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Tamoxifen Up-regulates Oestrogen Receptor-α, c-fos and Glyceraldehyde 3-Phosphate-dehydrogenase mRNAs in Ovine Endometrium

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Tamoxifen, the antiestrogen most widely used in medicine, was tested in ewes to determine whether it antagonizes oestradiol up-regulation of ER, PR, and other genes reported to be oestrogen-modulated (c-fos, oxytocin receptor (OTR), glyceraldehyde phosphate dehydrogenase (GAPDH), and apolipoprotein AI (apo AI)) in endometrium and liver. Ovariectomized ewes ($n = 6$ ewes per group) were injected with 20 mg tamoxifen (Tam) 24 h prior to tissue collection, 50 μg oestradiol (E2) 18 h prior to tissue collection, both drugs (T + E2) or drug vehicle (Con). E2 treatment resulted in 857 ± 93 pg oestradiol/g endometrium. Gross uterine characteristics of Tam- and T + E2-treated ewes were intermediate to those in Con and E2 groups. In endometrium, Tam treatment mimicked E2 treatment in up-regulating ER, c-fos, and GAPDH mRNAs two- or three-fold. However, neither E2 nor Tam treatments affected concentrations of OTR mRNA in endometrium, or ER, c-fos, GAPDH, OTR and apo AI mRNAs in liver. Like oestradiol, tamoxifen stabilized endometrial ER mRNA more than 3-fold in endometrial explants cultured with the transcription inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB). Thus, tamoxifen acts as an oestradiol agonist in ovine endometrium and shares a posttranscriptional mechanism with oestradiol in the up-regulation of ER gene expression.

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INTRODUCTION

Oestrogens are primary regulators of the expression of ER and PR genes, whose protein products mediate oestrogen and progesterone effects [1]. In response to the preovulatory oestrogen surge, concentrations of both ER and PR mRNAs and proteins increase in the endometrium of cycling female mammals [2–5]. We use ovariectomized ewes treated with a single, physiological dose of oestradiol [6] as a model to study the molecular mechanisms involved in the regulation of the ER and PR genes by the preovulatory surge of oestrogen. At 24 h post-injection, endometrial ER and PR gene expression increases in most uterine cells [7, 8]. While oestradiol up-regulates PR mRNA by increasing PR gene transcription, oestradiol up-regulates the expression of ER mRNA by enhancing its stability [9].

Antiestrogen drugs were developed to block the powerful effects of oestrogens. Tamoxifen is the antiestrogen most widely used in human medicine. However, its value in controlling the growth of breast cancers may be offset by an increase in the incidence of endometrial cancer in tamoxifen-treated patients [10]. Extensive characterization of tamoxifen actions, both *in vitro* and *in vivo*, has identified its oestrogen agonist activities along with its antagonist effects. The ultimate effect of tamoxifen, agonist vs antagonist, depends upon the gene, tissue, and species examined [11]. In uteri of most species, the agonist actions of tamoxifen predominate over antagonist actions.

Here, we tested whether one dose of tamoxifen (the same dose given to women once a day for the treatment of breast cancer [10]) could block the

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effects of a single dose of oestradiol in the endometrium of ovariectomized ewes. In addition to ER and PR mRNAs, four other gene products (c-fos, OTR, GAPDH, and apo AI mRNAs), reported to be oestrogen-responsive [12–15], were analyzed in endometrium. Expression of these genes was analyzed simultaneously in liver, which has the majority of the body's antiestrogen binding sites [1]. Since ER mRNA is up-regulated post-transcriptionally, our hypothesis was that tamoxifen would not up-regulate ER mRNA, but would likely act as an oestrogen agonist on other transcriptionally regulated genes [11].

MATERIALS AND METHODS

Animal treatments and sample collection

Crossbred Rambouillet ewes (*Ovis aries*) were ovariectomized during the breeding season after exhibiting estrous cycles of normal duration (16 to 18 days). Four weeks after ovariectomy, ewes were randomly assigned to four treatment groups ($n = 6$ ewes/group) as described in Fig. 1. Tamoxifen injection preceded that of oestradiol by 6 h so that available ER protein would be bound by tamoxifen. Oestradiol injection at 18 h prior to hysterectomy was chosen as a time of ER mRNA accumulation [7] so that the molecular mechanism involved could be tested in the final experiment. At the time of hysterectomy and liver biopsy, whole uteri and dissected endometrium were weighed. Liver and endometrium for steady state mRNA analyses were minced and snap-frozen in liquid nitrogen and stored at −80°C. Fresh endometrium from each ewe was cultured for determination of mRNA stabilities (see below). To assess whether oestradiol concentrations in endometrial tissue of E2-treated ewes were similar to those in ovary-intact, cycling ewes, five of the latter were checked for estrus daily with vasectomized rams. They were hysterectomized on day 15 ($n = 3$ ewes) or day 1 ($n = 2$ ewes) of the estrous cycle, which is 24 to 48 h before or after the peak of the preovulatory surge of oestrogen, respectively. All animal procedures were approved by the Texas A&M University Laboratory Animal Care and Use Committee. Unless otherwise indicated, chemicals were purchased from Sigma (St. Louis, MO).

Endometrial oestradiol concentrations

Endometrial samples (1 g) were homogenized in 5 ml phosphate-buffered saline (PBS, pH 7.0). Duplicate 1 ml aliquots were extracted with 4 ml anhydrous diethyl ether [16]. After drying the ether phases under a stream of nitrogen, samples were reconstituted in 200 μl PBS + glycerol and immediately assayed for oestradiol using the Diagnostic Systems Laboratories Ultra-Sensitive Estradiol Kit (Webster, TX). Briefly, duplicate 200 μl samples of standards (ranging from 0 to 750 pg/ml) or endometrial extracts were incubated 1 h with antiserum to oestradiol. Iodinated oestradiol (100 μl) was then added and samples were incubated another 2 h. Hormone-antibody complexes were collected as precipitates, counted, and analyzed with Assay Zap software (Biosoft, Cambridge, U.K.).

Cloning of ovine cDNAs

Ovine cDNAs for GAPDH, c-fos and apo AI were cloned in reverse transcriptase–polymerase chain reactions. Endometrial mRNAs for GAPDH and c-fos cDNAs and liver mRNA for apo AI cDNA were reverse transcribed with a dT_{17} primer as described previously [7]. PCR primers, designed to human cDNA sequences, were CATTGACCTCAACTACA and TAAGCAGTTGGTGGTGCAGG (GAPDH); GAGTCTGAGGAGGCCTTCAC and AGACGTGTAAGTAGTGCAGC (c-fos); TCCTTGACAACTGGGACAGC and GTCCAGGTAGGGCTGCACC (apo AI); in sense and antisense orientations, respectively. PCR products of expected sizes for c-fos and apo AI cDNAs were produced from 30 cycles of 95°C for 30 s, 55°C for 30 s, and 68°C for 2 min. The GAPDH cDNA required an additional round of PCR with a pair of nested primers: CACCATCTTCCAGGAGCGAG and TCATACTTCTCA-

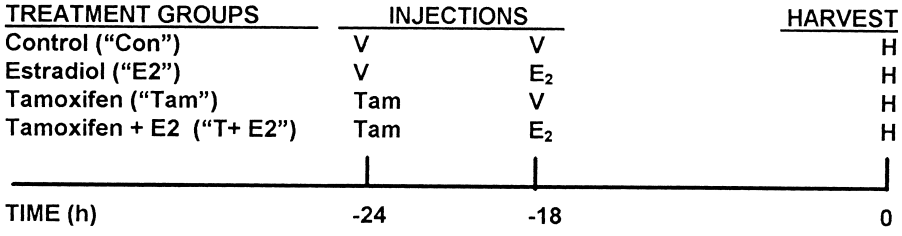

Fig. 1. A timeline describing the treatments administered to ovariectomized ewes. Groups of ewes, ovariectomized one month previously, received im injections of tamoxifen and/or oestradiol at 24 or 18 h prior to hysterectomy. V = vehicle (charcoal-stripped corn oil); E_2=50 μg oestradiol; Tam = 20 mg tamoxifen; H = hysterectomy. Each group consisted of six ewes.

TGGTTCAC, sense and antisense, respectively. The PCR products were cloned into the pCRII vector with the TA cloning kit (Invitrogen; San Diego, CA) and sequenced on both strands with Sequenase (USBiochemicals; Cleveland, OH). Ovine sequences were aligned and compared to those of other species with GCG software programs (Wisconsin Package Software; Madison, WI). GenBank accession numbers for ovine GAPDH, c-fos and apo AI cDNA sequences are U94718, U94719 and U94720, respectively.

Synthesis of cRNA probes

For probing RNA on Northern and slot blots, cRNA probes were generated from the ovine cDNAs described above, as well as ER, PR, and OTR cDNA clones [7, 17], and the human 18S rRNA probe (pTRI RNA 18S; Ambion; Austin, TX). ER and PR cRNA probes were generated with T7 RNA polymerase (Maxiscript kit; Ambion) as described previously [7]. Antisense apo AI, c-fos, and GAPDH cRNA probes were generated similarly from plasmids linearized with BamH I. The OTR plasmid was cut with Bgl II and SP6 RNA polymerase was used to generate antisense cRNA [17]. Because PR and c-fos mRNAs are relatively low in abundance, the specific activity of PR and c-fos cRNA probes was doubled by eliminating unlabelled UTP and doubling the ^{32}P-UTP in those *in vitro* transcription reactions. Unincorporated radionucleotides were removed on spin columns (Bio101; La Jolla, CA).

Preparation of RNA samples and Northern analyses

Total cellular RNA samples were purified from tissues with the TriPure Reagent (Boehringer Mannheim; Indianapolis, IN). Poly(A) + RNA samples were purified directly from tissue (0.5 g) with the Fasttrack kit (Clontech; Palo Alto, CA). Northern blot analyses of 5 μg poly(A) + RNA (ER) or 40 μg total cellular RNA (apo AI, c-fos, GAPDH and OTR) samples were performed as previously described [7]. Apo AI and OTR were only analyzed by Northern blotting in either liver or endometrium, respectively. High stringency was maintained throughout blot hybridization (55°C) and washing (68°C in 0.1 × SSC (1 × = 150 mM NaCl + 15 mM Na citrate) + 0.1% sodium dodecyl sulfate followed by treatment with 1 μg/ml RNase A for 10 min at 20°C). Blots were exposed to Fuji X-ray film for times ranging from 15 min (for ER) to 7 days (for c-fos) to record hybridization signals in the linear range of the film. Message sizes were calculated by comparing their migration distances to those of ethidium bromide-stained 28S and 18S rRNAs. The absence of non-specific bands on the Northern blots justified the use of slot blots in the following experiments.

Steady state mRNA analyses on slot blots

Slot blot analyses were performed with identical probes, hybridization and wash conditions to those of Northern analyses. Total cellular RNA samples (20 μg RNA, estimated from duplicate A_{260} measurements) from each treated and control ewe were denatured and immobilized in 4 μg aliquots (except c-fos blots had 8 μg samples) on nitrocellulose (Schleicher and Schuell, Keene, NH) as described by Sambrook *et al.* [18]. While liver samples were analyzed in singlicate, endometrial RNA preparations and analyses were performed in duplicate from two tissue samples per ewe. Replicate blots were hybridized with individual antisense cRNA probes, washed, and used to create autoradiographs, as described above for Northern analyses. Hybridization signals on autoradiographs were quantitated by densitometry using Adobe Photoshop scanning and Intelligent Quantifier (Bio Image, Ann Arbor, MI) quantitation software.

mRNA stability analyses from explant cultures

Finely minced endometrium (500 mg) was cultured in phenol red-free Ham's F12-Dulbecco's minimal essential medium at 37°C on a rocker platform under O_2:N_2:CO_2 (50:45:5%). Oestradiol (10^{-9} M) and/or hydroxytamoxifen (10^{-6} M) were added to the medium of explants from oestradiol- and/or tamoxifen-treated ewes, to approximate hormone and antihormone influences of the *in vivo* treatments. Transcription was inhibited with 75 μg/ml 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), which blocked 90% of transcription in the explant cultures [9]. Samples of explants (100 mg) were harvested after 0, 3, 6, 9, and 12 h of culture. Explant samples were snap frozen in liquid nitrogen and stored at −80°C until analysis on RNA slot blots, as described above.

Statistical analyses

Quantitative data were analyzed by least squares ANOVA using the General Linear Model procedures of SAS [19]. Results from treatment groups were compared: E2 vs Con for oestradiol effects, Tam vs Con for agonist effects of tamoxifen, and T + E2 vs E2 for antagonist effects of tamoxifen. Data are presented as least squares means and pooled standard errors for treatment groups. In mRNA analyses on slot blots, 18S rRNA hybridization signals were used to normalize hybridization signals, by division, to account for any discrepancies in RNA loading between samples. In steady state comparisons of mRNA concentrations, values were normalized to the average value of the control group to show changes relative to the control ewes. In mRNA stability data, values were normalized to the average time 0 value to the percent of initial mRNA remaining. Because

mRNA degradation is assumed to reflect first order kinetics [20], linear decay lines (not shown) on the semi-logarithmic plots were used to estimate mRNA half-lives (times to 50% mRNA remaining). Level of statistical significance is $p < 0.05$, unless otherwise stated.

RESULTS

Oestradiol concentrations in endometrium

To determine if the oestradiol injection resulted in physiological levels of oestradiol in the endometrium, oestradiol was measured in endometrial homogenates from control and E2-treated ewes. Oestradiol concentrations were undetectable in the endometrium of control ewes, while E2 treatment resulted in 857 ± 93 pg oestradiol per g of endometrium. For comparison, endometrium from ovary-intact ewes was analyzed on days 15 and 1 of the estrous cycle (before and after the preovulatory surge of oestrogen on day 16 of the 17 day long cycle). The oestradiol concentrations in the day 15 and 1 cyclic endometrium (333 ± 81 and 461 ± 114 pg oestradiol per g endometrium, respectively) were not different from each other. These data indicate that a single injection of 50 μg estradiol results in about two-fold higher concentrations of hormone in endometrial tissues than is present in cycling ewes, before or after the preovulatory surge of estrogens.

Oestradiol and tamoxifen effects on gross uterine characteristics

Uteri of ewes treated with tamoxifen, with and without oestradiol, were intermediate in outward appearance compared to the small, pale tracts of control ewes and the turgid, reddened uteri of the oestradiol-treated group. In general, uteri from ewes in the Tam group appeared more hydrated than control uteri but paler than E2 uteri, perhaps indicating less blood vessel enlargement. Uteri from the T + E2 treatment group appeared similar to those of the E2 group. One exception was that T + E2 ewes had very tough caruncles, which are evaginations of the endometrium of ruminants that are specialized for vascular attachment of the placenta.

Compared to controls, oestradiol treatment increased the total uterine weight by 60%, while dissected endometrial weights did not increase (Fig. 2). Uterine and endometrial weights of Tam ewes were not different from controls. The uterine weights of the T + E2 treated ewes were intermediate to and not different from control or E2 groups. However, the dissected endometrial weights showed a trend toward a 33% decrease in the T + E2 ewes compared to those of the E2 group ($p < 0.10$). Thus, tamoxifen appeared to exert partial agonist/partial antagonist effects on gross characteristics of sheep uteri.

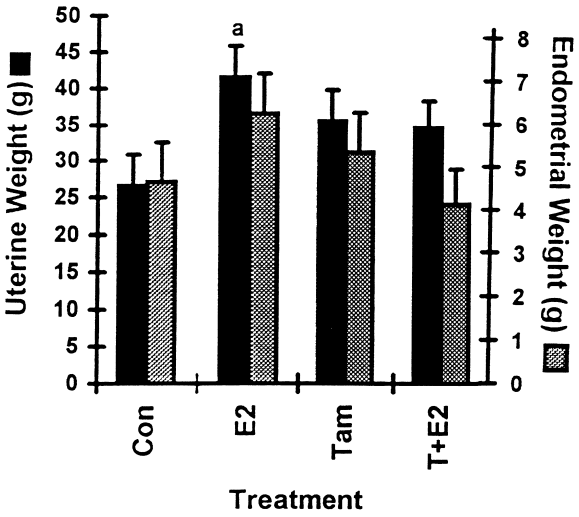

Fig. 2. Oestradiol and tamoxifen effects on uterine and endometrial weights. Weights of whole uteri (filled bars relate to left axis) and dissected endometrium (hatched bars relate to right axis) are presented as least squares means and standards errors for treatment groups described in Fig. 1. 'a' above the bar indicates a significant difference of oestradiol treatment compared to the control group.

Ovine mRNA and protein sequences are similar to human sequences

We cloned three new ovine cDNA fragments for c-fos, GAPDH and apo AI mRNAs that were 331, 201 and 170 base pairs, respectively. The percent of identical nucleotides between sheep and human c-fos, GAPDH and apo AI cDNAs was 89, 88 and 85%. Since all were within the coding regions of the mRNAs, sequences of 110, 67 and 57 amino acids were inferred for the ovine c-fos, GAPDH and apo AI proteins (not shown). Comparisons of the ovine and human amino acid sequences for c-fos, GAPDH and apo AI demonstrated that they were 92, 91 and 82% identical, respectively. As expected, the ovine GAPDH and apoAI cDNA sequences were more similar to those cDNA sequences known for other ungulates: 90 and 94% identical to porcine and bovine GAPDH cDNA sequences, respectively, and 91 and 96% identical to porcine and bovine apo AI cDNA sequences, respectively. These clones were used to produce cRNA probes for subsequent mRNA analyses.

Northern analyses of ER, apo AI, c-fos, GAPDH and OTR mRNAs in liver and endometrium

Northern blot analyses were performed to assess the specificity of the ER, apo AI, c-fos, GAPDH, and OTR cRNA probes in detection of mRNAs in liver and endometrium. Specifically hybridizing bands indicated ER, apo AI, c-fos, GAPDH and OTR mRNAs whose migration distances were consistent with sizes of 6.8, 1.0, 2.4, 1.8, and 6.8 kilobases, respectively (Fig. 3). Signal intensities were similar between liver

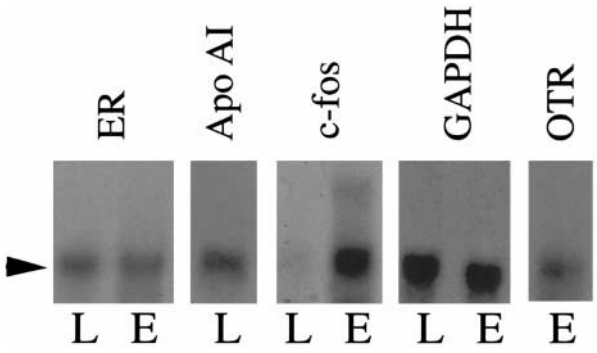

Fig. 3. Northern analysis of ER, apo AI, c-fos, GAPDH and OTR mRNAs in liver and endometrium. Ovine cRNA probes were used to probe liver (L) and endometrial (E) samples of total cellular RNA (40 μg; apo AI, c-fos, GAPDH and OTR) or poly(A) + RNA (5 μg; ER) on Northern blots. Single bands (indicated by the arrowhead) from the resultant autoradiographs are shown to demonstrate the specificity of the hybridization. Migration distances of the bands were compared to those of the 28S and 18S rRNAs to estimate message lengths: 6.8, 1.0, 2.4, 1.8 and 6.8 kilobases for ER, apo AI, c-fos, GAPDH and OTR mRNAs, respectively.

and endometrium for ER and GAPDH mRNAs, however, endometrium appeared to contain more c-fos mRNA than did liver.

Oestradiol effects on concentrations of endometrial mRNAs

In endometrium, oestradiol treatment up-regulated the expression of ER, PR, c-fos and GAPDH mRNAs, but not OTR mRNA (Fig. 4(A)). Oestradiol increased ER and GAPDH mRNA concentrations similarly, to 304 ± 36 and $374 \pm 32\%$ of control values, respectively. PR mRNA levels also rose in response to oestradiol, but to a lesser extent: $165 \pm 19\%$ of control values. The concentrations of c-fos mRNA rose to $193 \pm 37\%$ of control values ($p < 0.08$). In contrast, OTR mRNA concentrations were unchanged by oestradiol treatment. Thus, four out of the five genes analyzed were up-regulated by oestradiol in endometrium.

Tamoxifen effects on concentrations of endometrial mRNAs

To determine the effects of tamoxifen on the expression of the ER, PR, c-fos, OTR and GAPDH genes in endometrium, Tam ewes were compared to those of the Con group (Fig. 4(A)). Tamoxifen enhanced ER, c-fos and GAPDH mRNA concentrations: 200 ± 35, 258 ± 36, and $335 \pm 32\%$, respectively. Tamoxifen increased ER mRNA concentration in endometrium to a lower level than did oestradiol, while its up-regulation of GAPDH was as strong as that induced by oestradiol. There was also a trend toward an increase (45%) in endometrial PR mRNA concentrations in Tam ewes compared to Con ewes ($p = 0.1$). As with oestradiol treatment, tamoxifen did not affect OTR mRNA concentrations. Thus, tamoxifen acted as an oestrogen agonist on gene expression in sheep endometrium.

Comparisons of endometrial mRNA concentrations of the T + E2 and E2 groups determined whether tamoxifen acts as an oestrogen antagonist (Fig. 4(A)). T + E2 ewes increased ER and GAPDH mRNA concentrations by 342 ± 35 and $369 \pm 32\%$, respectively, compared to controls. The values were very similar to those of the E2 ewes. However, T + E2 treatment resulted in endometrial PR and c-fos mRNA concentrations that were intermediate to (and not different

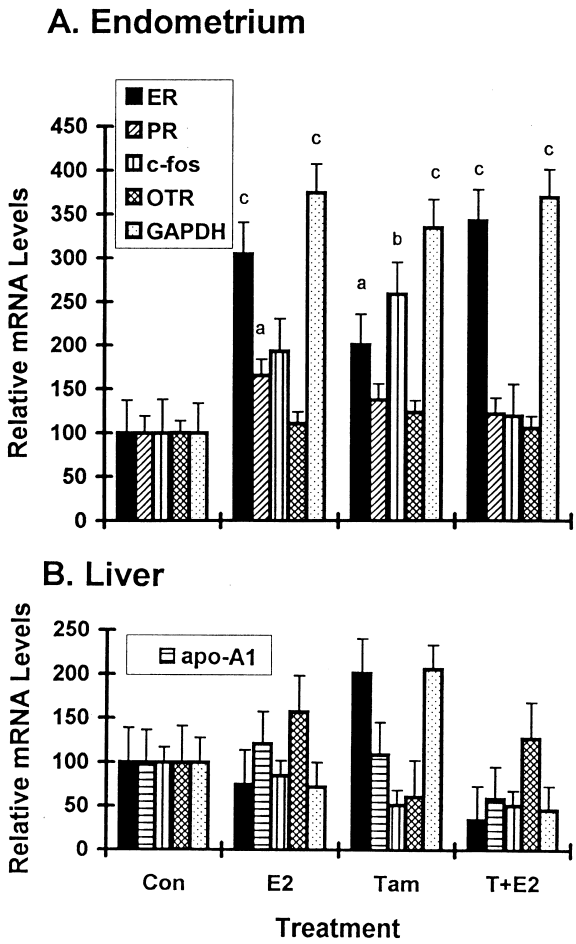

Fig. 4. Oestradiol and tamoxifen effects on concentrations of ER, PR, apo AI, c-fos, OTR and GAPDH mRNAs in endometrium and liver. (A) Endometrial RNA samples were analyzed on replicate slot blots. Relative mRNA levels for ER (filled bars), PR (diagonally hatched bars), c-fos (vertically hatched bars), OTR (cross-hatched bars), and GAPDH (stippled bars) are reported as least squares means and standard errors for the treatment groups described in Fig. 1. Differences between treated groups compared to the control group are indicated by a, b, and c above bars ($p < 0.05$, $p < 0.01$, $p < 0.001$, respectively). (B) Liver RNA samples were analyzed as in (A), but with apo AI mRNA (horizontally hatched bars) replacing PR mRNA.

from) those of Con and E2 groups. We conclude that tamoxifen antagonizes some actions of oestradiol on endometrial genes (preventing the up-regulation of PR and c-fos mRNAs), but not all of them (up-regulation of ER and GAPDH mRNAs).

Oestradiol and tamoxifen effects on concentrations of liver mRNAs

In liver, oestradiol had no effect on concentrations of ER, apo AI, c-fos, OTR, and GAPDH mRNAs (Fig. 4(B)). Similarly, Tam and T + E2 treatments evoked no changes in concentrations of apo AI and OTR gene products. However, Tam-treated ewes showed definite trends toward two-fold increases in ER ($p < 0.08$) and GAPDH ($p = 0.05$) mRNA concentrations compared to controls. Although T + E2 treatment did not significantly alter concentrations of ER and GAPDH mRNAs compared to controls, the combined treatment depressed concentrations of both messages compared to tamoxifen treatment alone. Both Tam and T + E2 treatments appeared to decrease c-fos mRNA concentrations 50% ($p < 0.06$). Thus, the E2 treatment did not up-regulate ER, c-fos and GAPDH mRNA concentrations in liver, in contrast to endometrial effects. However, Tam treatment appears to up-regulate ER and GAPDH mRNA concentrations in liver, similar to its actions in endometrium. These actions were antagonized by oestradiol in the T + E2 treatment group.

Tamoxifen increases ER mRNA stability in endometrium

The molecular mechanism by which oestradiol up-regulates endometrial ER mRNA is by enhancing the stability of the message [9]. To determine if tamoxifen acts similarly, endometrial explants from each ewe were cultured with DRB to inhibit transcription. The decay rates of ER and PR mRNAs over the 12 h culture period are shown in Fig. 5. Oestradiol stabilized ER mRNA at least 3-fold, from a half-life of 9 h in Con ewes to one ≥24 h in the E2 group. Tam treatment stabilized ER mRNA similarly. The fact that PR mRNA stability (Fig. 5), as well as GAPDH and c-fos mRNA stabilities (not shown), were unaffected by treatment demonstrates that the stabilization was specific for ER mRNA.

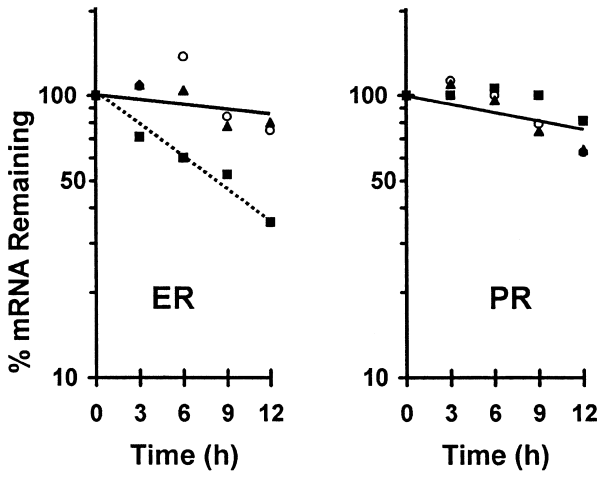

Fig. 5. Tamoxifen, like oestradiol, enhances ER mRNA stability. To determine effects on ER mRNA stability, transcription was inhibited with 75 μg/ml DRB in cultures of endometrial explants from Con, E2, and Tam ewes. Percents of mRNAs remaining, analyzed at 0, 3, 6, 9, and 12 h of culture, are presented as least squares means for Con (filled squares), E2 (open circles), and Tam (filled triangles) treatment groups. ER and PR mRNA data are presented in left and right panels, respectively. Standard errors are 17 for ER and 16 for PR measurements. Lines describe the decay of ER mRNA in Con (dashed) and E2 and Tam treatment groups (solid) and PR mRNA for all three treatments (solid). E2 and Tam treatments stabilized ER mRNA compared to Con, while neither E2 nor Tam affected the degradation of PR mRNA.

DISCUSSION

The systemic dose of oestradiol used here was modified from a steroid replacement regimen given to ovariectomized ewes to make them receptive to transferred embryos [21]. Oestradiol accumulated in endometrial tissues to two-fold higher concentrations than those in endometrial tissues of ewes and rhesus monkeys [22], and about six-fold higher than those of cows [23], in the post-ovulatory stage of their estrous/menstrual cycles. Our study and another using ovariectomized rhesus monkeys and rats [24] demonstrate the persistence of high oestradiol concentrations after circulating levels have fallen [7]. This probably explains why endometrial oestradiol concentrations were not different in ewe endometrium before and after the preovulatory surge of oestrogen. In addition, it indicates that oestradiol may be acting in tissues at times when circulating oestradiol concentrations are low.

Oestradiol induces uterine growth in all species, which is used as a bioassay for oestrogenic activity [1]. However, tamoxifen induction of uterine growth varies between species: from small in the rat and rabbit to large in the mouse and pig [25]. This is the first report of tamoxifen actions in sheep reproductive tissues. Uteri of the Tam group exhibited gross uterine characteristics that were intermediate to those of the Con and E2 groups, consistent with a partial agonist activity of tamoxifen. The trends of Tam increasing uterine weights and E2 and Tam increasing endometrial weights are likely to be real, but confounded by animal to animal variation in body size. The only gross indications that tamoxifen was a partial oestrogen antagonist in the sheep was that T + E2 ewes did not demonstrate the increased uterine weights compared to controls and had lower endometrial weights than did ewes in the E2 treatment group.

Tamoxifen's actions on endometrial gene expression were more striking. Contrary to our hypoth-

esis, tamoxifen acted like oestradiol in increasing the concentration of ER mRNA in ovine endometrium. Similar effects were seen on ER protein levels in endometrium of women given a single 20 mg dose of tamoxifen [26]. It is important to distinguish these studies from those using chronic tamoxifen treatments (20 mg per day), which result in ten-fold higher concentrations of tamoxifen the plasma of women than does a single dose [27]. Chronic treatments also paradoxically increase circulating levels of oestradiol, as well as down-regulate ER protein concentrations 3.5-fold in endometrium of women [28].

The regulation of PR gene expression by oestradiol is well-characterized, with transcriptional activation of its dual oestrogen-responsive promoters [29]. The modest increases in PR mRNA concentrations in E2-treated ewes correlate with moderate (30%) increases in the rate of PR gene transcription [7]. Tamoxifen, like oestradiol, modestly elevated endometrial PR mRNA concentrations compared to controls. This is consistent with data in other species [27]. Since acyclic women required more than 48 h to up-regulate PR protein concentrations in endometrium in response to a single dose of tamoxifen, we suspect that PR mRNA may have increased to a greater extent if sheep endometrium was collected at 36 or 48 h post-tamoxifen.

Tamoxifen, like oestradiol, rapidly up-regulates c-fos gene expression in rodent uteri to peak levels at 1–2 h post-treatment [12, 30]. Since effects are likely to be similar in sheep endometrium, the relatively late sampling times were probably responsible for the more modest changes in c-fos mRNA concentrations in the sheep endometrium. The Tam treatment appeared more effective in up-regulating endometrial c-fos concentrations than the E2 group probably because tamoxifen and tamoxifen-bound ER persist in endometrium longer than oestradiol and oestradiol-bound ER [26, 27]. The up-regulation of c-fos mRNA by oestradiol and tamoxifen was probably countered by autologous down-regulation by increased c-fos protein [12], which was most evident with the sequential treatments in the T + E2 group, so that no increase in c-fos mRNA levels were detected.

Tamoxifen treatment strongly enhanced GAPDH mRNA concentrations in endometrium, similar to ER mRNA concentrations. Up-regulation of the GAPDH gene by oestradiol was first demonstrated in the rat uterus [14]. The mechanism of GAPDH mRNA up-regulation by oestradiol and tamoxifen has yet to be determined. However, in ovine endometrium it is likely to be transcriptional, since no effects on mRNA stability were apparent.

OTR gene expression was not regulated in endometrium by oestradiol, although studies using intact animals suggested that it might be [13]. Of OTR, ER, and PR genes, all critical to maternal recognition of pregnancy in ruminants [2], only OTR was not up-regulated in endometrium by oestradiol. Zhang *et al.* found similar results in ovariectomized ewes [31], so progesterone pretreatment and withdrawal may be more critical to the up-regulation of endometrial OTR gene expression than is the presence of oestradiol. In light of this, it is not surprising that tamoxifen did not affect OTR gene expression.

Liver has only 20% of the ER protein concentration found in endometrium [27], but liver contains the majority of the tamoxifen binding sites in the body [32]. Because oestrogens and tamoxifen act on the liver to alter blood lipids in a way that protects women against heart disease [33, 34], we were interested in oestradiol- and tamoxifen-induced changes in gene expression of the liver. Estrogen replacement therapy increases circulating apo AI protein and the cardiovascular health of post-menopausal women [34]. While oestradiol did not affect ER, apo AI, c-fos or GAPDH mRNA concentrations at 18 h post-injection in this study, oestradiol reduced ER, apo AI, c-fos and GAPDH mRNA concentrations about 50% in liver at 24 h post-injection [35]. Here, at 24 h post-injection, tamoxifen similarly down-regulated c-fos mRNA. However, in contrast to oestradiol actions at 24 h post-injection [35], tamoxifen up-regulated ER and GAPDH mRNA concentrations in liver. These tamoxifen actions were overcome by oestradiol effects in the livers of T + E2 ewes, where ER and GAPDH mRNA concentrations were down-regulated. Thus, oestradiol and tamoxifen effects in the liver depend on the gene, time of analysis and the acute or chronic nature of the treatment.

The most profound change in gene expression evoked by a single dose of oestradiol that we have discovered is the 5-fold increase in endometrial ER mRNA concentrations at 24 h post-injection [7]. The molecular mechanism for this oestradiol action is entirely post-transcriptional, by enhancement of message stability [7, 9]. Here, tamoxifen also up-regulated ER mRNA concentrations in endometrium by enhancing the stability of ER mRNA. To our knowledge, this is the first demonstration of tamoxifen acting post-transcriptionally as an oestrogen agonist.

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