diol, Sigma Chemical, St. Louis, MO). All other reagents were of analytical grade, procured from local suppliers.

Animals

Immature female mice (*Mus musculus*, Swiss strain), aged 4–6 weeks, bred in our institute animal facility were used for all experiments. The experimental animals were housed at controlled temperature (27°C ± 1°C) and light (14 h light, 10 h dark) regimens at the facility.

Animals were divided into the following treatment groups: group I (vehicle alone, control), group II (17β-estradiol), group III (tamoxifen), group IV (RU-39411), group V (AF-45), and group VI (ICI-182780). Groups II–V were divided into three subgroups each for testing the dose response of the drugs under investigation. Thus, group II has three subgroups classified as low dose (0.24 μg/50 μL/day), medium dose (0.60 μg/50 μL/day), and high dose (1.50 μg/50 μL/day). AF-45, RU-39411, and ICI-182780 were all tested at three doses: low dose (8 μg/50 μL/day), medium dose (20 μg/50 μL/day), and high dose (50 μg/50 μL/day). The drugs were delivered through subcutaneous injections at 9:00 AM for 3 consecutive days and the animals were killed at 9:00 AM on day 4 by CO_2-asphyxiation. The uteri were excised and cleared of adhering fat tissues and blood vessels, and were then washed in five changes of physiological saline.

Assay of superoxide dismutase SOD activity

SOD was extracted as described in one of our earlier reports.[29] SOD activity was assayed according to the method of Marklund and Marklund,[32] which uses the inhibition in the rate of autoxidation of pyrogallol as a measure of the activity of SOD. Pyrogallol autoxidizes in solution with simultaneous production of $O_2^{\cdot}$-anion radical, which, in turn oxidizes other pyrogallol molecules. The oxidized form of pyrogallol gives an absorption band at 420 nm. Addition of SOD into the system greatly slows down the observed rate of autoxidation of pyrogallol, which is used in the assay of SOD by this method. The enzyme kinetics were monitored on an SLM-AMINCO DW-2000 spectrophotometer. All calculations were made based on per milligram fresh weight of the tissue.

Spin trapping of oxyradicals

The superoxide radical was detected by incorporating DDC as an inhibitor of SOD into the isolation medium and then trapping the free radical with PBN.[29] The hydride radical ($H^{\cdot -}$) was trapped as $H^{\cdot -}$-MNP (methyl nitroso propane) adduct, by incubating the tissue homogenate with 20 mmol/L MNP (final concentration) for 30 min. After incubation, 50-μL aliquots were transferred into glass capillaries and one end flame-sealed. EPR spectra of PBN-$O_2^{\cdot -}$ adduct and H-MNP adduct were recorded on a Varian E-104 EPR with TM_{110} cavity. Instrumental settings employed were: scan range 100 G, time constant 1 sec, modulation amplitude 4G, receiver gain $2.5 \times 10^4 \times 10$, microwave power 5 mW, field set 3237G, scan time 8 min, modulation frequency 100 Hz, temperature 27°C, and microwave frequency 9.01 GHz. The EPR absorption line intensities of the low field, mid-field, and high field lines were calculated employing the equation $I = kw^2h$ (where $k = 6.51 \times 10^{-10}$, w = line width, h = line height,[29] which served as a measure to compare the quantity of the radical trapped.

Measuring NADPH oxidase activity

The NADPH oxidase activity was measured using a direct method by estimating the NADPH-induced enhancement in the superoxide production. Each tissue homogenate was divided into two aliquots. One aliquot was incubated with a spin trap PBN for quantifying the basal superoxide anion radical production. The second aliquot was incubated with PBN in the presence of an excess supplement (20 mmol/L, final concentration) of NADPH. In both cases, incubation was carried out for 1 h, after which the samples were loaded into glass capillary tubes and the superoxide quantification was performed using standard spin resonance enhancement protocols. The difference in the spectral response between the NADPH-deficient and NADPH-supplemented incubations was indicative of the NADPH-oxidase activity.

Spin labeling protocols

The uterine horns (50 mg of tissue) were mildly sheared in 1 mL of HBSS. A 200 μL aliquot of each of the preparations was incubated with 2.6×10^{-4} mol/L (final concentration) of the spin label 5-doxyl stearate. After 10 min, $NiCl_2$ at a final concentration of 50 mmol/L was added into the incubation to remove the unincorporated spin label signals and the incubation was continued for another 20 min. After the incubation, the preparations were diluted to 2 mL with HBSS and were centrifuged at 300 *g* for 10 min to harvest the cells. The pellet was resuspended in 200 μL HBSS. From this suspension, approximately 50 μL was transferred into glass capillary tubes and was flame-sealed. The EPR spectra were recorded on a Varian E-104 EPR with TM_{110} cavity. Instrumental settings employed were: scan range 100 G, time constant 1.0 sec, modulation amplitude 2 G, receiver gain $2.5 \times 10^4 \times 10$, microwave power 5 mW, field set 3237 G, scan time 4 min, modulation frequency 100 Hz., temperature 27°C, and microwave frequency 9.01 GHz. The rotational correlation time t_c was calculated using the relation $t_c = kw_o[(h_o/h_{+1)}1/2-1]$, where w_0 is the width, h_o and h_{+1} are the mid field and low field line heights, and k is a constant with a numerical value of 6.51×10^{-10}.

Statistical analysis

All the experiments were repeated five times. Observations from the estradiol-treated group were compared with their respective controls. Values obtained in the antiestrogen and RU-39411 groups were compared with their respective dosage counterparts from the estradiol-treated group. One-way analysis of variance (ANOVA) was run using Introductory Statistical Software Package, version 1.0.[33]

Results

The total SOD activity in the uteri of immature mice was lowered due to administration of estradiol. Although the lowest dose (0.24 μg/day/animal) of estradiol did not bring about a statistically significant decline in SOD activity, the higher doses brought about significant reduction in the activity of this enzyme. Tamoxifen, ICI-182780, and AF-45 reflected a dose-dependent increase in SOD activity. Interestingly, RU-39411 also caused an increase in the SOD activity in the uterus of immature mice in a dose-dependent fashion (Figure 1).

On the contrary, estradiol resulted in an increase in the NADPH oxidase activity when compared with the respective control values. The antiestrogens were effective in suppressing the NADPH oxidase activity in a dose-related fashion. RU-39411 was ineffective in affecting the NADPH oxidase activity at the lowest concentration tested on comparison with estradiol treated groups. However, at higher doses, RU-39411 also introduced inhibition in NADPH oxidase activity (Figure 2).

The quantitative line intensity responses of PBN-$O_2^{\cdot -}$ adduct are plotted graphically in Figure 3. As could be seen in this exhibit, estradiol produced significant augmentation in the superoxide radical profiles in uterine cell preparations when compared with the control levels. All the other compounds tested significantly lowered ($p < 0.01$) the superoxide levels in the test set-up and their effects were dose-dependent.

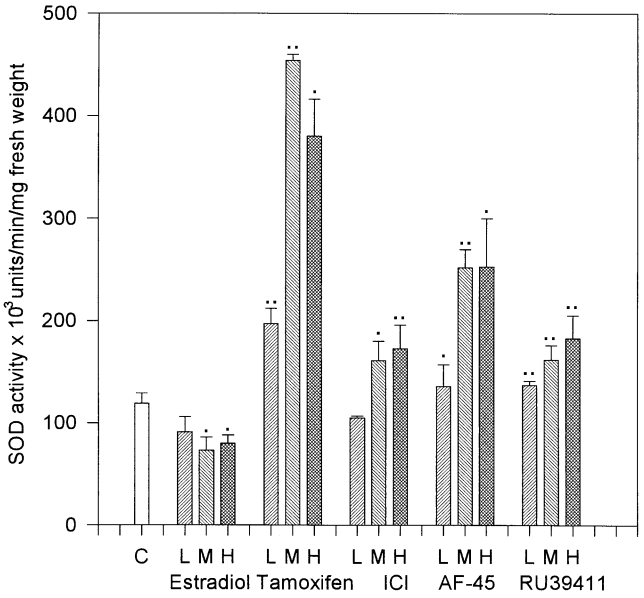

Figure 1. Effect of lower (L), medium (M), and higher (H) doses of each of estradiol, tamoxifen, ICI-182780, AF-45, and RU-39411 on uterine SOD activity. Values indicated are mean ± SEM of five independent observations. ˙p <0.05 and ¨p <0.01.

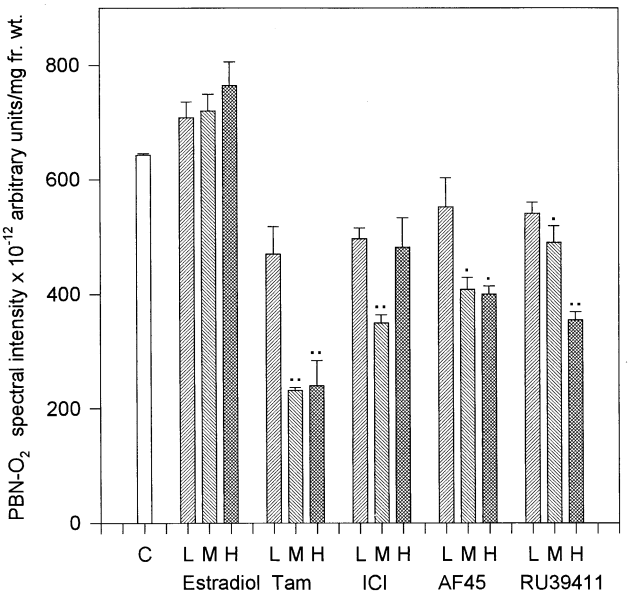

Figure 3. EPR spectral intensities of PBN-O_2^- adduct measured from immature mice uteri in response to the lower (L), medium (M), and higher (H) doses of each of estradiol, tamoxifen, ICI-182780, AF-45, and RU-39411. Error bars represent standard error of five independent observations. ˙p <0.05 and ¨p <0.01.

Spin trapping with MNP appeared to produce a composite spectrum of two radical species. As expected, MNP-dienyl radical generated a typical three-line spectrum with aN = 17.1 G. Estradiol administration augmented the dienyl radical production. The antiestrogens resulted in dose-dependent inhibition in the amount of dienyl radical formed. It is noteworthy that RU-39411 had a stimulatory effect on the production of dienyl radicals (Figure 4).

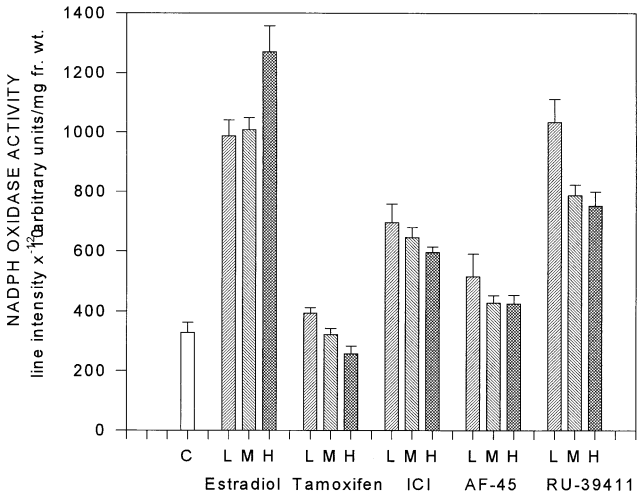

Figure 2. Effect of lower (L), medium (M), and higher (H) doses of each of estradiol, tamoxifen, ICI-182780, AF-45, and RU-39411 on uterine NADPH oxidase activity. Values indicated are mean × SEM of five independent observations. ˙p <0.05 and ¨p <0.01.

Superimposed over the MNP-dienyl radical spectrum, we also observed a four-line spectrum with a line intensity ratio of 1:2:2:1 signifying a spectral quality of aN = aHb = 14.5G. This signal appeared to have originated from a possible reaction between reduced MNP with hydride radical ($H^{\cdot -}$). To verify this assumption, we prepared mouse liver mitochondria into which MNP was introduced (a known system to bring about MNP reduction followed by $H^{\cdot -}$ trapping). A representative spectrum obtained is shown in Figure 5a. Similar trace originating from a mouse uterine preparation also is provided for comparison (Figure 5b). Treatment with estradiol brought about gross augmentation in the generation of MNP-$H^{\cdot -}$ adduct formation. Tamoxifen and ICI-182780 augmented MNP-$H^{\cdot -}$ adduct formation at lower doses, whereas the effect was reversed at higher doses. RU-39411 resulted in dose-dependent reduction in the amount of $H^{\cdot}$ generated (Figure 6).

An analysis of the membrane fluidity profile in terms of the rotational freedom of a stearic acid spin label inserted into the lipophilic domains of the uterine cell membrane showed a significant enhancement in membrane fluidity in estradiol-treated group

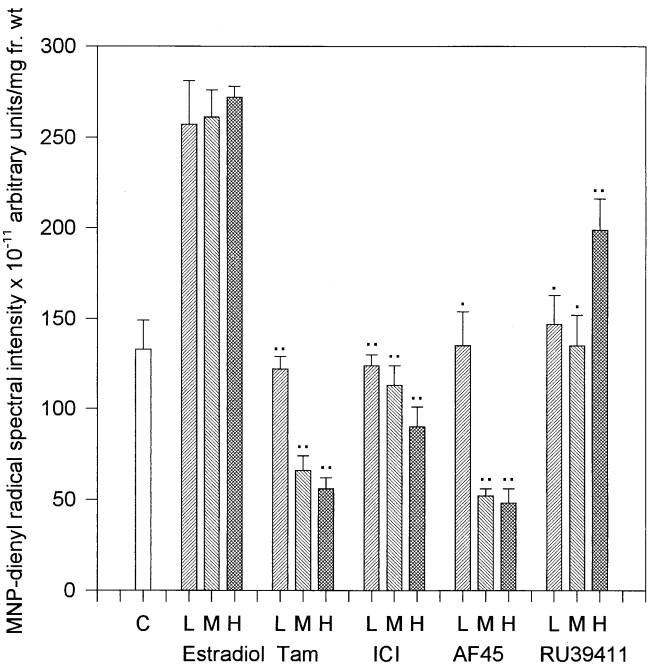

Figure 4. EPR spectral intensities of MNP-dienyl radical adduct measured from immature mice uteri in response to the lower (L), medium (M), and higher (H) doses of each of estradiol, tamoxifen, ICI-182780, AF-45, and RU-39411. Error bars represent standard error of five independent observations. ˙p <0.05 and ¨p <0.01.

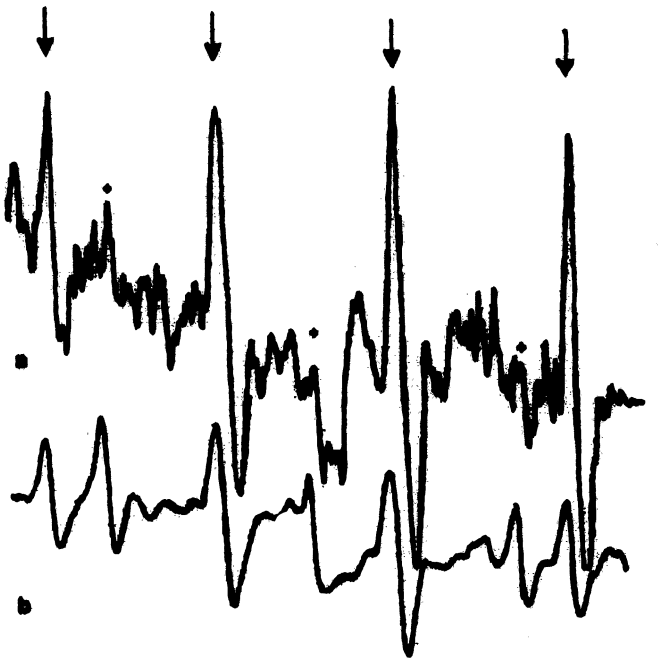

Figure 5. EPR spectra of MNP-H˙ radical adduct from (a) mouse liver mitochondria (a known spectrum for MNP reduction following hydride radical trapping) and (b) from mouse uterine preparations. Peaks representing MNP-$H^{\cdot -}$ radical intensities are shown by arrows and the one with MNP-dienyl radical adducts are marked with a + sign.

in a precise dose-dependent manner. All the other test compounds had a reverse action. The data are summarized in Figure 7.

Discussion

The overall aim in this study was to quantitate the profiles of SOD, NADPH oxidase, superoxide anion, hydride radical, and uterine membrane fluidity associated with the uterine cells of immature mice primed with 17β-estradiol. We also attempted to compare these observations with the observation in the effects of selected antiestrogens AF45, tamoxifen, ICI182780, and RU39411, known to have both estrogenic as well as antiestrogenic activity, on the above said parameters. We could make a major interpretation that all the three antiestrogenic compounds (AF45, tamoxifen, and ICI182780) failed to show biochemically similar effects as were introduced by estradiol on these parameters in our assays. The main manifestations were that estradiol could augment the levels of NADPH oxidase activity, superoxide anion radical, dienyl radical levels, and uterine membrane fluidity and could lower the SOD activity. On the contrary, the antiestrogenic compounds tested brought about exactly opposite effects in a dose-dependent fashion. With the exception that it stimulated dienyl radical generation, RU-39411 appeared to show antiestrogen-like activity in all the other parameters studied.

The MNP-$H^{\cdot -}$ radical adduct could be formed in the system as follows:

$$\text{MNP} \xrightarrow[\text{reduction}]{\text{microsomal}} \text{MNP}^{+} + e^{-}$$

$$e^{-} + H^{+} \longrightarrow H^{\cdot -}$$

$$\text{MNP}^{+} + H^{\cdot -} \longrightarrow \text{MNP} - H^{\cdot -}$$

Based on our understanding, we would like to state that this is the first report documenting hormonal dependence of hydride radical generation in a biological system. A recent investigation documented a membrane-bound energy-transducing nicotinamide nucleotide transhydrogenase that catalyzes the direct transfer of a hydride ion between NAD(H) and NADP(H) in a reaction that is coupled to transmembrane proton translocation.[34]

The parallel changes in the levels of NADPH oxidase and superoxide anion radical profiles might suggest the activation of NADPH oxidase by estradiol,

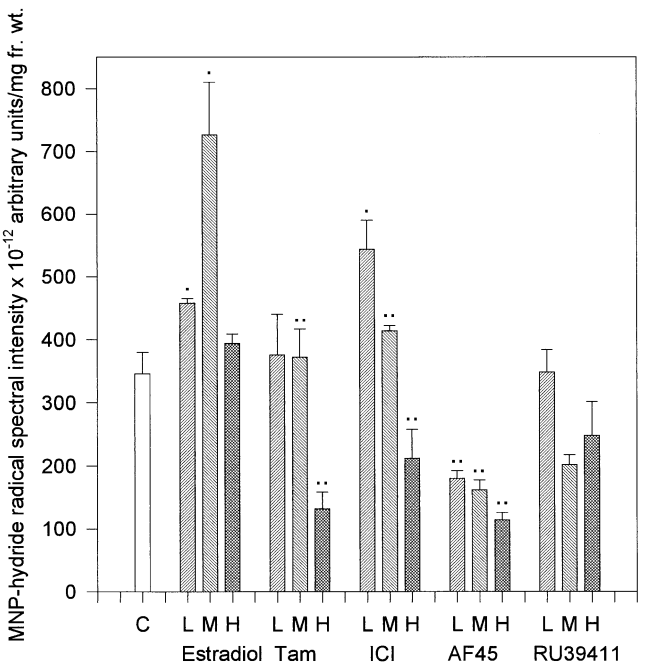

Figure 6. EPR spectral intensities of MNP-H·radical adduct measured from immature mice uteri in response to the lower (L), medium (M), and higher (H) doses of each of estradiol, tamoxifen, ICI-182780, AF-45, and RU-39411. Values indicated are mean ± SEM of five independent observations. · p <0.05 and ¨ p <0.01.

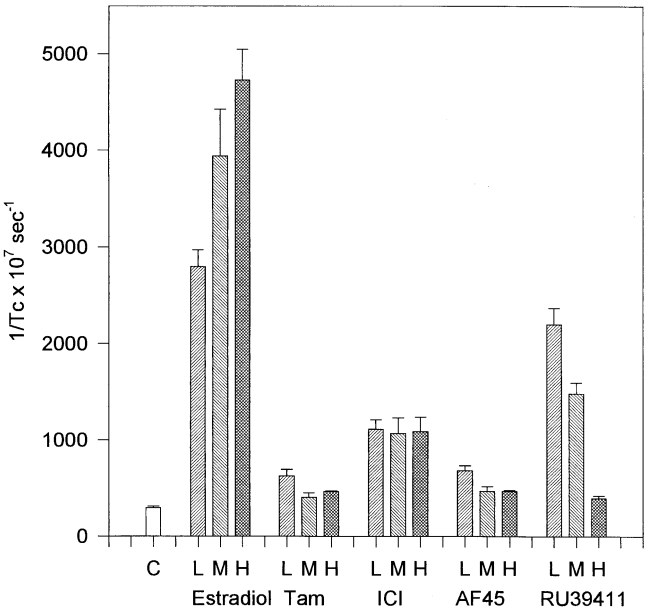

Figure 7. Effect of lower (L), medium (M) and higher (H) doses of each of estradiol, tamoxifen, ICI-182780, AF-45, and RU-39411 on uterine membrane fluidity. Values indicated are mean ± SEM of five independent observations. · p <0.05 and ¨ p <0.01.

leading to the observed augmentation of superoxide anion radical generation. The negative modulation of SOD activity could enhance the kinetics of superoxide accumulation in response to a given amount of estradiol. In that event, the accumulating $O_2^{\cdot -}$ could mediate membrane modifications as demonstrated by us in our earlier report[30,31] introducing phase transitions. An ESR study had demonstrated that 17β-estradiol is metabolized by lactoperoxidase to its phenoxyl radical intermediate, which could abstract hydrogen from NADH to form $NAD^{\cdot -}$ and that $NAD^{\cdot -}$ radical reduces molecular oxygen to superoxide radical.[35] A study on the estrogenic induction of NADPH diaphorase activity in the preoptic neurons containing estrogen receptor immunoreactivity in female rats showed that NADPH diaphorase could be positively regulated by estradiol in neurons containing estrogen receptor.[36] In nonpregnant sheep, the NADPH diaphorase activity was enhanced by estrogen in the uterine arteries.[37]

Studies have shown that both estrogens and antiestrogens bind at the same site of the steroid binding domain of the estradiol receptor (at the extreme carboxyl terminus, presumably at cystein 530),[38] the antiestrogens can modulate estradiol-dependent growth and differentiation of estradiol responsive cells through two known mechanisms: 1) altering the estradiol receptor conformation and reducing its affinity for estradiol,[39] and 2) failing to stimulate estradiol-dependent transcription at the response elements on the target genes.[40]

Studies carried out to explain the extra-estrogen receptor action of tamoxifen on protein kinase C (PKC) inhibition revealed that tamoxifen partitioned into the membrane, and that there was no appreciable covalent association of tamoxifen with cellular proteins. Various antioxidants (vitamin E, vitamin C, β-carotene, catalase, and SOD) inhibited all these cellular effects of tamoxifen, suggesting that tamoxifen, by initially partitioning into the membranes, induced a generation of transmembrane signals and an oxidative stress to elicit the membrane association of PK.[41] Based on our results, we could interpret that both estradiol and the antiestrogenic agents partitioned into the endometrial membranes inducing perturbations in their lipophilic microenvironment. Estradiol induced a profound melting of endometrial membranes, whereas the antiestrogens lacked this property (Figure 7).

A recent report demonstrated that treatment of cultured rat granulosa cells with SOD, FSH, or forskolin resulted in a small but significant increase in cGMP concentrations, and that cotreatment of cells

with FSH plus SOD as well as forskolin plus SOD had a synergistic effect on cGMP content, increasing cGMP levels >100-fold The findings that SOD and activators of the cAMP-dependent signaling pathway synergistically increase the levels of the second messenger cGMP and that dibutryl cGMP attenuated FSH-, forskolin-, and cAMP-induced aromatase activity suggested a potential mechanism of SOD action and demonstrated the antagonistic action of cGMP on cAMP-mediated estrogen production.[42] In our studies, we observed a significant decrease in SOD activity in the uterine tissue after the administration of estradiol and a significant elevation in SOD activity as a result of antiestrogen treatment (Figure 1). Thus, it appears likely that the SOD and estradiol levels are connected via a negative feedback regulation. But, at this point of time, it is difficult to explain the observed down-regulation of SOD by estradiol. But it is quite likely that the estrogen receptor bound to antiestrogen could have subtle conformational changes when compared to estrogen receptor bound to estrogen. The antiestrogen induced conformation might bind specifically to some response elements to modulate transcription.[43]

Considering our observations collectively, estradiol action on mammalian uterus elevates NADPH oxidase activity, augments superoxide anion, dienyl, and hydride radical production, lowers SOD activity, and enhances the membrane fluidity. Tamoxifen, AF-45, and ICI-182780 did not exhibit estrogen-like action in terms of the parameters of our assays. We believe that the biological activity of these antiestrogens should be examined using these novel estradiol assay parameters.

Acknowledgments

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References

1. Yamamoto KR. Steroid receptor regulation of transcription of specific genes and gene networks. Ann Rev Genet 1985;19:209–52.
2. Newbold RR, Hanson RB, Jefferson WN. Immature mouse uterine tissue in organ culture: estrogen-induced growth, morphology and biochemical parameters. In Vitro Cell Dev Biol 1994;30A:519–28.
3. Katzenellerbogen BS, Gorski J. Estrogen actions on synthesis of macromolecules in target cells. In: Litwack G, ed. Biochemical actions of hormones. New York: Academic Press; 1975:187.
4. Markaverich BM, Upchurch S, Clark JH. Two binding sites for estradiol in rat uterine growth. In: Bresciani F, ed. Perspective in steroid receptor research. New York: Raven Press; 1980.
5. Harris SE, Rosen JM, Means AR, O'Malley BW. Use of a specific probe for ovalbumin messenger RNA to quantitate estrogen-induced gene transcripts. Biochemsitry 1975;14:20272–81.
6. Deeley RG, Goldberg RF. Regulation of expression of the vitellogenin gene in avian liver. In: Hamilton TH, Clark JH, Sadle WA, eds. Ontogery receptors and reproductive hormone action. New York: Raven Press; 1979.
7. Westley B, Rochefort H. A secreted glycoprotein induced by estrogen in human breast cancer lines. Cell 1980;20:353–62.
8. Noteboom WD, Gorski J. An early effect of estrogen on protein synthesis. Proc Natl Acad Sci USA. 1963;50: 250.
9. Lyttle CR, DeSombre ER. Uterine peroxidase as a marker for estrogen action. Proc Natl Acad Sci USA. 1977;74:3162–6.
10. Finlay TH, Katz J, Levitz M. Purification and properties of an estrogen-stimulated hydrolase from mouse uterus. J Biol Chem 1982;257:10914–9.
11. Kneifel MA, Leytus SP, Fletcher E, Weber T, Mangel WF, Katzenellenbogen BS. Uterine plasminogen activator activity modulation by steroid hormones. Endocrinology 1982;111:493–9.
12. Moulton BC, Barker KL. Synthesis and degradation of glucose-6-phosphate dehydrogenase in the rat uterus. Endocrinology 1971;89:1131–6.
13. Sarff M, Gorski J. Control of estrogen-binding protein concentration under basal conditions and after estrogen administration. J Biochemistry 1971;10:2557–63.
14. Leavitt WW, Toft DO, Strott CA, O'Malley BW. A specific progesterone-receptor in the hamster uterus: physiologic properties and regulation during the estrous cycle. Endocrinology 1974;94:1041–53.
15. Manak R, Wertz N, Slabaugh M, Denari H, Wang J-T, Gorski J. Purification and characterization of the estrogen-induced protein (i.p.) of the uterus. Mol. Cell Endocrinol. 1980;17:119–32.
16. Skipper JK, Eakle SD, Hamilton TH. Modulation by estrogen of synthesis of specific uterine proteins. Cell 1980;22:69–78.
17. Reiss NA, Kaye AM. Identification of the major components of the estrogen-induced protein of rat uterus as the BB isozyme creatine kinase. J Biol Chem 1981;256: 5741–9.
18. Fisher B, Constantino JP, Redmond CK, Fisher ER, Wickerham DL, Cronin WMJ. Endometrial cancer in tamoxifen-treated breast cancer patients: findings from the national surgical adjuvant breast and bowel project (NSABP) B-14. Natl Cancer Inst 1994;86:527–37.
19. Kedar RP, Bourne TH, Powles TJ, Collins WP, Ashley SE, Cosgrovo DO, Campbell S. Effects of tamoxifen on uterus and ovaries of postmenopausal women in a randomised breast cancer prevention trial. Lancet 1994; 343:1318–21.
20. Wakeling AE. Pharmacology of antiestrogens. Furr BJA, Wakeling AE, eds. Pharmacology and clinical uses of inhibitors of hormone secretion and action. London: Bailliere Tindall, 1987:1–19.
21. Jordan VC. Biochemical pharmacology of antiestrogen action. Pharmacol Rev 1984;36:245–76.
22. Wakeling AE. Use of pure antiestrogens to elucidate the mode of action of estrogens. Biochem Pharmacol 1995; 49:1545–9.

23. Wakeling AE, Bowler J. Novel antiestrogens without partial agonist activity. J Steroid Biochem 1991;31:645–53.
24. Wakeling AE, Dukes M, Bowler J. A potent specific pure antiestrogen with clinical potential. Cancer Res 1991;51:3867–73.
25. Chin-ping K, Ming-kang C, Hsui-chuan C, Shihi-hsing C, Ta-wei P, Kang T. Pharmacological studies of a contraceptive drug anordrin. Chinese Med J 1976;2: 177–4.
26. Crabbe P, Fillion H, Letourneaux Y, Diczfalusy E, Aedo AR, Goldzieher JW, Shaikh AA, Castracane VD. Chemical synthesis and bioassay of anordrin and dinordrin I and II. Steroids 1979;33:85–96.
27. Mehta RR, Jenco JM, Chatterton RTJ. Antiestrogenic and antifertility actions of anordrin (2-alpha, 17-alpha diethynyl-A-nor-5 alpha-androstane-2beta-diol 2,17-dipropionate). Steroids 1981;38:679.
28. Kaplankraicer R, Kostovetsky I, Bardin CW, Shalgi R. Tamoxifen and RU39411 synergise with mifepristone to produce preimplantation pregnancy loss by increasing embryo transport in rat. Contraception 1996;53: 367–73.
29. Laloraya M, Kumar GP, Laloraya MM. Changes in the levels of superoxide anion radical and superoxide dismutase during the estrus cycle of *Rattus norvegicus* and the induction of superoxide dismutase in rat ovary by lutropin. Biochem Biophys Res Commun 1988;157: 146–53.
30. Jain S, Thomas M, Kumar GP, Laloraya M. Programmed lipid peroxidation of biomembranes generating kinked phospholipids permitting local molecular mobility: a peroxidative theory of fluidity management. Biochem Biophys Res Commun 1993;195:574–80.
31. Laloraya M. Fluidity of Phopholipid bilayer of the endometrium at the time of implantation of the blastocyst—a spin-label study. Biochem Biophys Res Commun 1990;167:561–7.
32. Marklund S, Marklund G. Involvement of superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur J Biochem 1974;47:469–74.
33. Frankenbergre W, Blackmore T. Introductory Statistics Software Package, Verson 1.0, Reading, MA: Addison-Wesley Publishing, 1985.
34. Hatefi Y. Nicotinamide nucleotide transhydrogenase—a model for utilization of substrate binding energy for protein translocation. FASEB J. 1996;10:444–52.
35. Sipe HJJ, Jordan SJ, Hanna PM, Mason RP. The metabolism of 17-beta-estradiol by lactoperoxidase: a possible source of oxidative stress in breast cancer. Carcinogenesis. 1994;15:2637–43.
36. Okamura H, Yokosuka M, Hayashi S. Estrogenic induction of NADPH-diaphorase activity in the preoptic neurons containing estrogen receptor immunoreactivity in the female rat. J Neuroendocrinol 1994;6: 597–601.
37. Veille JC, Li P, Eisenach JC, Massmann AG, Figueroa JP. Effects of estrogen on nitric oxide biosynthesis and vasorelaxant activity in sheep uterine and renal arteries in vitro. Am J Obstet Gynecol 1996;174:1043–9.
38. Jordan VC, Murphy CS. Endocrine pharmocology of the antiestrogens as anti tumor agents. Endocr Rev 1990; 11:578–612.
39. Sasson S, Notides AC. Mechanism of the estrogen receptor interaction with 4-hydroxytamoxifen. Mol Endocrinol 1988;2:307–12.
40. Fowell SE, White R, Howe S, Syndeham M, Page M, Parker MG. Inhibition of the estrogen receptor-DNA binding by the "pure" antiestrogen ICI-164, 384 appears to be mediated by impaired receptor dimerization. Proc Natl Acad Sci USA 1990;87:6883–92.
41. Gundimeda U, Chen ZH, Gopalakrishna R. Tamoxifen modulates protein kinase C via oxidative stress in estrogen receptor-negative breast cancer cells. J Biol Chem 1996;271:13504–14.
42. Lapolt PS, Hong LS. Inhibitory effects of superoxide dismutase and cyclic guanosine 3′5′-monophosphate on estrogen production in cultured rat granulosa cells. Endocrinol. 1995;136:5533–9.
43. Macgregor JI, Jordan VC. Basic guide to the mechanism of antiestrogen action. Pharmacol Rev 1998;50:152–96.