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# ACTIVATION OF 'IMMEDIATE-EARLY' GENES BY ESTROGEN IS NOT SUFFICIENT TO ACHIEVE STIMULATION OF DNA SYNTHESIS IN RAT UTERUS

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SUMMARY: 17ß-estradiol, a long acting estrogen that is mitogenic for rat uterus in vivo, or the short acting estrogens estriol and 16α-estradiol, not mitogenic on their own, were injected into adult, castrated rats and their effect on uterine gene expression and rate of DNA synthesis were compared. All three compounds increased steady-state mRNA concentration of c-*fos* , c-*jun* and c-*myc* proto-oncogenes to comparable levels (2 hrs after treatment), whereas only 17ß-estradiol was found to stimulate significantly DNA synthesis (20-22 hrs later). Based on the different retention time of the tested estrogens in rat tissues, it is concluded that a short exposure to the hormone is sufficient to render uterine cells competent to progress through the cell cycle, via activation of 'immediate-early' genes expression, but that stimulation of DNA synthesis requires further changes, achieved via a prolonged exposure of the cells to the estrogenic stimulus. 

In adult rodents, 17ß-estradiol (17ß-$E_2$) stimulates DNA synthesis and cell proliferation in the luminal epithelium of the uterus (1-3) but not in the liver (4). This is preceded, within the first 2 hours, by transient stimulation of 'immediate-early' genes expression, including c-*fos* (3), c-*myc* (3) and c-*jun* (5-6) proto-oncogenes, in the uterus but not in the liver (4). This has led to the conclusion that 'immediate-early' genes activation is linked to the proliferative response of uterine cells to the hormone (3, 6). Estriol ($E_3$) and 16α-estradiol (16α-$E_2$) are short acting estrogens (7-8), that bind with high affinity to the estrogen receptor and induce formation of transcriptionally active nuclear hormone-receptor complexes (9-10), but are weakly mitogenic in vivo, unless administered in multiple, sequential doses to the animals (9 and ref. therein). Stack and Gorsky (11) demonstrated that short acting estrogens induce, within the first 3-4 hours, stable changes in rat uterine cells that are essential, but not sufficient, to achieve increased DNA synthesis. The nature of these changes is however still unknown.

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The effect of a single injection of 17ß-$E_2$, $E_3$ or 16α-$E_2$ on c-*fos* , c-*myc* and c-*jun* uterine mRNA levels was measured in adult, castrated rats and this was correlated with changes in the rate of uterine DNA synthesis. Results show that all three estrogens increase expression of the tested genes within the first 2 hours, whereas only 17ß-$E_2$ stimulates significantly DNA synthesis rate 20 to 22 hours later. These results suggest, for the first time, that like in the case of 'competence' growth factors, 'immediate-early' genes activation by estrogen is not sufficient to drive uterine cells into the S-phase and that further regulatory events are required to achieve a full proliferative response to the hormone.

## MATERIALS AND METHODS

Animals. Adult female Sprague Dawley rats (225-250 g) from Charles River Italia, ovariectomized under light ether anesthesia 10 to 14 days earlier, were injected ip (in groups of 6 or 10 animals) with 1.5 µg/100 g body weight of the indicated steroid in 0.15 ml vehicle solution (10% ethanol:90% PBS), or with the vehicle alone. At the indicated time (2 hours after injection for RNA determination and 20-24 hours after injection for assessment of DNA synthesis rate), animals were killed by cervical dislocation, uteri were dissected and immediately frozen in liquid nitrogen, pulverized and freeze-dried for RNA extraction or, alternatively, chilled in ice-cold buffer for isolation of nuclei.

RNA purification and Northern blot analysis. Total uterine RNA was extracted with guanidinium thiocyanate and purified by ultracentrifugation through a dense cushion of cesium chloride. Polyadenylated (poly-$A^+$) RNAs were purified by affinity chromatography on oligo(dT)-cellulose, glyoxalated and analysed by agarose gel electrophoresis, followed by blotting onto nitrocellulose filters and hybridization with $^{32}$P-labelled DNA probes as described (3, 6). Plasmids used for preparation of DNA probes were the following: p-fos-1 (v-*fos* ; 12), pMC38 *Pst* IB (v-*myc* ; 13), pUN 121(human c-*jun* cDNA; 14) and pT2 (chicken ß tubulin cDNA; 15). Filters were washed four times with 1X SSC, O.1% SDS at 42°C for 30 min and then exposed to autoradiographic film for 14 to 72 hours. Densitometric scanning was performed with a Ultroscan-XL (LKB) densitometer and are expressed as O.D. units of proto-oncogene mRNA hybridization signal / O.D. units of ßtubulin mRNA hybridization signal obtained on the same filter.

Purification of uterine nuclei. Uteri, at 0-4°C, were minced and homogenized in 1ml / uterus (6 ml total) buffer A (500 mM hexylene glycol, 1 mM EGTA, 2.5 mM $MgCl_2$,10 mM HEPES: pH 7.5). The resulting homogenate was filtered through 4 layers of cheese-cloth and then through 1 layer of nylon cloth (100 µ mesh), underlaid with 3 ml buffer B (buffer A containing 0.8 M sucrose) and nuclei were pelleted by centrifugation at 5000 X g for 10 min (4°C). The pellet was resuspended with 3 ml buffer C (buffer A containing 1.9 M sucrose), underlaid with 3 ml of the same and nuclei were pelleted again by centrifugation at 10,000 X g for 30 min (4°C). The pellet obtained was resuspended with 1.5 ml buffer D (250 mM sucrose, 2.5 mM $MgCl_2$, 10 mM HEPES: pH 7.8) and used immediately for DNA synthesis in vitro, or frozen in aliquots for further use.

DNA synthesis in isolated nuclei. DNA synthesis in isolated nuclei was measured as described by Stack and Gorski (16), with minor modifications. In triplicate, 0.1 ml aliquots of nuclear suspension was throughly mixed with 0.1 ml of reaction buffer (17.5 mM $MgCl_2$, 190 mM HEPES: pH 7.8, 20 mM ATP, 20 µM each of dATP, dCTP and dGTP). 10µCi $^3$H-dTTP (60-65 Ci/mmol; New England Nuclear, Italy) was added, the reaction was allowed to proceed at 30°C for 10 min and stopped by addition of 1 ml ice-cold 10% (w/v) TCA+10 mM Na-pyrophosphate, incubation at

0°C for 20 min and centrifugation at 10,000 rpm for 3 min in an Eppendorf 5415 centrifuge. The pellets were washed three times with 1 ml 5% TCA+5 mM Na-pyrophosphate and hydrolyzed in 1 ml 0.5 N $HClO_4$ at 70°C for 20 min. 0.25 ml duplicate aliquots were taken for DNA assay by the diphenylamine procedure (17) and for determination of incorporated $^3$H-dTMP (16).

## RESULTS

Ovariectomized adult rats were injected ip with 1.5 µg/100 g bw 17ß-$E_2$, $E_3$ or 16α-$E_2$. Under these conditions, plasma levels of hormone peak within the first 30 min followed, within the first 2 hrs, by the appearance of active hormone-receptor complexes in target cells nuclei (9, 18 and data not shown) and, in the case of 17ß-$E_2$, by increased transcription of proto-oncogenes in the uterus (3, 6). Animals were sacrified either before (time 0) or 2 hrs after injection of estrogen, or of the estrogen vehicle alone, and uterine RNA was extracted, purified and polyadenylated mRNA species were analysed by 'Northern blot', using labelled DNA probes specific for each of the tested genes. The results of a representative experiment are reported in Fig. 1, as actual autoradiographs of nitrocellulose filters, and in Table I, as quantitative densitometric scanning of the autoradiographic signals, corrected on the basis of ß-tubulin mRNA concentration in the same blot. 17ß-$E_2$ induces a substantial increase of the steady-state concentration of c-*fos* , c-*myc* and c-*jun* mRNA (17 fold, 4 fold and 5 fold respectively, compare lanes 1 and 3 in Fig. 1 and see Table I), but not of ß-tubulin mRNA. The proto-oncogenes tested respond also to $E_3$ and 16α-$E_2$ (lanes 4 and 5) and the level of induction is similar to that

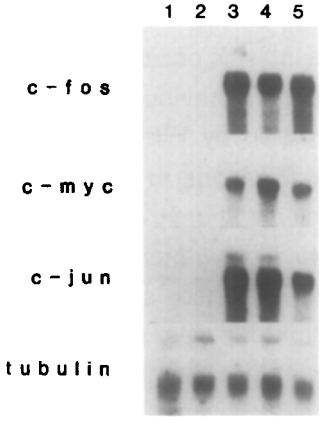


Fig. 1. Effect of 17ß-estradiol, Estriol and 16α-estradiol on steady-state concentration of c-*fos* , c-*myc* and c-*jun* mRNA in the uterus of ovariectomized rats. 10 µg uterine poly(A)$^+$ RNA was analysed by Northern blot using v-*fos* , v-*myc* , human c-*jun* and chicken ß-tubulin DNA probes, as described in Materials and Methods section. Autoradiographs of the blots are presented.
**1** Control (uninjected); **2** Vehicle (10% ethanol); **3** 17ß-estradiol; **4** Estriol; **5** 16α-estradiol.

Table I: Effect of 17ß-estradiol, Estriol and 16α-estradiol on DNA synthesis and c-onc mRNA levels in rat uterus

| Estrogen | DNA Synthesis* DPM [3H]TMP incorporated | c-fos** | c-myc** | c-jun** |
|---|---|---|---|---|
| None | 464 | 0.12 | 0.23 | 0.40 |
| (Vehicle) | 510 | 0.12 | 0.28 | 0.36 |
| 17ß-estradiol | 1170 | 1.95 | 0.89 | 2.21 |
| Estriol | 528 | 1.41 | 0.93 | 2.04 |
| 16α-estradiol | 474 | 1.76 | 0.75 | 1.57 |

* Detected by 'in vitro' [3H]thymidine incorporation in nuclei isolated from the uterus of animals sacrified 20-24 hrs after injection and expressed as DPM [3H]TMP incorporated/hr/µg DNA. Results are representative of two, separate experiments.

** O.D. units, corrected for ßtubulin mRNA hybridization signal.

achieved with 17ß-$E_2$. Interestingly, the degree of proto-oncogene induction does not correlate with the mitogenic potency of $E_3$ and 16α-$E_2$, that is considerably lower than that of 17ß-$E_2$ (7, 11). Injection of the vehicle alone does not induce significant changes in proto-oncogene mRNA levels (lane 2).

To determine the effect of estrogen on uterine DNA synthesis under the same experimental conditions used for proto-oncogene mRNA assessment, the incorporation of $^3$H-labelled thymidine by uterine nuclei (16), isolated before or 22 hours after injection of estrogen, was measured (Table I). This time-point was selected since maximal DNA synthesis occurs in the uterus 20 and 24 hrs after 17ß-$E_2$ injection (3, 16). In line with previously reported results, the rate of DNA synthesis is increased in the uterus by 17ß-$E_2$ (2 to 3 fold), whereas $E_3$ or 16α-$E_2$ do not induce detectable modifications of this parameter, suggesting that DNA synthesis is, in this case, dissociated from c-*fos* , c-*myc* and c-*jun* genes activation. It must be noted that the relatively low increase of DNA synthesis in response to 17ß-$E_2$ treatment is reproducible, and is probably due to the fact that these experiments were performed in adult rats, since it has been demonstrated that in these animals only the endometrial cells, that constitute a minority of the uterine cells, respond to the hormone with increased proliferation (2, and references therein). In line with these findings, using immunocytochemistry for detection in situ of the c-*fos* gene product in the uterus of adult rats, we have demonstrated that estrogen treatment is accompanied by appearance of the FOS protein only in the nuclei of endometrial cells (M. Papa, A.Weisz and F. Bresciani, manuscript in preparation).

## DISCUSSION

In vertebrates, estrogen deprivation determines an arrest of the proliferative activity of target cells and estrogen administration to immature or castrated animals increases cell proliferation by inducing the recruitment of quiescent cells in cycle, while at the same time shortening the G1 and S phases (19). At the molecular level, this is accompanied by rapid and transient stimulation of 'immediate-early' genes expression followed, 15 to 20 hrs later, by a surge of DNA synthetic activity.

The pre-replicative period of cell cycle have been divided broadly in two distinct and temporally ordered phases: 'competence' and 'progression' (20), with regulatory factors acting specifically on each of these two phases (21). Induction of 'immediate-early' genes expression by growth factors occurs during the 'competence' phase and is necessary but not sufficient to achieve increased DNA synthesis rate. The identity between the cellular response to estrogen and to polypeptide growth factors have lead to the conclusion that they both exhert their regulation acting on the same set of cellular genes, and gene products, that constitute the cellular program for growth control (3). The use of in vivo short acting estrogens revealed that a prolonged exposure of uterine cells to the hormone is required to achieve the full mitogenic response, and that cells undergo changes, during the early phases of the response to the hormone, that predispose them to further modifications required to enter the S phase (9, 11). The results of the present study offer a molecular explanation for these changes, since it can be now postulated that, in analogy with 'competence' growth factors, transient expression of 'immediate-early' genes by estrogen render the cell competent to progress through the cell cycle and that other genes, or gene products, must exist that are target for estrogen regulation during the late G1 and, possibly, also during S and G2 phases. The identification of these genes will help to define more precisely the ordered series of events that control cell proliferation, and how this is regulated by estrogen and other mitogens.

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