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Oestradiol Up-regulates Oestrogen Receptor, Cyclophilin, and Glyceraldehyde Phosphate Dehydrogenase mRNA Concentrations in Endometrium, but Down-regulates Them in Liver

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Oestradiol regulates reproductive physiology and cardiovascular health in women. In the endometrium of ovariectomized ewes, previous work demonstrated that a single dose of oestradiol (50 μg) up-regulates oestrogen receptor-α (ER) and progesterone receptor (PR) gene expression within 24 h. Here we compared responses to different doses of oestradiol and different dosing regimens in two diverse tissues: endometrium and liver. ER, c-fos, cyclophilin and glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA concentrations were analyzed on replicate RNA slot blots in both tissues, while PR and apolipoprotein AI (apo AI) mRNA concentrations were only analyzed in endometrium or liver, respectively. Along with ER mRNA, oestradiol strongly up-regulated GAPDH and cyclophilin mRNA concentrations in endometrium. In liver, however, oestradiol down-regulated them, along with apo AI mRNA. Responses to different doses and dose regimens, including repeated 50 μg doses, were similar to those evoked by a single 50 μg dose of oestradiol. Thus, oestradiol appears to have all-or-none effects which include up-regulation of ER, cyclophilin and GAPDH gene expression in endometrium and down-regulation of ER, apo AI, cyclophilin and GAPDH gene expression in liver. These results illustrate the sharp contrast between two mammalian tissues in their responses to physiological levels of oestradiol. **© 1998 Elsevier Science Ltd. All rights reserved.**

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INTRODUCTION

Oestradiol modulates expression of a variety of genes in many mammalian tissues to have wide-ranging effects, such as regulation of reproductive physiology and cardiovascular health. As a primary target tissue, endometrium is exquisitely sensitive to oestrogen, which alters the expression of many endometrial genes to adapt tissue functions to the stage of reproduction. Mammalian liver is a very different organ compared to the uterus, but it also responds to oestrogen, perhaps due to evolutionary conservation from egg-laying animals. In the latter, the liver plays a major role in female reproduction by synthesizing egg proteins in response to oestrogen. Oestrogen regulation of liver genes involved in lipoprotein synthesis and metabolism, such as apo AI, may explain how oestrogen alters blood lipid profiles to protect women from heart disease [1].

A major factor in determining the tissue-specificity of oestrogen actions is the concentration of the ER protein. Its ligand, oestrogen, is the primary stimulator of ER gene expression in mammalian reproductive tissues. For example, the preovulatory surge of oestrogen up-regulates ER gene expression in the endometrium of all female mammals investigated,

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including the ewe and woman [2, 3]. Oestrogen is the only gonadal hormone responsible for these effects because ovariectomized animals treated with physiological doses of oestradiol demonstrate similar up-regulation of ER gene expression in their uteri [4, 5]. However, the action of physiological levels of oestradiol on ER gene expression in the mammalian liver is unknown.

Over the last five years, we have studied the regulation of ER and PR gene expression in the endometrium of ovariectomized ewes treated with oestradiol. In frog liver, a single dose of oestradiol increases ER gene expression for months, establishing a positive, autoregulatory loop [6]. Our hypothesis was that oestradiol would up-regulate ER mRNA and apo AI gene expression in liver, to enhance oestrogen responsiveness and recreate the effects in women that improve cardiovascular health [1].

Here, we directly compared oestradiol effects on expression of the ER gene between liver and endometrial tissues in three experiments. The effects of oestradiol on apo AI, PR, GAPDH, cyclophilin, and c-fos gene expression were also assessed. In the first experiment, the responses to a single physiological dose of oestradiol (50 μg; Ref. [4]) were compared to those of 50 μg divided into graded doses and administered over 32 h, to create a more natural oestrogen surge. Both single and graded doses were adapted from long term treatments given to ovariectomized ewes to make them successful recipients for transferred embryos [7]. The second experiment was a dose–response study with doses two-fold higher and lower than the 50 μg dose. The final experiment investigated whether oestradiol effects could be enhanced by second 50 μg dose of oestradiol administered at a time when ER protein levels were greater in endometrium [8].

MATERIALS AND METHODS

Animals, steroid treatments, and tissue 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 assigned randomly to a treatment group in one of three experiments. In Experiment I, ewes injected with a single 50 μg dose of oestradiol (0.5 ml, "E2" group) were compared to ewes receiving a sequence of graded oestradiol doses (5, 10, 20, 10, and 5 μg oestradiol; "GE2" group) distributed over 32 h (see Fig. 1). Tissues were collected 18 h after the single or peak injection of oestradiol. Ewes in the control groups ("Con" and "GCon") received equivalent

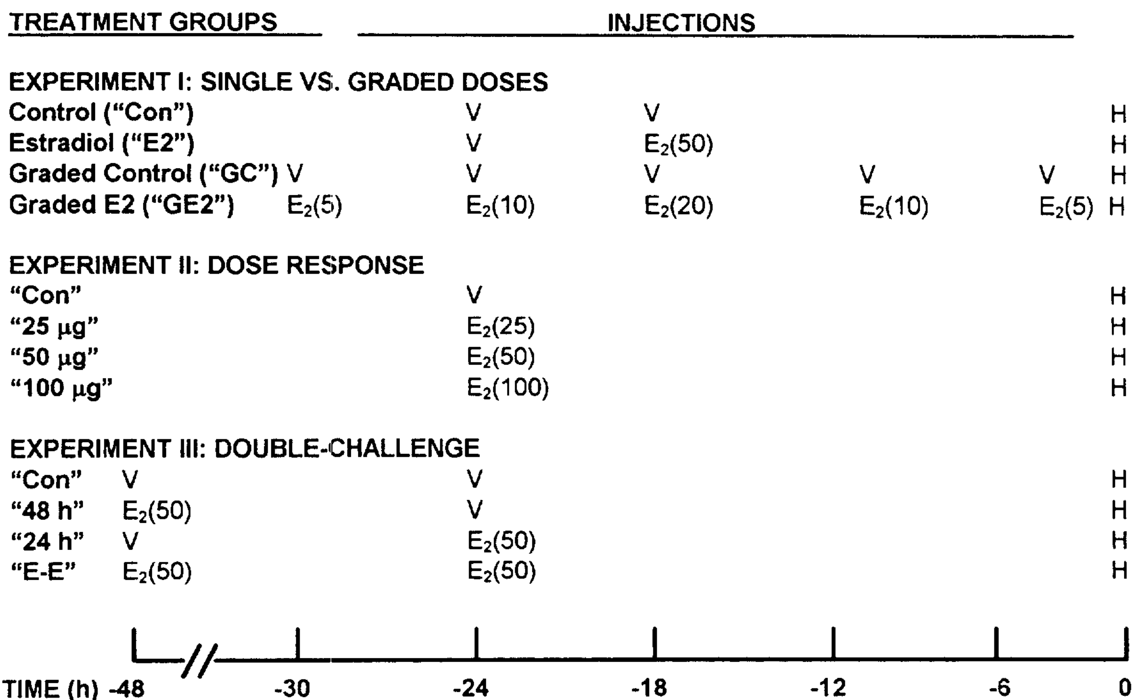

Fig. 1. A timeline describing the treatments administered to ovariectomized ewes. Groups of ewes, ovariectomized one month previously, received i.m. injection(s) of oestradiol (E2; followed by the dose in μg in parentheses) or drug vehicle (V; charcoal-stripped corn oil). The times indicated on the timeline below are relative to the time of hysterectomy (H), when liver and endometrium were collected. Treatment groups were composed of 6 ewes except for the GE2, GCon, 25 μg, and 48 h groups, which were composed of 4, 3, 5 and 3 ewes, respectively.

volumes of vehicle (charcoal-stripped corn oil; Kodak, Rochester, NY) at the appropriate time(s). Experiment II ewes in "Con", "25 μg," "50 μg," and "100 μg" treatment groups received a single i.m. injection containing 0, 25, 50 or 100 μg of oestradiol at 24 h prior to hysterectomy. In Experiment III, ewes that received a double-challenge ("E–E"; 50 μg oestradiol at 48 and 24 h prior to hysterectomy) were compared to three control groups: ewes that received vehicle at both times ("Con"), or single doses at 48 h ("48 h") or 24 h ("24 h") prior to hysterectomy. Treatment groups were composed of 6 ewes except for GE2, GCon, 25 μg, and 48 h groups, which were composed of 4, 3, 5 and 3 ewes, respectively. Samples of endometrium and liver were harvested surgically. Whole uteri and dissected endometrium were weighed. Endometrium and liver tissues were minced, snap frozen in liquid nitrogen, and stored at −80°C. Unless otherwise indicated, chemicals were purchased from Sigma (St. Louis, MO).

Synthesis of radiolabelled cRNA probes

All cRNA probes were generated from ovine cDNAs cloned in our lab [4, 10] except for the human cyclophilin and 18S rRNA probes (Ambion; Austin, TX). GenBank accession numbers for ovine ER, PR, GAPDH, c-fos, apo AI cDNA sequences are U30299, U30300, U94718, U94719, and U94720, respectively. ER and PR cRNA probes were generated with T7 RNA polymerase (Maxiscript kit; Ambion), as described previously [4]. Antisense GAPDH, c-fos, and apo AI probes were generated similarly from plasmids linearized with *Bam*HI. Because PR and c-fos mRNAs are relatively low in abundance, the specific activities of PR and c-fos cRNA probes were doubled by eliminating unlabelled UTP and doubling ^{32}P-UTP in those *in vitro* transcription reactions. Unincorporated radionucleotides were removed on spin columns (Bio101; La Jolla, CA).

Preparation and slot blot analysis of mRNAs

Total cellular RNA was purified from endometrium and liver samples (0.5 g) with Tripure reagent (Boehringer Mannheim; Indianapolis, IN). Samples of 20 μg RNA, estimated by A_{260} measurements, were denatured and immobilized in 4 μg aliquots (except c-fos blots which had 8 μg aliquots) on nitrocellulose (Schleicher and Schuell, Keene, NH) slot blots [9]. Replicate blots were hybridized with antisense cRNA probes for ER, PR (endometrium only), GAPDH, apo AI (liver only) and c-fos mRNAs as well as 18S rRNA. Blot hybridization and washing conditions were stringent, as described for Northern analyses which demonstrated a single hybridizing band of appropriate size for each cRNA probe [4, 10]. Blots were exposed to Fuji X-ray film (VWR Scientific; Houston, TX) for 30 min to 4 days, depending on the probe, to get an autoradiographic exposure in the linear range of the film. Hybridization signals on autoradiographs were quantitated by densitometry using Adobe Photoshop scanning and Intelligent Quantifier (Bio Image, Ann Arbor, MI) quantitation softwares.

Statistical analyses

Quantitative data were analyzed by least squares ANOVA using the General Linear Model procedures of SAS [11]. Results from treatment groups were compared by orthogonal contrasts to the control group. Level of significance was taken to be $p < 0.05$ except where noted. Data are presented as least squares means and standard errors for treatment groups. 18S rRNA hybridization signals were used to normalize signals from mRNAs to account for unequal RNA loading between slots. Hybridization signals of mRNAs were expressed relative to the average of the appropriate control group to show treatment-induced changes in the mRNA concentrations.

RESULTS

Oestradiol increases uterine weights

Overall, oestradiol treatment increased the size, redness, and tone of the uteri. Within the uteri of ewes that received the graded oestradiol treatment regimen, evaginations of the endometrium, called "caruncles," were turgid, resembling the kernels of corn-on-the-cob. These observations correlated with increased uterine weights, from 25 to 40 ± 4 g, in response to single and graded dose treatments (Fig. 2). There was also a trend toward increased endometrial weights with both oestradiol treatments, from 3.8 to 5.6 ± 0.9 g ($p < 0.06$). Thus, both single dose and graded dose treatments with oestradiol altered the gross character of the uteri.

Effects of graded doses of oestradiol on endometrial gene expression

A single dose of oestradiol increased ER, PR and GAPDH mRNA concentrations in endometrium, while concentrations of c-fos mRNA were unchanged compared to controls (Fig. 3). Graded doses of oestradiol evoked a similar pattern of change in endometrial mRNA concentrations. The values for the two control groups did not differ. However, the graded doses of oestradiol increased GAPDH mRNA concentrations to a greater extent than did the single dose: $569 \pm 45\%$ compared to $358 \pm 38\%$ ($p < 0.001$). In conclusion, a single 50 μg dose of oestradiol generated similar effects when divided into graded doses; however, the latter increased GAPDH mRNA concentrations to a greater extent.

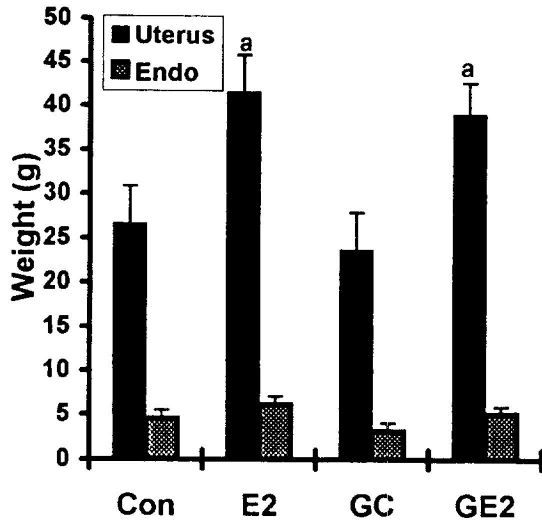

Fig. 2. Oestradiol effects on uterine and endometrial weights. Weights of whole uteri (filled bars) and dissected endometrium ("Endo," hatched bars) are presented as least squares means and standards errors for treatment groups described in Fig. 1. "a" Above the bar indicates that E2 and GE2 treatments increased uterine weights compared to the control groups ($p < 0.05$). In addition, both treatments tended to increase endometrial weights ($p < 0.06$).

Effects of different doses of oestradiol on gene expression in endometrium

Using single doses of oestradiol that were within the physiological range, a dose–response study was performed in ovariectomized ewes. In endometrium, all doses of oestradiol enhanced ER mRNA concentrations, an average to 208 ± 38% of control values [Fig. 4(A)]. There was no dose–response effect of estradiol on ER mRNA concentrations in endometrium ($p = 0.2$). PR mRNA concentrations appeared to increase in response to oestradiol (66% with 50 μg), but values for each treatment group were not significantly different from those of control ewes. Oestradiol strongly up-regulated cyclophilin and GAPDH mRNA concentrations, to 360 ± 48% and 478 ± 66% of control levels, respectively [Fig. 4(A)]. In sharp contrast to the other four gene products analyzed, c-fos mRNA concentrations were reduced by an average of 60 ± 17% by all three doses of oestradiol. In conclusion, physiological doses of oestradiol up-regulated expression of ER, cyclophilin and GAPDH genes, but down-regulated expression of the c-fos gene in endometrium.

Oestradiol effects on gene expression in liver

The effects of oestradiol on gene expression in the liver were quite distinct from those in endometrium. In contrast to ER mRNA up-regulation in endometrium, all doses of oestradiol reduced ER mRNA concentrations by 61 ± 11% in liver [Fig. 4(B)]. Apo AI and cyclophilin mRNA concentrations were also reduced by all doses of oestradiol, by an average of 35 ± 19% and 48 ± 16%, respectively. However, the reductions in mRNA concentrations did not increase with greater doses of estradiol. These results demonstrate that physiological doses of oestradiol evoke opposite effects on expression of ER and cyclophilin genes in liver and endometrium.

Effects of oestradiol double-challenge on gene expression in endometrium

Since 50 μg of oestradiol up-regulates ER protein within 24 h in the majority of endometrial cells [8], we investigated whether a second dose of oestradiol 24 h after the first would have even greater effects due to the greater concentration of ER. The effects of oestradiol double-challenge were compared with those of a single 50 μg dose at 24 or 48 h prior to tissue collection, as well as the vehicle-treated control group (Fig. 1).

In endometrium, E–E, 24 h and 48 h treatment groups increased ER mRNA concentrations similarly, an average of 228 ± 44% [Fig. 5(A)]. Likewise, all oestradiol treatments increased endometrial cyclophilin and GAPDH mRNA concentrations (343 ± 56

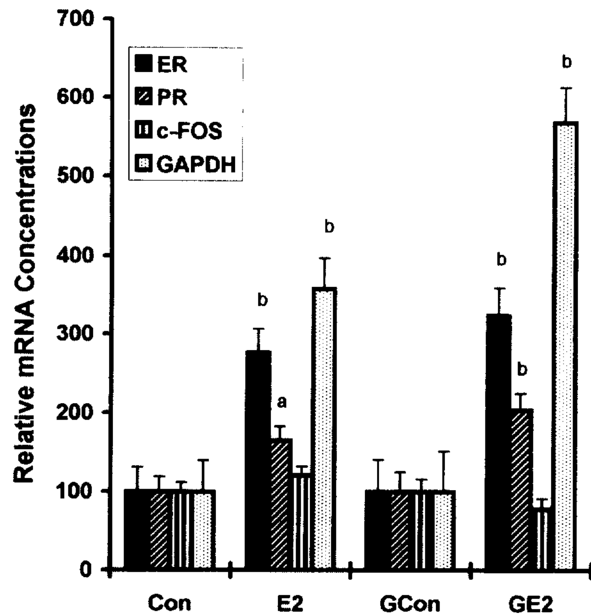

Fig. 3. Oestradiol effects on concentrations of ER, PR, c-fos and GAPDH mRNAs in endometrium. Duplicate endometrial RNA samples were analyzed for ER, PR, GAPDH, and c-fos mRNA and 18S rRNA concentrations on replicate slot blots. Hybridization signals for mRNAs were normalized to 18S rRNA signals, and to the average value of the appropriate control group. Relative mRNA levels for ER (filled bars), PR (diagonally hatched bars), c-fos (vertically hatched bars), and GAPDH (stippled bars) are reported as least squares means and standard errors for the Experiment I treatment groups described in Fig. 1. Oestradiol increased ER, PR, and GAPDH mRNA concentrations ("a" and "b" above bars indicate differences with $p < 0.05$ and $p < 0.01$, respectively). The GE2 treatment increased GAPDH mRNA more than did the E2 treatment ($p < 0.001$).

and 461 ± 80%, respectively). PR mRNA concentrations were not altered significantly by any treatments. However, all oestradiol treatments appeared to reduce c-fos mRNA concentrations an average of 41 ± 21% compared to controls ($p < 0.1$). None of the oestradiol effects were significantly greater in the E–E treatment group compared to the groups that received a single dose. In summary, the effects of repeated challenge with oestradiol did not differ from

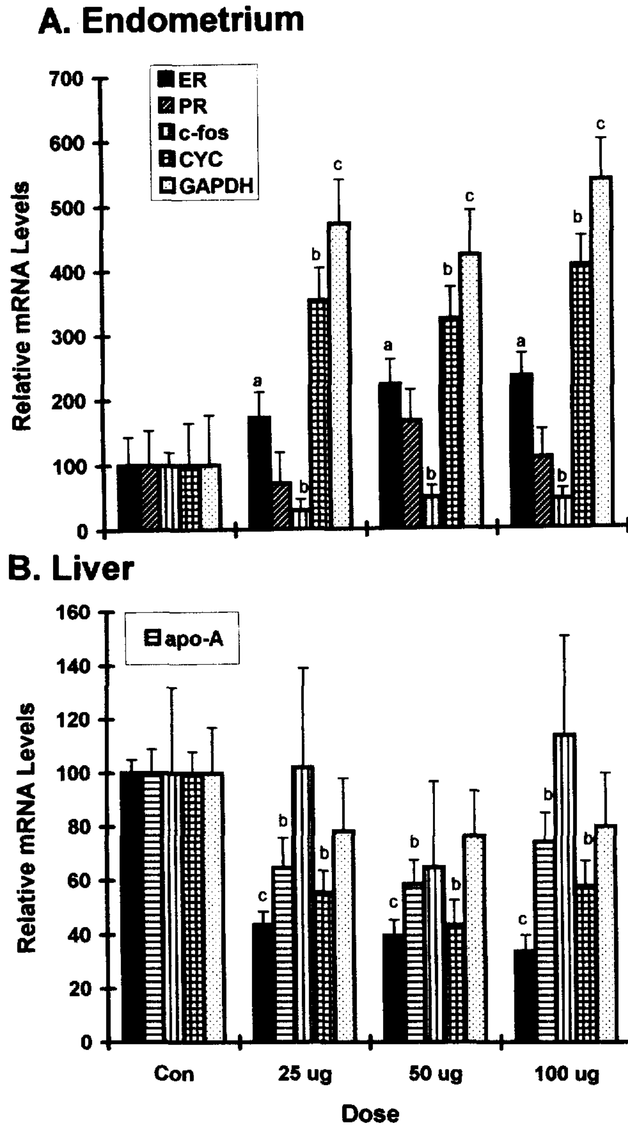

Fig. 4. Effects of different doses of oestradiol on ER, PR, apo AI, c-fos, cyclophilin and GAPDH mRNA concentrations in endometrium and liver. Panel A. Endometrial RNA samples were analyzed for ER, PR, c-fos, cyclophilin and GAPDH mRNA and 18S rRNA concentrations on replicate slot blots. Hybridization signals for mRNAs were normalized to 18S rRNA signals, and to the average value of the appropriate control group. Relative mRNA concentrations for ER (filled bars), PR (diagonally hatched bars), c-fos (vertically hatched bars), cyclophilin (cross-hatched bars), and GAPDH (stippled bars) are reported as least squares means and standard errors for the Experiment II treatment groups described in Fig. 1. Differences of 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). Panel B: Liver RNA samples were analyzed as in panel A, but with apo AI mRNA (horizontally hatched bars) replacing PR mRNA.

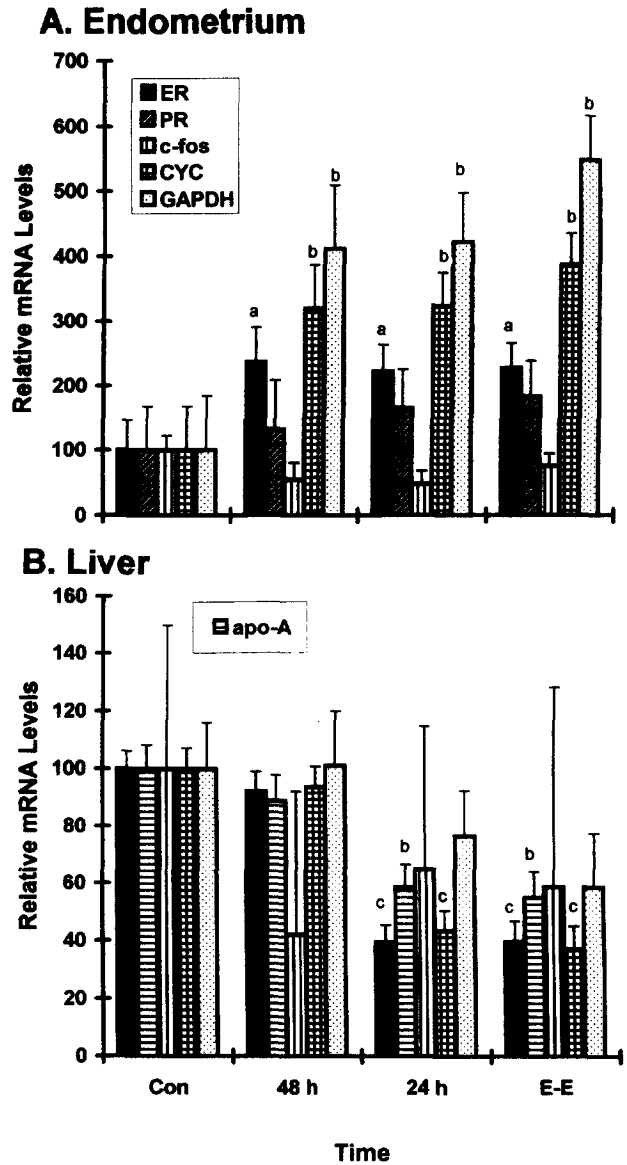

Fig. 5. Effects of oestradiol double challenge on ER, PR, apo AI, c-fos, cyclophilin and GAPDH mRNA concentrations in endometrium and liver. Panel A. Endometrial RNA samples were analyzed for ER, PR, c-fos, cyclophilin and GAPDH mRNA and 18S rRNA concentrations on replicate slot blots. Hybridization signals for mRNAs were normalized to 18S rRNA signals, and to the average value of the appropriate control group. Relative mRNA levels for ER (filled bars), PR (diagonally hatched bars), c-fos (vertically hatched bars), cyclophilin (cross-hatched bars), and GAPDH (stippled bars) are reported as least squares means and standard errors for the Experiment III treatment groups described in Fig. 1. Panel B: Liver RNA samples were analyzed as in panel A, but with apo AI mRNA (horizontally hatched bars) replacing PR mRNA. Differences of 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). E–E treatment showed a trend toward reducing GAPDH mRNA concentrations in the liver ($p < 0.1$).

those of a single injection at either 24 or 48 h prior to tissue collection.

Effects of oestradiol are of shorter duration in liver

The effects of repeated challenges with oestradiol on gene expression were also investigated in liver. As in endometrium, the effects of two 50 μg doses at 24 and 48 h prior to tissue collection were similar to the effects of a single dose at 24 h [Fig. 5(B)]. Both reduced the concentrations of ER, apo AI, and cyclophilin mRNAs in liver by an average of 60 ± 7, 43 ± 9, and $60 \pm 8\%$, respectively. GAPDH mRNA concentrations tended to be $42 \pm 16\%$ lower in the E–E treatment group than in Con and 48 h treatment groups ($p < 0.1$). The concentrations of c-fos mRNA in liver were low and highly variable, perhaps masking oestradiol effects. Interestingly, the effects of a single dose of oestradiol on ER, apo AI, cyclophilin, and GAPDH mRNA concentrations were absent by 48 h post-injection. This indicates that oestradiol effects on gene expression, in addition to being opposite in liver and endometrium, are also of shorter duration in liver.

DISCUSSION

Here we report acute responses, ranging from alterations in gross tissue characteristics to changes in gene expression, brought about by one or more physiological dose(s) of oestradiol. Oestradiol responses were measured in two mammalian tissues of diverse function: endometrium and liver. The single 50 μg oestradiol dose, used here, was chosen from a steroid treatment regimen used to prepare ovariectomized ewes as embryo transfer recipients [7]. We have demonstrated that this dose results in concentrations of oestradiol in plasma and endometrial tissues similar to those in ovarian veins and endometrium of ovary-intact ewes at proestrus and estrus [4, 10].

Most treatment regimens that seek to simulate the preovulatory surge of oestrogen provide increased plasma levels of oestradiol for more than 24 h. Therefore, we tested the effects of a graded dose regimen [7] against those of a single 50 μg dose of oestradiol in Experiment I. In a second experiment, responses to two-fold lower and higher doses of oestradiol were assessed. In the third experiment, responses to a second dose of oestradiol, administered at a time of increased ER protein in endometrium [8], were compared to those of the single dose. Surprisingly, oestradiol responses were quite similar between all treatment groups. Thus, the oestradiol effects appear to be "all or none": independent of dose and dose frequency. The similarity between responses to all of the oestradiol treatments indicates that maintaining high plasma concentrations of oestradiol is not crucial for effectiveness. High concentrations of oestradiol accumulate locally in uterine tissues by binding ER and low affinity, high capacity oestrogen-binding sites ("type II receptors"), thus serving as a reservoir of ligand which maintains oestradiol effects after circulating concentrations fall [12, 13].

The only exception to the apparent all-or-none effects of oestradiol on gene expression was their shorter duration in liver compared to endometrium. In liver, concentrations of ER, apo AI, and cyclophilin mRNAs returned to levels in control ovariectomized ewes within 48 h. In endometrium, however, oestradiol responses were as strong at 48 h as they were at 24 h post-injection. Perhaps oestradiol was depleted faster in the liver because of its lower concentration of oestradiol binding sites and/or its rapid metabolism in that tissue [14].

The most remarkable feature of oestradiol-induced alterations in gene expression was their tissue-specificity. In endometrium, oestradiol increased ER and PR gene expression as predicted from previous studies [4, 8]. The up-regulation was similar to that described in ovary-intact sheep and other mammals during proestrus [5, 15]. In liver, on the other hand, oestradiol decreased ER mRNA concentrations. This is consistent with findings in rat liver, where ER protein increased following ovariectomy [16]. It is possible that oestradiol effects on ER gene regulation differ between liver and endometrium because of a liver-specific promoter on the ER gene and/or tissue-specific splicing of ER gene products [17, 18]. We plan to investigate estrogen receptor-β gene expression in these endometrial and liver samples for completeness, but the ER-α gene appears to be the dominant form expressed in both tissues in the rat [19].

Like ER mRNA concentrations, oestradiol strongly up-regulated GAPDH and cyclophilin mRNA concentrations in endometrium, but not liver. Also similar to ER gene expression, liver cyclophilin mRNA concentrations were down-regulated by oestradiol. In Experiments II and III, GAPDH mRNA concentrations in liver demonstrated a consistent trend toward reduction in all groups treated with oestradiol within 24 h of tissue collection ($p < 0.1$). Thus, cyclophilin and GAPDH mRNA concentrations in liver and endometrium were affected by oestradiol, clearly indicating that they are regulated and not constitutively-expressed genes. If concentrations of an mRNA of interest were normalized to either, results would artifactually show oestradiol effects where none existed: specifically, down-regulation in endometrium and up-regulation in liver. Because we have not identified a suitable, oestradiol-insensitive mRNA, we normalize mRNA hybridization signals to those of 18S rRNA, an indicator of the total mass of RNA in the analysis.

In contrast to other gene products, c-fos mRNA concentrations were reduced by most oestradiol treat-

ments in both endometrial and liver tissues. Only the E2 treatment group of Experiment I demonstrated no effect of estradiol on endometrial c-fos mRNA concentrations. Our results contrast with those of others who demonstrated that c-fos mRNA increases in endometrium of sheep during proestrus [20]. The sampling regimen in Experiments II and III may have missed a transient up-regulation of c-fos mRNA concentrations, which peaks 1 to 2 h post-oestradiol in the uteri of ovariectomized rodents [21]. The graded dose treatment in Experiment I, however, included an estradiol injection only 4 h prior to tissue collection. Perhaps c-fos protein concentrations increased after each estradiol injection in the endometrium of these ewes and this repressed c-fos gene transcription, thus preventing an increase in c-fos mRNA concentrations [21].

Oestradiol also down-regulated apo AI gene expression in liver. This is consistent with results in the rat, where apo AI mRNA levels rose in response to ovariectomy [22]. However, these findings contrast to the fact that oestrogen therapy increases circulating concentrations of apo AI in women [1], as well as the expression of transcription factors critical to apo AI gene expression in liver cells [23]. Perhaps the higher dose(s) of oestradiol or longer treatment would enhance apo AI gene expression in sheep liver. Alternatively, plasma levels of apo AI in women may increase from translational or posttranslational regulation of gene expression.

In conclusion, although oestradiol binding to ER protein is common to most tissues of the body, the subsequent cascade of effects is both tissue- and gene-specific. This includes the autologous regulation of ER gene expression by oestradiol. In endometrium, oestradiol rapidly and powerfully enhances expression of the ER gene. In contrast, oestradiol down-regulates ER gene expression in liver, probably limiting oestrogen action. This may explain, in part, the opposite effects of oestradiol on ER, cyclophilin and GAPDH gene expression in endometrium and liver.

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