Dose-dependent effects of 4-hydroxytamoxifen, the active metabolite of tamoxifen, on estrogen receptor-α expression in the rat uterus

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Tamoxifen, a selective estrogen receptor modulator, has agonist or antagonist activity, depending on the target tissue. The estrogen-like agonist effects of tamoxifen in the uterus are mediated primarily by 4-hydroxytamoxifen (4OH), the major active metabolite. Tamoxifen, 4OH and estradiol-17β (E_2) all bind to estrogen receptors (ERα and ERβ), but with different affinities, suggesting that these ligands are capable of producing differential *in vivo* effects on the uterus. However, differences in short-term effects of tamoxifen, 4OH and E_2 on the uterus have not been compared in the rat *in vivo*. Thus, we treated adult, ovariectomized rats (225–250 g) with vehicle (sesame oil), tamoxifen (1 mg/kg body weight), 4OH (0.01, 0.1 or 1.0 mg/kg body weight), E_2 (40 μg/kg body weight), estradiol valerate (a long-lasting estrogen; 40 μg/kg body weight) or ICI 182,780 (a pure anti-estrogen; 1 mg/kg body weight). Animals were sacrificed at 0, 3, 6, 12 or 24 h post-injection, and protein and mRNA levels for ERα and two estrogen-regulated early response genes, c-*fos* and *jun*-B, were examined. Administration of E_2 and 4OH (1 mg/kg body weight dose) resulted in down-regulation of uterine ERα protein in the uterine luminal and glandular epithelium by 6 h post-treatment. In contrast, no change in ERα level was observed after treatment with tamoxifen. Rapid (by 3 h) and transient increases in c-*fos* and *jun*-B mRNA levels were observed after E_2 treatment; however, c-*fos* and *jun*-B induction by 4OH was highly dose dependent, and higher 4OH doses induced rapid but persistent proto-oncogene expression *in vivo*. Our results demonstrate that tamoxifen and its major metabolite have differential effects on uterine gene expression, and 4OH is highly estrogenic in the rat uterus. *Anti-Cancer Drugs* 16:559–567 © 2005 Lippincott Williams & Wilkins.

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Introduction

Tamoxifen is a non-steroidal anti-estrogen used in treatment of estrogen receptor (ER)-α positive breast cancer. The drug has shown promising results in treatment and prevention of the disease [1]. Due to the estrogen agonist activity of the compound in the uterus, women taking tamoxifen are at increased risk for developing endometrial cancer [2].

The uterine activity of tamoxifen is mediated primarily by the active metabolite, 4-hydroxytamoxifen (4OH), and binding of 4OH to ERα stimulates RNA synthesis and subsequent uterine tissue growth [3,4]. The uterine growth response to tamoxifen involves increased expression of genes that play a role in cell cycle control, including the *fos* and *jun* immediate-early proto-oncogenes [5–8]. These proto-oncogenes are directly inducible by ERα and subsequently interact with a specific DNA regulatory sequence, activator protein (AP)-1, found in many genes, by means of either a Jun–Jun homodimer or Jun–Fos heterodimer. The AP-1 transcription factor is involved in a variety of cellular processes, including proliferation, differentiation and transformation [9,10]. It has been shown that tamoxifen treatment alters *fos* and *jun* expression patterns in the rodent uterus [7,8,11], suggesting that AP-1 transcription factors play a role in uterine responses to the anti-estrogen.

The effect of tamoxifen or 4OH on ERα itself in the uterus, however, is not as well understood. Estradiol-17β (E_2) the natural ligand for ERα, causes rapid down-regulation and proteolysis of ERα [12,13]. In contrast, tamoxifen binding appears to stabilize ER levels *in vitro* [14,15]. It has been previously demonstrated the partial agonist activity of the parent compound, tamoxifen, in the rodent uterus was associated with stabilization of ERα levels, rather than receptor degradation [7,8,11,16]. However, the effect of the major metabolite 4OH on ERα levels has not been previously investigated in the rat uterus *in vivo*.

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In the present study, we demonstrate that 4OH rapidly decreases receptor protein levels and induces ERα target genes, c-*fos* and *jun*-B. The effects of 4OH were highly dose dependent. However, at higher doses, 4OH showed striking similarity to E_2 on ERα kinetics and ERα target gene expression.

Methods

Animals

All animal studies were performed under protocols and procedures approved by the local Institutional Animal Care and Use Committee and in accord to NIH standards established by the Guidelines for the Care and Use of Experimental Animals and by the American Veterinary Medical Association. Mature female Sprague-Dawley rats (225–250 g) were obtained from Harlan Sprague-Dawley (Indianapolis, IN). Rats were given standard chow and water *ad libitum*, and maintained on a 12-h light–dark cycle, with lights on at 0700 h. Animals ($n = 90$; three per group) were ovariectomized under general anesthesia upon arrival and allowed to recover for 2 weeks. Animals received a single s.c. injection in the periscapular region of tamoxifen (1 mg/kg body weight), 4OH (0.01, 0.1 or 1 mg/kg body weight), ICI 182,780 (ICI; 1 mg/kg body weight), E_2 (40 μg/kg body weight), estradiol valerate (EV; 40 μg/kg body weight) or sesame oil vehicle. Treatment injection preparation has been described previously [16,17]. Animals were sacrificed at 0, 3, 6, 12 or 24 h post-injection. Uteri were harvested, trimmed of fat and mesentery, and then flash frozen in liquid nitrogen. An approximately 2-cm piece from the middle of one uterine horn per animal was fixed in 4% paraformaldehyde and paraffin embedded for immunohistochemical analysis (see below).

RNA isolation and Northern blot analysis

Total RNA was isolated from one uterine horn of individual animals using TRI reagent (Molecular Research Center, Cincinnati, OH), following the protocol provided by the manufacturer. Northern blot analysis was performed as previously described [16,17]. The constructs employed in this study have been described previously [16,18]. Full-length rat ERα, rat c-*fos* and mouse *jun*-B cDNAs were labeled with [^{32}P]CTP using the Rediprime II kit (Amersham Life Sciences, Arlington Heights, IL). Loading differences were corrected for using expression of 36B4, a constitutively expressed gene in the rat uterus [18].

Protein isolation and Western blot analysis

Whole-cell extracts were prepared from one horn of each rat uterus using a general lysis buffer containing 25 mM HEPES, pH 7.4, 1% Triton X-100, 0.5% SDS, 0.5% deoxcholate and 1 mM PMSF, as well as a cocktail of protease inhibitors: 1% aproprtin, 0.1% leopeptin and 10 μM pepstain. Tissue was homogenized in 500 μl lysis buffer using a 1-ml Dounce Homogenizer, centrifuged at 12 000 *g* for 15 min at 4°C and the protein content in the supernatants was determined using the Bio-Rad (Hercules, CA) protein assay kit. Protein extracts were mixed with one-quarter volume of 5 × electrophoresis sample buffer and heated for 5 min at 90°C. Western analysis was performed as previously described [17,19]. Briefly, protein extracts (50 μg/well) were resolved on a 12% polyacrylamide gel, transferred to a PVDF-Plus membrane (Osmonics, Westborough, MA) and probed with antibodies. Primary antibody was detected by horseradish peroxidase-conjugated second antibody and visualized using enhanced SuperSignal West Pico Chemiluminescent Substrate. The band density of exposed film was evaluated with ImageJ software (http://rsb.info.nih.gov/ij/).

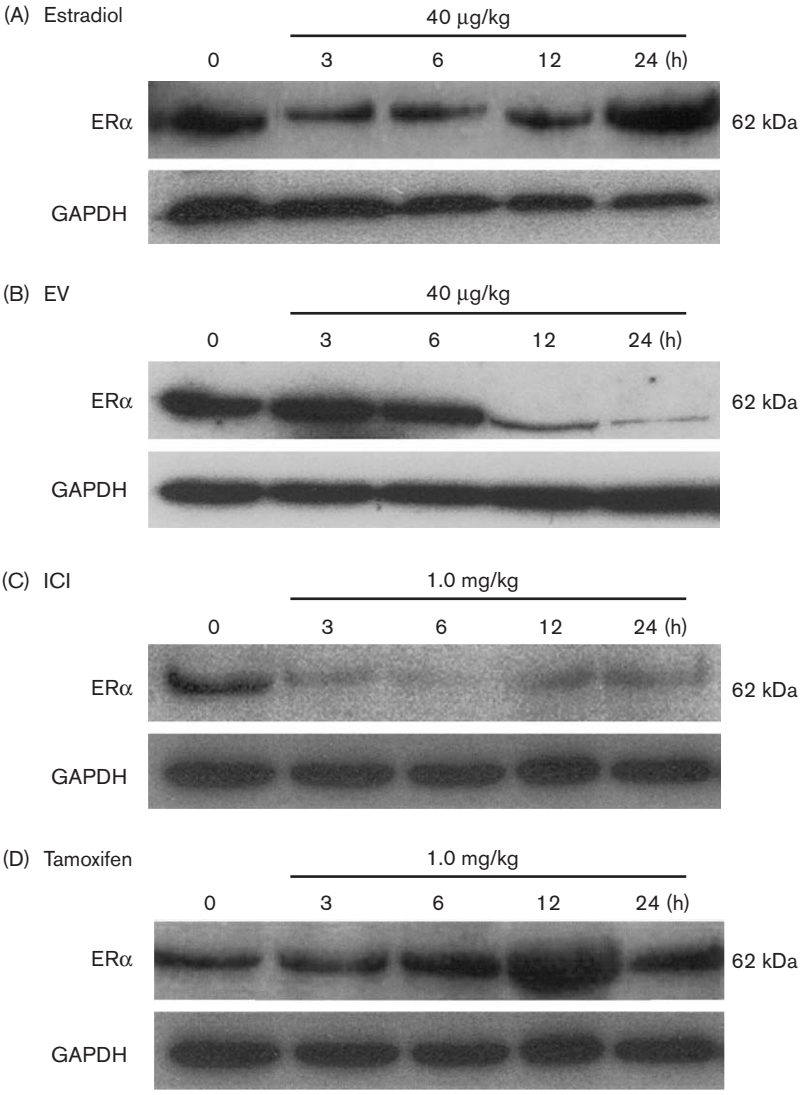
Immunohistochemistry

Innumohistochemistry was preformed on uteri samples as described previously [17,20]. Paraffin-embedded tissue was sectioned (6 μm), mounted on Superfrost Plus slides, dried to room temperature and hydrated in a series of washes from xylene to dH_2O. Antigenicity was enhanced by heating sections in a 1 × Dako Target Retrieval solution (Dako, Carpenteria, CA) to boiling. Sections were allowed to cool for 20 minutes, and washed in 1 × PBS plus 0.1% Tween 20 (PBST 3 × 10 min each). After washing, tissues were blocked with undiluted normal goat serum (Sigma-Aldrich) at room temperature for 20 min, washed in PBST (3 × 5 min each) and incubated overnight at 4°C with anti-ERα rabbit polyclonal antibody (1:100, PA1-309; Affinity Bio Reagents, Golden, CO). The following day, sections were washed in PBST (3 × 10 min each) and subject to reaction with secondary antibody (1:400 goat anti-rabbit; Vector, Burlingame, CA) at room temperature for 1 h. After washing in PBST (3 × 5 min each), sections were reacted with ABC Elite reagent (Vector) for 1 h, washed in PBST (3 × 5 min each) and reacted with nickel-intensified diaminobenzidine reagent (DAB kit; Vector) for 2.5–3 min. Sections were then rinsed twice in dH_2O and dehydrated in graded alcohols through xylene, and coverslipped. Control sections were obtained from non-treated animals. Positive controls were incubated with primary antibody, while negative controls were incubated without primary antibody.

Results

The effects of estrogens and anti-estrogens on uterine ERα protein levels were examined in adult rats. Animals were given a single injection of sesame oil vehicle, E_2, tamoxifen, 4OH, EV and ICI, and sacrificed at various times post-treatment. To determine treatment effects on levels of target proteins of interest, Western blot analysis was performed. ERα protein levels in the whole uterus were high in the untreated controls, and subsequently decreased between 3 and 12 h after E_2 treatment (Fig. 1A). By 24 h, ERα levels were again high, similar to 0 h (Fig. 1A). Treatment with EV, a long-acting

Fig. 1

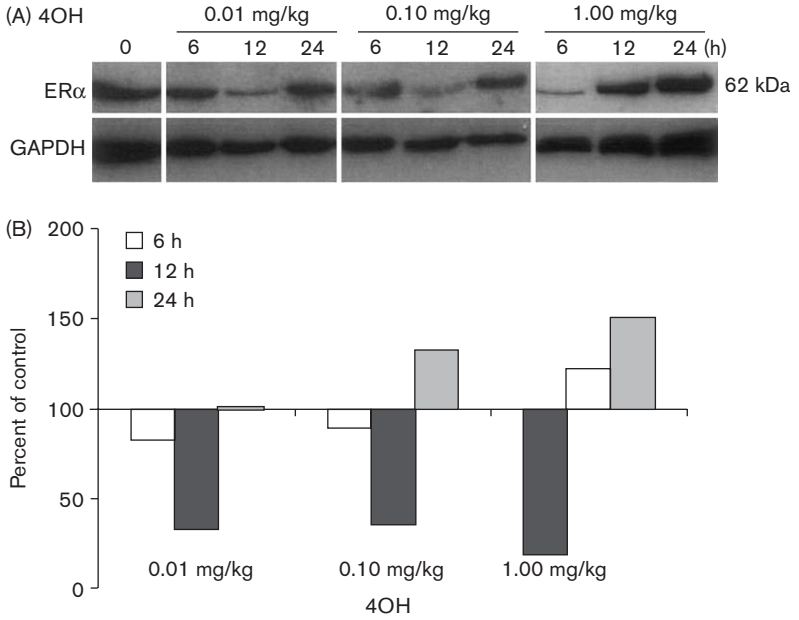


Effects of estrogen and anti-estrogen treatment on ERα protein levels in the rat uterus. Protein (50 µg/lane) was isolated from one horn of each uterus after administration of (A) E_2, (B) EV, (C) ICI or (D) tamoxifen for the indicated times to ovariectomized adult rats. ERα protein levels were analyzed by immunoblotting. GAPDH was used as an internal control to correct for SDS–PAGE loading. A representative gel is shown.

estrogen [21,22], also decreased ERα protein levels (Fig. 1B). However, the effect of EV on the levels of ERα was delayed compared to the E_2 group and down-regulation of ERα was not seen until 12 h post-EV injection. ERα levels remained low at 24 h after administration of this long-acting estrogen. Treatment with the pure anti-estrogen ICI produced a rapid (by 3 h) decrease in ERα protein (Fig. 1C). By 24 h post-treatment with ICI, ERα expression was detectable, but remained well below control levels.

In contrast to the above, at all time points examined after administration of tamoxifen, uterine levels of ERα remained unchanged or were higher compared to controls (Fig. 1D). Because the *in vivo* actions of tamoxifen are mediated primarily by the 4OH metabolite, it was of interest to thoroughly examine the effect of this potent metabolite on ERα protein levels in the rat uterus. Administration of low doses of 4OH (0.1 and 0.01 mg/kg) caused a decrease in uterine ERα levels, but the time course of inhibition was delayed by 6 h, with maximal inhibition occurring by 12 h; at 24 h, ERα levels were similar or higher than control levels (Fig. 2A and B). Treatment with 1 mg/kg 4OH, however, yielded a result more similar to that of the E_2 group than the rats given the parent drug: ERα protein decreased dramatically at 6 h, but returned to levels similar or higher than control levels by 12 and 24 h, respectively (Fig. 2A and B).

Fig. 2



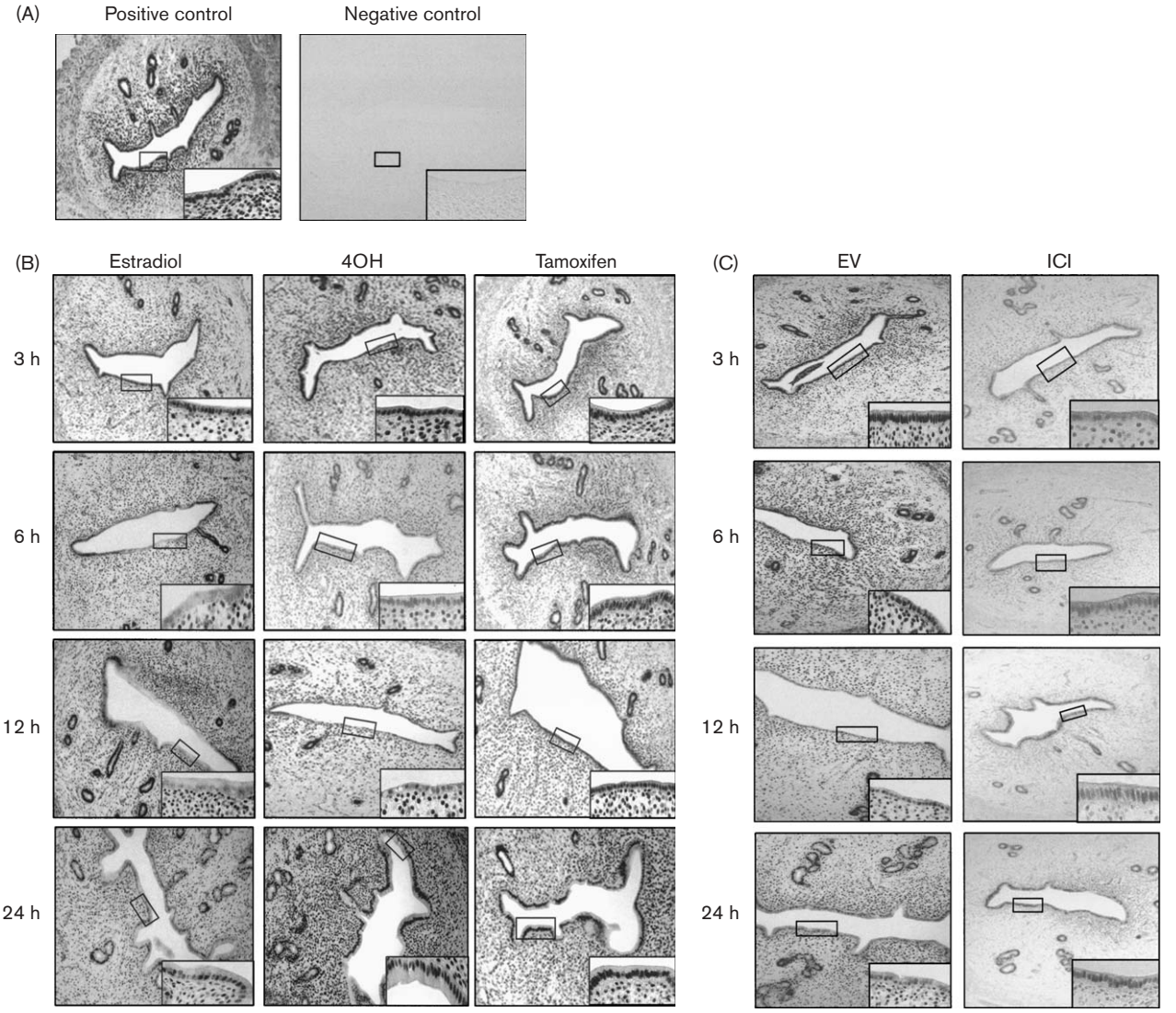
Effects of various doses of 4OH on ERα protein levels in the rat uterus. Protein (50 μg/lane) was isolated from one horn of each uterus after administration of 4OH for the indicated times to ovariectomized adult rats (A). ERα protein levels were analyzed by immunoblotting. GAPDH was used as an internal control to correct for SDS–PAGE loading. A representative gel is shown. The histogram (B) shows the relative ERα levels compared to control (0 h) after 4OH treatment for 6 (white), 12 (black) or 24 (gray) h.

Immunohistochemistry was used to determine the uterine tissue compartments exhibiting ERα down-regulation. Intense staining in the luminal and glandular epithelia of the positive control animals was observed, and numerous ERα-positive cells were present throughout the stroma, particularly in the peri-luminal area (Fig. 3A). Immunostaining in the luminal epithelium decreased by 6 and 12 h after administration of E_2; by 24 h post-E_2 treatment, ERα staining intensity was similar to controls (Fig. 3B). Similarly, ERα expression in the uterine luminal epithelium was decreased by treatment with 4OH (1 mg/kg) at 6 and 12 h, and ERα immunostaining had returned to control levels by 24 h after administration of 4OH (Fig. 3B). In contrast, after treatment with the parent compound, tamoxifen, expression of ERα in the uterine tissue compartments remained essentially unchanged at all time points examined and appeared similar to vehicle-treated animals. After administration of the long-acting estrogen EV, decreased ERα immunostaining was observed at 12 and 24 h (Fig. 3C). Treatment with ICI rapidly (by 3 h) and dramatically decreased ERα levels in all uterine tissue compartments (Fig. 3C). Overall, the results of the immunohistochemical analyses were in agreement with those using Western blotting to examine whole-organ levels of ERα (Figs 1 and 2 versus 3).

To examine steady-state ERα mRNA levels, Northern blot analysis of the whole uterus after treatment with estrogens and anti-estrogens was performed (Fig. 4). Previous studies in our laboratory, using the identical experimental system, demonstrated a significant reduction in uterine ERα mRNA levels at 6 h post-injection of estradiol (E_2; 40 μg/kg body weight); however, ERα mRNA levels were greater than control by 24 h post-injection [20]. In contrast, tamoxifen (1 mg/kg body weight) treatment did not decrease ERα mRNA levels in the uterus until 24 h post-injection [16]. In the present study, ERα mRNA levels after treatment with 0.01 mg/kg dose of 4OH remained relatively unchanged (Fig. 4A and B). Administration of 0.1 mg/kg dose of 4OH caused ERα mRNA levels to decrease by 12 h and the highest dose of 4OH (1.0 mg/kg) resulted in a decrease in ERα transcript levels at 6 h (Fig. 4A and B). The expression pattern of ERα mRNA levels after treatment with 0.1 and 1.0 mg/kg 4OH was identical to the ERα protein expression pattern post-treatment with these same doses (Figs 2 and 3 versus 4). In contrast, no reduction in mRNA levels at any time point was seen after administration of the long-acting estrogen EV or the potent anti-estrogen ICI (Fig. 4C and D). These results indicate that the patterns of ERα protein turnover observed with EV and ICI are not reflected at the level of ERα mRNA.

To examine the effects of estrogens and anti-estrogens on expression of ERα target genes, c-*fos* and *jun*-B mRNA levels were examined in whole uterine extracts (Fig. 5).

Fig. 3



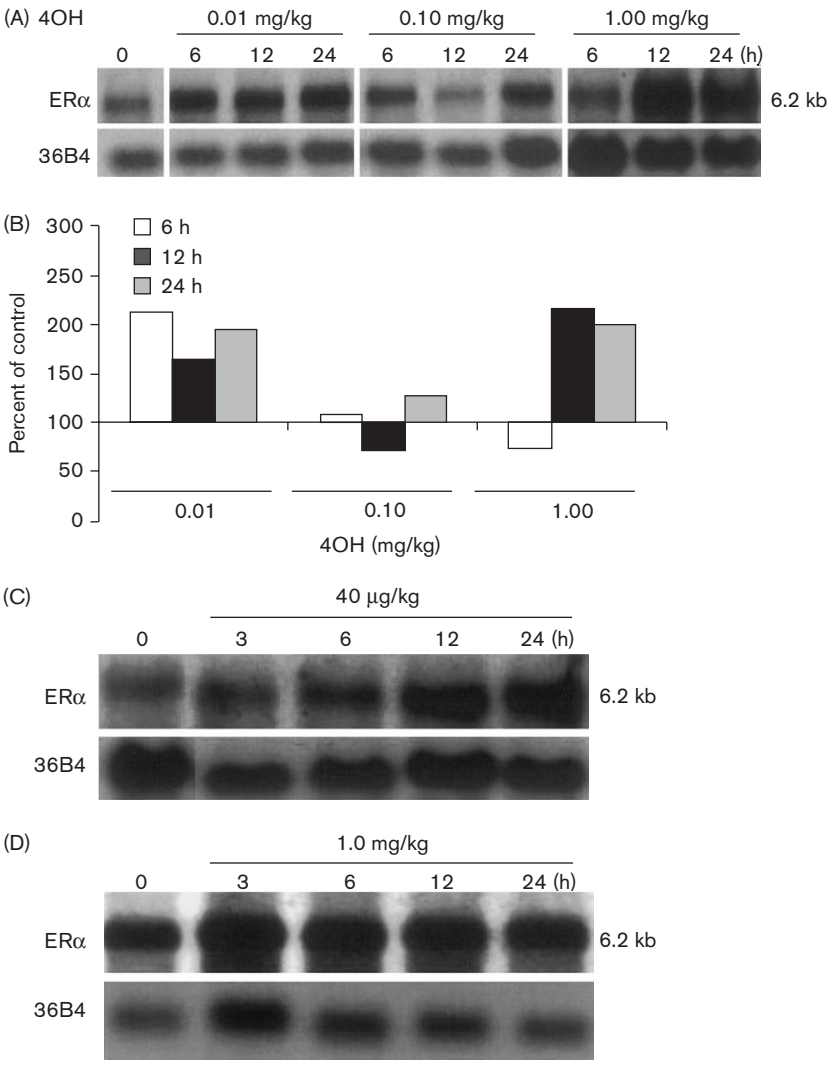
Effects of estrogen and anti-estrogen treatment on ERα immunostaining in the rat uterus. (A) Positive control (0 h) shows strong ERα immunostaining in luminal and glandular epithelium (box). Negative controls (0 h) were incubated with secondary antibody alone and showed no staining. (B) ERα immunostaining after treatment with estradiol (40 μg/kg body weight), 4OH (1 mg/kg body weight) or tamoxifen (1 mg/kg body weight) for 3, 6, 12 and 24 h. Representative micrographs are shown. (C) ERα immunostaining after treatment with EV (40 μg/kg body weight) or ICI (1.0 mg/kg body weight) for 3, 6, 12 and 24 h. Representative photomicrographs are shown.

Previous reports, using this experimental model, have shown that E_2 induces rapid (by 3 h), but transient, proto-oncogene expression of in the rat uterus [5,23,24]. Tamoxifen also induces expression of c-*fos* and other early response genes in the rat uterus, but with altered kinetics compared to E_2 [7,8,24]. In the current study, 4OH (1 mg/kg body weight) induced both c-*fos* and *jun*-B mRNA in a manner similar to E_2 treatment, except the response was prolonged and proto-oncogene mRNA levels, although reduced, remained above control values at 24 h post-treatment (Fig. 5A). Lower doses of 4OH (0.01 and 0.1 mg/kg) also induced c-*fos* mRNA; however, the response was delayed and not prolonged at the low dose (0.01 mg/kg; Fig. 5B). After treatment with EV, c-*fos* induction was delayed and prolonged compared to E_2; an increase in c-*fos* expression was not detected until 6 h post-treatment, and remained above controls at 12 and 24 h post-EV administration (Fig. 5C).

Discussion

The anti-estrogen tamoxifen displays complex pharmacology *in vivo*. The drug has agonist or antagonist activities, depending on the target tissue. In the uterus,

Fig. 4

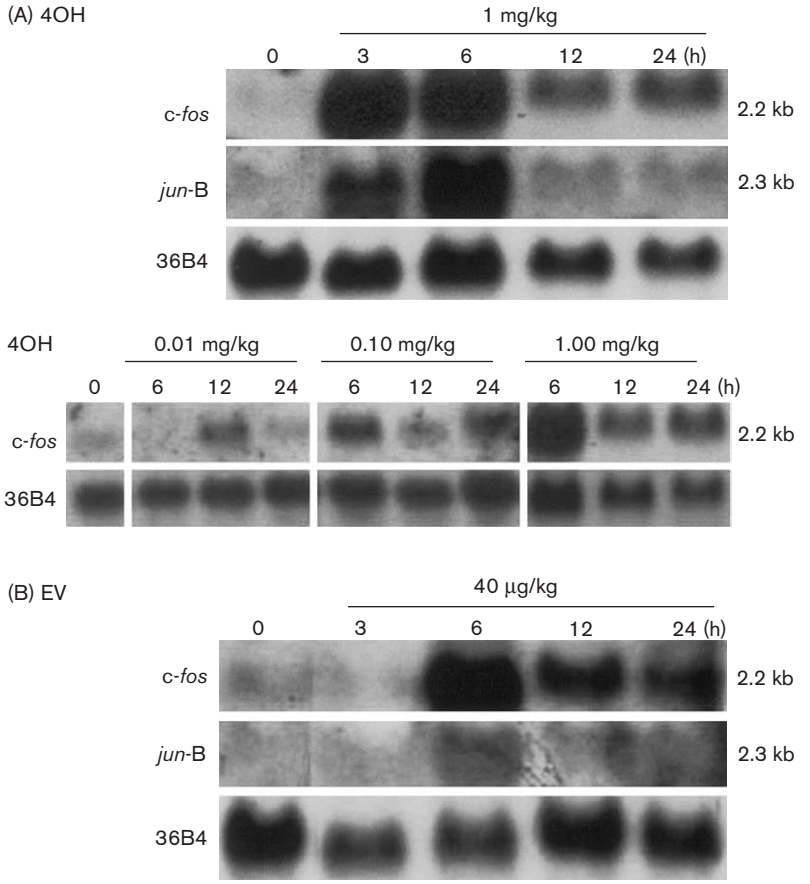


Effects of estrogen and anti-estrogen treatment on ERα mRNA levels in the rat uterus. Total RNA (20 μg/lane) was isolated from one horn of each uterus after administration of (A) 4OH, (C) EV or (D) ICI for the indicated times to adult ovariectomized rats. Levels of ERα mRNA were analyzed by Northern blot. The housekeeping gene, 36B4, was used as an internal control to correct for agarose gel loading. The histogram (B) shows the relative ERα levels compared to control (0 h) after 4OH treatment for 6 (white), 12 (black) or 24 (gray) h. A representative gel is shown.

the partial estrogen agonist activity of tamoxifen has been associated with an increased risk of endometrial cancer in women taking the drug for breast cancer treatment and prevention [1,2,25]. To gain a better understanding of the agonist activity of tamoxifen, we compared uterine effects of the parent compound and various doses of the active metabolite (4OH) to the naturally occurring hormone, E_2, a long-acting estrogen (EV) and a pure anti-estrogen (ICI). Differential effects of these ligands on uterine levels of ERα and receptor target gene were observed. Perhaps most notably, increasing doses of 4OH induced changes in uterine ERα protein levels that were similar to those induced by E_2, the cognate ligand of ERα, i.e. rapid and transient down-regulation of receptor mRNA and protein.

Ligand binding has been shown to influence the stability of steroid hormone nuclear receptors. E_2 reduces the half-life of ERα from 4–5 to 3 h [15] and ICI dramatically decreases the half-life of ERα protein to less than 1 h in cultured cells [26]. In the rodent uterus, it has been well established that E_2 rapidly and transiently down-regulates ERα expression at both the mRNA and protein levels (Fig. 1A) [20,27–30]. In contrast, tamoxifen prevents ERα protein turnover in both breast cancer cells [15] and uterine cells (Fig. 1D), suggesting that the tamoxifen–ERα complex formed in breast and uterine cells is more stable than either the E_2– or ICI–ERα complex (Figs 1 and 3). Furthermore, a functional relationship between ERα turnover and transcriptional activity exists, and blocking ERα turnover appears to

Fig. 5



Effects of estrogen and anti-estrogen treatment on c-*fos* and *jun*-B mRNA levels in the rat uterus. Total RNA (20 µg/lane) was isolated from one horn of each uterus after administration of (A) 4OH or (B) EV for the indicated times to ovariectomized adult rats. Levels of c-*fos* and *jun*-B mRNA were analyzed by Northern blot. The housekeeping gene, 36B4, was used as an internal control to correct for agarose gel loading. A representative gel is shown.

inhibit ERα transcriptional activity [31]. Thus, a stabilized tamoxifen–ERα complex may contribute to the anti-estrogen activity of the drug in breast cancer cells and its partial agonist activity in the uterus. Furthermore, the effects of 4OH on ERα protein turnover and ERα target gene expression (Figs 2 and 5) were clearly dose dependent, but support the notion that receptor degradation is linked to receptor transactivation function.

No agonist activity of ICI in any tissues has been reported, including the uterus [26]. Our results together with those of Gibson *et al.* [32] in mice show pure anti-estrogens cause rapid degradation of ERα protein in the rodent uterus and support that the mechanism of ICI action at the level of ERα includes increasing the rate of ERα degradation [13,33]. Furthermore, treatment with ICI had no effect on ERα mRNA levels (Fig. 4D), suggesting that the decrease in ERα protein is not due to effects on ERα transcription. Although administration of ICI caused nearly a complete loss of uterine ERα protein (Fig. 1C), the ICI–ER interaction was not functional and did not induce proto-oncogene expression. Degradation of ERα protein occurs via the ubiquitin–proteasome pathway [15,31] and treatment with the ICI has been shown to induce hyper-ubiquitination of ERα, while the more stable ERα–tamoxifen complex is associated with hypoubiquitination [15]. Our observations that different ERα ligands have various effects on uterine receptor protein levels support the concept that ligand-induced conformational changes in ERα can in turn alter receptor interactions with the ubiquitin–proteasome degradation system.

Previous studies have shown that uterine ERα levels must decrease before the onset of DNA synthesis and cellular growth [34], suggesting that receptor down-regulation levels play an important role in uterine cell proliferation. While tamoxifen causes dramatic increases in uterine weight and uterine cell hypertrophy, the drug

does not induce uterine cell proliferation to the extent of E_2 [24,35]. The dose of tamoxifen appears to be important in eliciting a uterine proliferative response [36], and our data on 4OH dose-dependent effects on ERα and receptor target gene expression support this notion, and further suggest that the amount of 4OH available for receptor binding determines the extent of the uterine response to the drug. 4OH has been shown to stimulate growth of the Ishikawa human endometrial adenocarcinoma cell line and the stimulation of growth was positively correlated with 4OH concentrations in the range of 10 nM to 1 μM [37]. 4OH induced growth of Ishikawa cells in a similar manner to E_2 and was not antagonistic when used in combination with E_2 [38]. Taken together, these observations indicate that 4OH, when present in a sufficient amount, may be capable of inducing E_2-like cell proliferation in the uterus.

In conclusion, the results of this study demonstrate that tamoxifen and its major metabolite, 4OH, have differential effects on the uterus. Through the ability of 4OH to mimic E_2 in a dose-dependent manner, effects of 4OH on ERα down-regulation and receptor target gene expression could contribute to the mechanism of proliferation of the uterine endometrium, and increased incidence of endometrial cancer in women taking tamoxifen for breast cancer treatment and prevention. It is well known that metabolic differences in drug metabolism exist among women [39,40], and differences in tamoxifen metabolism among women may contribute to altered tamoxifen activity and variability in side effects in patients taking the drug [41,42]. Genetic mutations have been associated with increased risk in endometrial cancer risk for women on hormone-replacement therapy [43] and differences in genetic background have been shown to lead to increased tamoxifen sensitivity in some tissues, including breast and uterus [17,44]. It seems reasonable to speculate that differences in tamoxifen metabolism could contribute to the development of endometrial cancer in some women taking tamoxifen for breast cancer treatment or prevention.

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