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DIFFERENTIAL EFFECTS OF OVARIAN STEROIDS AND TRIPHENYLETHYLENE COMPOUNDS ON MACROMOLECULAR UPTAKE AND THYMIDINE INCORPORATION IN THE MOUSE UTERUS

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Summary—In the rodent uterus, estrogen elicits a biphasic response i.e. an early phase (Phase I) and a late phase (Phase II). Estradiol-17β (E_2) and estriol (E_3), as well as triphenylethylene (TPE) compounds, CI-628 and clomiphene citrate (CC), were used to characterize Phase I and Phase II responses in uterine preparation for implantation in the mouse. While uterine macromolecular uptake (vascular permeability), a Phase I response, was studied in progesterone (P_4)-primed animals, uterine [^{3}H]thymidine incorporation (DNA synthesis), a Phase II response, was investigated with and without P_4-priming. In the P_4-primed uterus, all compounds, except CC, significantly increased uterine macromolecular uptake as determined by interstitial tissue accumulation of [^{125}I]bovine serum albumin ([^{125}I]BSA). DNA synthesis as determined by cellular incorporation of [^{3}H]thymidine was modulated by P_4, estrogens and TPE compounds in a cell-type specific and temporal manner. As a single injection and in the absence of P_4, E_2 induced [^{3}H]thymidine incorporation in the luminal and glandular epithelium at 18 and 24 h. E_3 was inferior to E_2 in this response. On the other hand, treatment with P_4 for 1 day or 4 days induced [^{3}H]thymidine incorporation primarily in stromal cells. However, stromal cell incorporation was potentiated when P_4 treatment was combined with estrogens or TPE compounds. These results reveal the relative importance of Phase I and cell-type specific Phase II responses in uterine preparation for implantation.

INTRODUCTION

In the mouse, embryo implantation requires complex interactions between embryonic and uterine cells in response to ovarian estrogen and P_4. Ovariectomy before the presumed estrogen surge on day 4 of pregnancy results in dormancy of the blastocyst and delayed implantation. Implantation, however, can be initiated by a single injection of estrogen in a P_4-primed uterus [1, 2]. One of the earliest prerequisite events in the initiation of implantation is the increased endometrial vascular permeability at the location of the blastocyst. This event is then followed by stromal cell decidualization [3]. The mechanism by which preimplantation ovarian estrogen secretion initiates this early event of the implantation process in a P_4-primed uterus is not clearly defined. In the uterus, estrogen elicits a biphasic action i.e. an early phase (Phase I) and a late phase (Phase II) [4–7]. Phase I uterine responses occur within 6 h of estrogen administration and a few of the well characterized responses are increased water imbibition, macromolecular uptake (vascular permeability), prostaglandin release and induced protein synthesis. Phase II uterine responses occur between 12–24 h of estrogen treatment and represent the "true growth" phase in which cellular proliferation (DNA synthesis) and hypertrophy occur [6–9]. Recently, we have characterized several estrogens, and TPE compounds, commonly known as antiestrogens, with regard to their biphasic responses in the P_4-primed uterus and determined their relative importance in implantation [10]. In this study, uterine wet and dry weights at 6 and 24 h were used to determine Phase I and Phase II responses. These results indicated that Phase I of estrogen action is important for implantation [10]. In addition, none of the test compounds increased uterine dry weights at 24 h above that of the P_4 treatment indicating that Phase II responses may not be required for the initiation of implantation. However, we studied but one of the various Phase I and Phase II responses, and thus it was necessary to assess other specific responses of these phases to demonstrate more definitely their importance in implantation. In the present investigation, we have studied [^{125}I]BSA uptake (an indicator of vascular permeability and a Phase I response) and nuclear incorporation of [^{3}H]thymidine (an indicator of DNA synthesis and a

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Phase II response) in the uterus following single injections of P_4 or estrogens, as well as in the P_4-primed uterus following single injections of estrogens and TPE compounds. P_4-priming is necessary for the induction of implantation by these compounds [3, 10].

EXPERIMENTAL

Animals and reagents

Charles River (CD-1) female mice (48 days old, 20–25 g) were ovariectomized without regard to the stages of estrous cycle. After 7 days of rest, animals received either a single injection of E_2 (Sigma Chemical Co., St Louis, Mo.), E_3 (Sigma), or P_4 (Sigma). Other groups of animals were treated with P_4 for 4 days and then on the 4th day of P_4 treatment, they received either a single injection of E_2, E_3 or the TPE compounds, CI-628 (Warner-Lambert/Parke-Davis, Ann Arbor, Mich.), or CC (Richardson–Merrill, Inc., Cincinnati, Ohio). E_2, E_3 and P_4 were dissolved in sesame oil (Sigma) and injected subcutaneously. CI-628 and CC were dissolved in 0.15 M NaCl solution and injected intraperitoneally (i.p.). The doses of steroids and TPE compounds are based on our previous work [10].

Interstitial tissue accumulation of [^{125}I]BSA

To determine uterine macromolecular uptake at 1 and 6 h (a Phase I response) after administration of the test compounds on the 4th day of P_4 treatment, 0.5 μCi [^{125}I]BSA per 0.1 ml saline (SA: 1.64 μCi/mg, NEN Research Products, Boston, Mass) was injected intravenously 15 min before animals were killed. The interstitial tissue accumulation of [^{125}I]BSA was determined by counting the tissue samples in a gamma counter [11]. The specificity of uterine-uptake of [^{125}I]BSA following the various treatments was determined by comparison with the uptake in other tissues, such as thigh muscle, liver, and kidney. The plasma distribution of radioactivity was also monitored.

DNA synthesis

To determine DNA synthesis (a Phase II response), nuclear incorporation of [^{3}H]thymidine was monitored at 6, 12, 18 and 24 h. Animals received an i.p. injection of [methyl-^{3}H]thymidine (25 μCi/0.1 ml saline, SA: 40 mCi/mmol, Research Products International Corp., Mount Prospect, Ill.) 2 h before they were killed. Uterine pieces (4–6 mm) were fixed in 4% paraformaldehyde in phosphate buffered saline and processed for paraffin embedding. Uterine sections (7 μm) were mounted on poly-L-lysine-coated slides. Slides were deparaffinized, air dried, dipped in NTB-2 emulsion (Eastman Kodak, Rochester, N.Y.), exposed for 14–18 days and developed using Developer D-19 (Eastman Kodak). All apparent cell types in the uterus were examined for [^{3}H]thymidine incorporation. Representative autoradiograms showing clusters of silver grains in the nuclei are indicative of localization of [^{3}H]thymidine incorporation.

RESULTS

Estrogen and TPE compounds increased uterine accumulation of [^{125}I]BSA above that of P_4 at 1 and 6 h (Fig. 1). CC was the least effective in this response. The uptake induced by other agents was significantly greater than CC at both the hours with the exception of E_2 at 1 h. Furthermore, all the compounds, except CC, maintained increased levels of [^{125}I]BSA at both time points examined (Fig. 1). There was no difference in [^{125}I]BSA uptake in other tissues among various treatment groups (data not shown).

As shown in Table 1, a single injection of E_2, E_3, or P_4 showed little or no incorporation of [^{3}H]-thymidine in any uterine cell-types at 6 h. At 12 h and at subsequent time points, a cell-type specific incorporation was evident following the various treatments. Under our experimental conditions, E_2-induced nuclear [^{3}H]thymidine incorporation in the luminal and glandular epithelium was maximal at 18 h, and the response was greater in the luminal epithelium (Table 1 and Fig. 2a). This is consistent with earlier reports that estrogens primarily induce cellular proliferation in the uterine luminal and glandular epithelium in the adult mouse [12, 13]. As expected, E_3 was a poor inducer of [^{3}H]thymidine

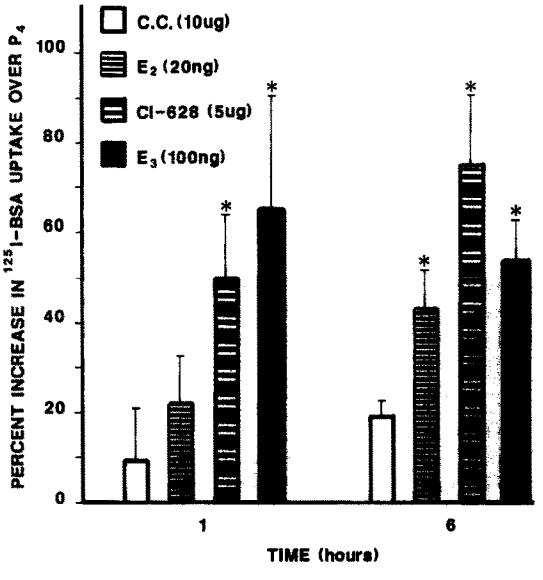

Fig. 1. Effects of estrogens and TPE compounds on uterine accumulation of [^{125}I]BSA at 1 and 6 h (a Phase I response). Percent increase in [^{125}I]BSA accumulation over P_4 was calculated by dividing the difference in radioactivity of the individual experimental uteri and mean radioactivity of P_4-treated uteri by the latter and multiplying the value by 100. Results shown are means ($\pm$SEM) for 4–8 animals per group. *$P < 0.05$ compared to the CC treated values (ANOVA). All animals were primed for 4 days with P_4 (2 mg/mouse/day).

Table 1. [^{3}H]Thymidine incorporation in the mouse uterus following administration of P_4, E_2 or E_3

	Time (h)	Epithelium		Stroma	Myometrium	
		Lumen	Gland		Longitudinal	Circular
P_4	6	1 ± 2^a	6 ± 7	0	0	0
	12	0	0	18 ± 5^{16}	0	2 ± 1
	24	3 ± 3	1 ± 1	104 ± 35^{17}	0	0
E_2	6	1 ± 1	8 ± 1	0	0	0
	12	$20 \pm 16^{1,3,6}$	$24 \pm 11^{9,10,15}$	0	0	0
	18	580 ± 200^2	$241 \pm 102^{9,10,15}$	10 ± 5	7 ± 1	2 ± 1
	24	$40 \pm 6^{3,7}$	$78 \pm 8^{9,13,14}$	0	2 ± 2	1 ± 1
E_3	6	$0^{4,1}$	0^8	0	0	0
	12	$1 \pm 1^{4,1}$	$18 \pm 15^{8,10,11,12}$	0	0	0
	18	$15 \pm 3^{5,6}$	84 ± 20^9	0	1 ± 1	0
	24	$21 \pm 15^{1,4,5,7}$	$28 \pm 14^{10,13}$	23 ± 7	6 ± 2	3 ± 2

[a]Mean number (±SEM) of autoradiographically labeled nuclei in 5 uterine sections from individual animals ($n = 5$). P_4(2 mg/0.1 ml oil/mouse), E_2 (20 ng/0.1 ml oil/mouse) and E_3 (100 ng/0.1 ml oil/mouse) were injected subcutaneously. Statistical analysis was computed between and within the E_2- and E_3-treated groups at all time points in the luminal or glandular epithelium, and for P_4 at 12 and 24 h in stroma (one-way analysis of variance, ANOVA). Values with different superscripts are different from each other ($P < 0.05$).

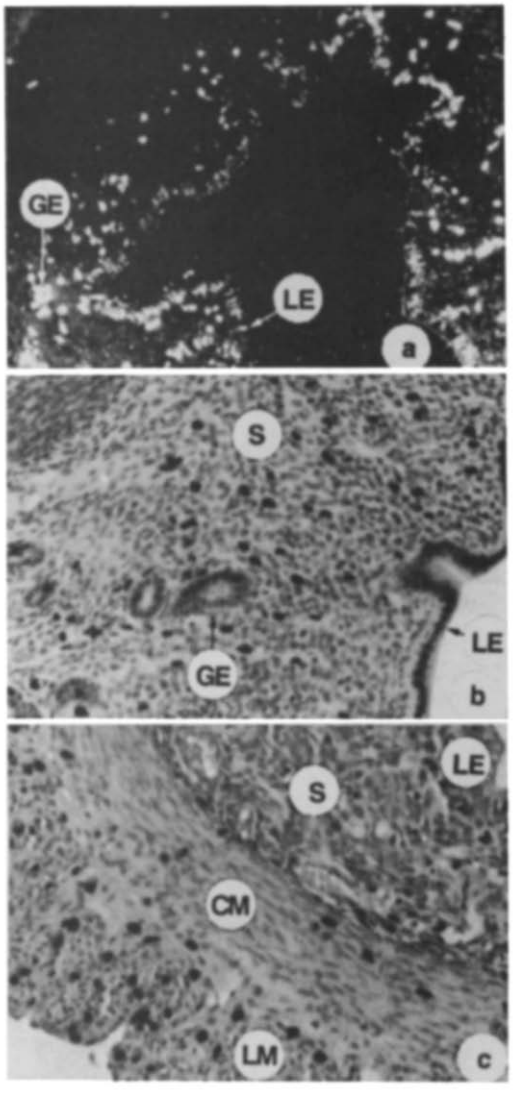

Fig. 2. [^{3}H]thymidine incorporation in the mouse uterus following administration of (a) E_2 at 18 h, (b) $P_4 + E_2$ at 24 h and (c) $P_4 + E_3$ at 24 h. E_2 treatment shows autoradiographically labeled nuclei localized solely to the luminal epithelium (LE) and glandular epithelium (GE), whereas E_2 superimposed on 4 days of P_4 treatment shows labeling primarily to stromal cells (S). E_3 in the P_4-primed uterus shows a large number of labeled nuclei in longitudinal muscle (LM) with a few in circular muscle (CM); (a) dark field, (b) and (c) bright field ×200.

incorporation in the luminal and glandular epithelium. However, the glandular epithelium was more responsive than the luminal epithelium to E_3 (Table 1). On the other hand, the incorporation was primarily limited to stromal cell nuclei 24 h following a single injection of P_4 (Table 1). Mice treated for 4 days with P_4 maintained comparable levels of stromal cell incorporation at each time point tested (Table 2). Superimposition of E_2 on P_4 treatment potentiated the stromal cell incorporation at 24 h as compared to P_4 alone (Table 2 and Fig. 2b). Although E_3 and CC in the P_4-primed animal significantly increased stromal cell incorporation at 24 h above that of P_4 alone, the response was not as great as that of E_2. CI-628 was the most potent agent in stimulating stromal cell incorporation of [^{3}H]thymidine in the P_4-primed uterus (Table 2). As single injections P_4, E_2, or E_3 showed few [^{3}H]thymidine-labelled cells in the myometrium (Table 1). However, E_3 strikingly increased [^{3}H]thymidine incorporation in myometrial cell nuclei at 24 h following 4 days of P_4 treatment (Table 2 and Fig. 2c).

DISCUSSION

Several interesting observations were noted in this study. Our present finding of increased macromolecular accumulation in the P_4-primed uterus in response to E_2, E_3, and CI-628 correlates well with our earlier observation of increased water imbibition under the same conditions [10]. On the other hand, CC is a poor inducer of these responses and was not very effective in initiating implantation [10]. In our study, the initiation of implantation was defined as the appearance of increased capillary permeability at the site of the blastocyst [3]. On the basis of our present and earlier works [9, 10], one could suggest that Phase I of estrogen action appears to be important for implantation. However, one must realize that the failure of CC to induce these responses does not necessarily indicate that these specific Phase I responses are obligatory for implantation. It is possible that CC may interfere with other components of the Phase I response which were not determined, but may be

Table 2. [^{3}H]Thymidine incorporation in the P_4-primed mouse uterus following administration of E_2, E_3, CI-628 or CC

	Time (h)	Epithelium		Stroma	Myometrium	
		Lumen	Gland		Longitudinal	Circular
P_4	6	0[a]	0	125 ± 17	10 ± 1	2 ± 1
	24	0	0	65 ± 13	25 ± 6	4 ± 1
E_2	6	0	0	77 ± 25	4 ± 2	5 ± 2
	24	0	0	261 ± 43*	48 ± 18	22 ± 7
E_3	6	0	0	74 ± 39	2 ± 2	0
	24	0	0	163 ± 29*	160 ± 31**	64 ± 8**
CI-628	6	0	0	84 ± 13	10 ± 6	3 ± 2
	24	0	0	845 ± 52*	34 ± 17	25 ± 12
CC	6	0	0	40 ± 19	3 ± 2	4 ± 1
	24	0	0	178 ± 70*	2 ± 2	6 ± 2

[a]Mean number (±SEM) of autoradiographically labeled nuclei in 5 uterine sections from individual animals ($n = 5$). Animals were treated with P_4 (2 mg/mouse) for 4 days. Doses for E_2 and E_3 are as in Table 1. CI-628 (5μg/mouse) and CC (10 μg/mouse) were injected i.p. in 0.15 M NaCl solution. *$P < 0.05$ (ANOVA) compared to P_4. **$P < 0.05$ (ANOVA) compared to all treatment groups at the same time point.

required for implantation. In this respect, we have recently shown that prostaglandins (PGs) and leukotrienes (LTs) could be mediators of Phase I of estrogen action in implantation in the mouse [9]. In this study, although inhibitors of cyclooxygenase and lipoxygenase pathways did not significantly alter macromolecular accumulation and water inhibition, they did interfere with implantation. Therefore, it could be speculated that the ineffectiveness of CC in the initiation of implantation is due to its possible failure to stimulate the synthesis of PGs and/or LTs in the target tissues. Again, it is to be recognized that compounds which interfere with implantation may not necessarily exert their effects via alterations in uterine macromolecular accumulation and/or water inhibition. This, however, does not indicate that these components of Phase I are not important for implantation. It is more likely that interactions among specific components of Phase I response are required for successful implantation.

The Phase II of estrogen response in the uterus requires further clarification. In the mature mouse, it is primarily the luminal and glandular epithelia which proliferate in response to estrogen. On the other hand, stromal cell proliferation requires P_4 and this response is potentiated by estrogen [12, 13] One interesting observation deserves mention. Preferential stimulation of [^{3}H]thymidine incorporation in P_4-primed myometrial cells by E_3 could be associated with the necessary growth of the myometrium during pregnancy to accommodate the developing fetus. This could be of particular importance in human pregnancy, where E_3 is the primary estrogenic product of the placenta. Therefore, Phase II responses are cell-type specific and subject to steroid hormonal modulation. Because uterine dry weight does not ascertain cell-type specific changes and because there is cellular partitioning of the Phase II response under different hormonal conditions, uterine dry weight is not an accurate determination of Phase II responsiveness. This statement is consistent with our present findings of cell-type specific differences in [^{3}H]thymidine incorporation by estrogens or TPE compounds in the presence or absence of P_4-priming. Although estrogens and TPE compounds in conjunction with P_4 failed to increase uterine dry weight above that of P_4 alone [10], they all stimulated nuclear incorporation of [^{3}H]thymidine in stromal cells, but to different degrees. Similar observations have previously been reported in which a TPE compound, nafoxidine, did not increase uterine dry weight in spite of increased cell proliferation [7]. Because uterine "true growth" represents both hypertrophy and hyperplasia, the failure of estrogens and TPE compounds to induce this "true growth" in a P_4-primed uterus (as measured by dry weight) could be due to the lack of cellular hypertrophy.

The next question to be addressed is whether Phase II action is important for the initiation of implantation. Because CC is a poor inducer of implantation [10] inspite of its ability, like other TPE compounds and estrogens, to stimulate stromal cell [^{3}H]thymidine incorporation in a P_4-primed uterus, we can suggest that cellular hyperplasia alone is not sufficient for the initiation of implantation. Furthermore, uterine dry weight was not increased (perhaps due to the lack of cellular hypertrophy) by any of the estrogens or TPE compounds in the P_4-primed animal [10], although they all increased stromal cell [^{3}H]thymidine incorporation to different degrees and that most compounds, except CC, induced implantation [10]. Therefore, we can further suggest that perhaps cellular hypertrophy is not required for the initiation of implantation i.e. increased endometrial vascular permeability at the site of the blastocyst. This, however, does not exclude the possible involvement of the late effects of estrogen, i.e. cellular hyperplasia and hypertrophy in the subsequent process of decidualization following initiation of implantation.

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