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STIMULATION OF EARLY PROTEIN SYNTHESIS IN THE UTERUS OF THE OVARIECTOMIZED RAT, BY CONTINUOUS INFUSION OF [^{3}H]-ESTRADIOL-17β *IN VIVO*—I: RELATIONSHIP WITH INFUSION TIME AND TISSUE CONCENTRATION

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SUMMARY

Intravenous infusions of [^{3}H]-estradiol-17β ($E_2{}^3H$) were performed in ovariectomized rats by a sequential short term-high rate and a long term-low rate infusion. With this procedure the uterine level of $E_2{}^3H$ was increased rapidly during the first 30 min of infusion, and remained constant thereafter. The time course of estrogen induced protein synthesis (I.P.) has been studied by the differential *in vitro* uptake of [^{3}H]- or [^{14}C]-leucine in uteri of stimulated and control animals respectively, according to the method of Katzenellebogen and Gorski[5]. Despite the maintenance of a constant tissue level of $E_2{}^3H$, it was observed that I.P. synthesis increased up to 90 min after the beginning of the infusion and decreased thereafter to a low value by the 4th h. This decrease in the I.P. response with time did not correspond to the loss of the tissue responsiveness, as shown by the possibility of eliciting a secondary I.P. response by infusing a larger amount of $E_2{}^3H$ while the primary I.P. response was declining. The experiments show that the I.P. response to the estrogen stimulation is not dependent on the maintenance of the stimulus in the tissue, and, conversely, that the maintenance of estradiol in the tissue at a constant level neither increases nor maintains the primary I.P. response, and does not exhaust the capacity of response of the tissue to a second and stronger stimulus. This aspect will be discussed further in the next paper [12].

INTRODUCTION

Estradiol-17β induces in the rat uterus the synthesis of specific proteins (induced proteins or I.P.), measurable in the cytosol as early as 40 min after the administration of the hormone *in vivo* [1–3]. The synthesis of these proteins has also been reported *in vitro* [4, 5]. I.P. synthesis has also been observed in cycling rats, being maximal in proestrus [6]. The synthesis of I.P. on the other hand appears to be an early and transient response to estrogens. Indeed, after estradiol injection, I.P. synthesis increases for about 2 h, and decreases thereafter as a consequence of decreased I.P. synthesizing capacity of the tissue [2, 4, 7]; the latter eventually enters a refractory state, during which no I.P. synthesis can be stimulated by further estradiol injection [6, 7]. The time sequence of the I.P. response has been studied so far with acute injections of estradiol, where the true effect of time on the elicited I.P. synthesis may be partially obscured by a dose effect, since the estradiol level in the tissue is likely to be modified throughout the period of I.P. synthesis [7].

In the present work, we have tried to obtain a steady level of the hormone in the tissue as rapidly as possible and to maintain it for the complete duration of the study. A continuous infusion technique eventually allowing the establishment of such a constant level of estradiol regulated by an adequate infusion rate, was adopted [8]. However, since, the time, i.e. 3 h [9], required to reach equilibrium with a single constant infusion was too long, and was unsuitable for the study of a genuine time effect, we realised a two step infusion method, where a short term-high rate preceded a long term-low rate infusion, in order to speed up the establishment of the equilibrium in the tissue, and realise a square wave of estradiol level in the uterus from the 30th min onwards.

MATERIALS AND METHODS

Materials. Wistar female rats, 60 days old, were ovariectomized bilaterally and used either between 10–12 or 21–28 days later, in separate experimental groups.

Estradiol-2,4,6,7[^{3}H] ($E_2{}^3H$) (85–105 Ci/mmol) was obtained from New England Nuclear, and checked for radio-chemical purity (at least 95%) on Sephadex LH_{20} column chromatography.

The infusing solution of $E_2{}^3H$ was prepared in 0.9% saline, containing 5% ethanol and 1 g % BSA (bovine serum albumin) in order to insure complete solubility of estradiol. Infusing vols were 4.5 ml/h during 8 min for the high rates infusion, and 0.45 ml/h for the low rates infusion.

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L-leucine -4,5[^{3}H] (30–50 Ci/mmole) and *L*-leucine-[^{14}C] (U) (more than 270 mCi/mmol) were obtained from New England Nuclear and used without further purification.

Electrophoresis was performed on Cellogel R.S. strips 5 × 24 cm, on Multiphor type 2117 (LKB) electrophoresis apparatus on a water cooled plate, in VT ter buffer (Chemetron).

Radioactivity counting was performed in Instagel (Packard) with a Tri-carb Packard Spectrometer. After electrophoresis, cellogel strips were cut into segments of 5 mm and first soaked with 0.5 ml soluene (Packard) before addition of 5 ml instagel. Quenching correction was done by external standard.

Methods. Intravenous infusion of $E_2{}^3H$ was performed through the jugular vein as previously described [8, 10]. At the end of the infusion time the animal was killed by decapitation. Blood was collected and the uterus was rapidly removed. An aliquot was taken for tissue radioactivity content as previously described [8]. Two thirds of the uteri from three rats, infused similarly, were pooled for leucine incorporation. The uteri were slit open, cut into smaller pieces and incubated at 37 for 1 h in the presence of 25 μCi *L*-leucine-4,5 [^{3}H] in 1 ml PBS buffer (Difco). Two uteri from control rats (infused with saline) were incubated similarly with 5 μCi *L*-leucine-[^{14}C]. The incubating flasks were saturated with O_2–CO_2 (95–5) and maintained at 37 in a waterbath under gentle shaking.

At the end of the incubation the uteri were blotted with filter paper, and rinsed thrice in 25 ml Tris–HCl buffer pH 7.5 at 0°. Stimulated and control uteri were then homogenized together with an all glass homogenizer in 0.6 ml Tris 10 mM-HCL–sucrose 0.25 M buffer, pH 7.5, and the low speed (800 *g*) supernatant was obtained as previously described [9]. The low speed supernatant was centrifuged again at 106,000 *g* for 15 min to obtain the high-speed supernatant (cytosol). One hundred ng of estradiol were added to the cytosol in order to compete with $E_2{}^3H$ for the specific binding sites, and reduce to negligible levels the contribution of $E_2{}^3H$ to the amount of tritium associated with the proteins (unpublished). The cytosol was then dialyzed overnight at 4 against 50 ml of Tris–HCL–sucrose buffer.

50 μl Of the dialysed cytosol were then applied to a cellogel R.S. strip and electrophoresis was run for 70 min at 430 volts and 1.2 to 1.5 mA, with VT ter transporting buffer. The cellogel strips were dried on Whatman filter paper No. I and cut into 5 mm pieces for tritium and ^{14}C counting. I.P. synthesis was calculated from the ^{3}H/^{14}C ratio, according to Mayol [11], as the % increase above background (Fig. 1):

$$\text{I.P.} = \sum \frac{^3\text{H}/^{14}\text{C in peak area} - \text{basal } ^3\text{H}/^{14}\text{C}}{\text{basal } ^3\text{H}/^{14}\text{C}} \times \text{number of values in peak}$$

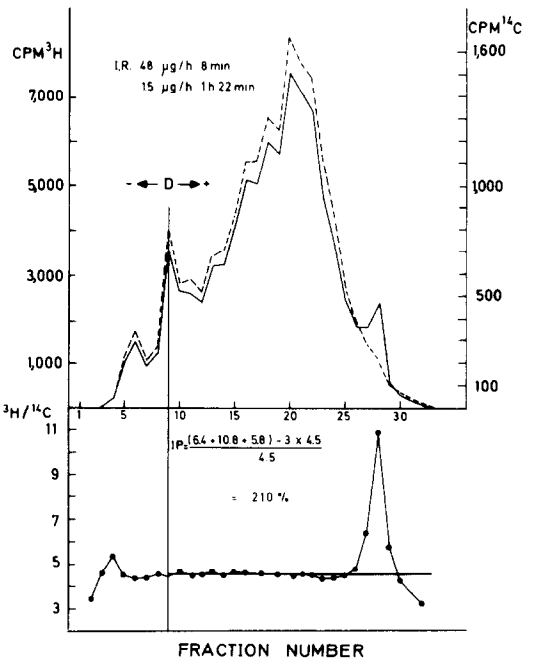

Fig. 1. Labeling pattern of soluble uterine proteins separated by cellogel electrophoresis after an *in vivo* short term-high rate (48 μg/h for 8 min) and long term-low rate (1.5 μg/h up to 90 min) $E_2{}^3H$ infusion to ovariectomized rats. D indicates the point where the sample was deposed. Treated uteri incorporated [^{3}H]-leucine. Control uteri incorporated [^{14}C]-leucine. Lower panel shows the ^{3}H/^{14}C ratio for the different slices of the cellogel strip after electrophoresis. The % increase in I.P. synthesis was calculated according to the formula derived from Mayol[11]. A numerical example is shown.

RESULTS

1. *Concentration of $E_2{}^3H$ in the uteri in function of the infusion time*

Aliquots of uteri from animals infused with $E_2{}^3H$ were analyzed for radioactive content as previously described [8]. Non metabolized $E_2{}^3H$ accounts for about 90% of the radioative material [8]. Hence, the latter was expressed in terms of weight of $E_2{}^3H$ per g of wet tissue.

Figure 2 shows the $E_2{}^3H$ concentrations for varying times of infusion ranging from 8–240 min, at different rates. In all these experiments, the short term-high rate infusion lasted for 8 min, followed by a low rate infusion of varying durations. It can be seen that for infusion rates below 10.8 μg/h + 500 ng/h, a constant tissue level of $E_2{}^3H$ was maintained from the 30th minute up to the fourth hour. For higher rates, the tissue levels decreased significantly during the long term infusion ($P < 0.005$ between 30 and 240 min). Part of this decrease can be accounted for by water imbibition, but it mainly represents a loss of loosely bound hormone taken up initially in excess of the tissue binding capacity. This aspect will be discussed in the second part of this work [12].

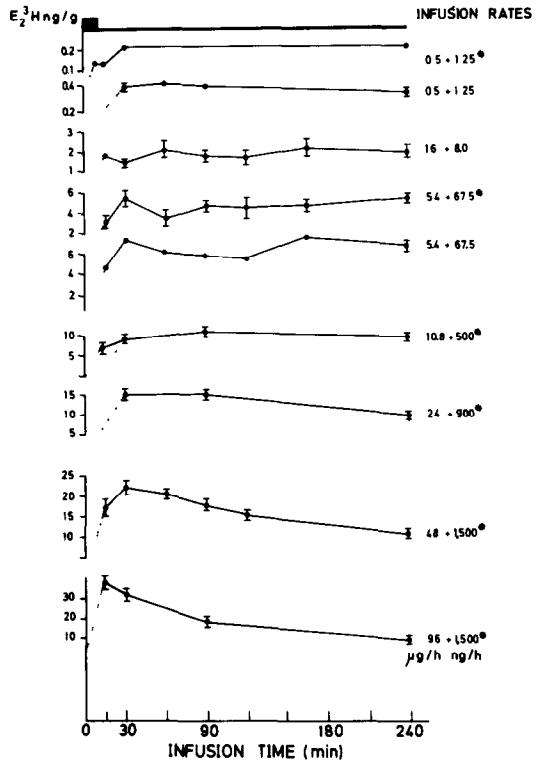

Fig. 2. Uterine $E_2{}^3H$ concentration (ng/g wet weight) at varying times, ranging from 8–240 min, in course of continuous infusion of $E_2{}^3H$ at different rates. Ovariectomized rats 10–12 days or 21–28 days (*) postovariectomy were subjected to an initial short term-high rate (0.5–96 μg/h for 8 min) followed by a long term-low rate (1.25–1,500 ng/h) *in vivo* infusion with $E_2{}^3H$. At the indicated times the animals were killed and the uteri removed for measurement of the radioactivity, which was expressed as ng/g of wet weight. Each point represents the mean ± 1 S.D. of at least 5 animals. Points without S.D. are the means of 3 animals.

2. *Time sequence of I.P. response in the presence of a constant level of $E_2{}^3H$ in the uterus*

I.P. response was measured at several times after the initiation of the $E_2{}^3H$ infusion. Figure 3 shows the progressive increase of I.P. response from 15 minutes up to 90 minutes and the subsequent decrease with time down to almost zero at 4 h whilst the tissue level of $E_2{}^3H$ remained constant at about 6 ng/g. The indicated time refers to the duration of the infusion period; it should be recalled that incubation of uteri in the presence of leucine, following the infusion period, lasted for 1 h.

Figure 4 shows the time sequence of I.P. synthesis for varying rates of infusion, ranging from 0.5 μg/h (short term-high rate) + 1.25 ng/h (long term-low rate) to 96 μg/h + 1,500 ng/h. At all these rates, I.P. synthesis was maximal at 90 min, and decreased thereafter in a rather parallel way. Both the maximal level of I.P. (Fig. 4) and the constant level of $E_2{}^3H$ prevailing in the uteri (see Fig. 2) were different for all these infusion rates.

From this set of experiments, it appeared that the maintenance of the $E_2{}^3H$ level in the uterus did not increase any further or even maintain I.P. synthesis. The experiment described on Fig. 5 was designed to test the importance of the degree of "impact"* of the hormone, as compared to its long term maintenance in the tissue, on the I.P. response. In this experiment the high rate infusion (96 μg/h) was reduced to 1.5 min and the low rate infusion (500 ng/h) was pursued during only 13.5 min in one group (B) or up to 180 min in the other group (A). When the infusion was stopped after 15 min (1.5 + 13.5), the tissue levels of $E_2{}^3H$ were significantly lower at 90 and 120 min as compared to the animals maintained under continuous infusion. However, the I.P. response noted at 90, 120 or 180 min respectively were not different between the two groups.

3. *Time sequence of I.P. response and I.P. synthesizing potentiality*

Figure 6 compares two experiments, in which similar tissue levels of $E_2{}^3H$ were achieved. In experiment A, the short term-high rate infusion was omitted and the tissue level increased slowly to its maximal level at 3 h. In experiment B, the maximal level was already

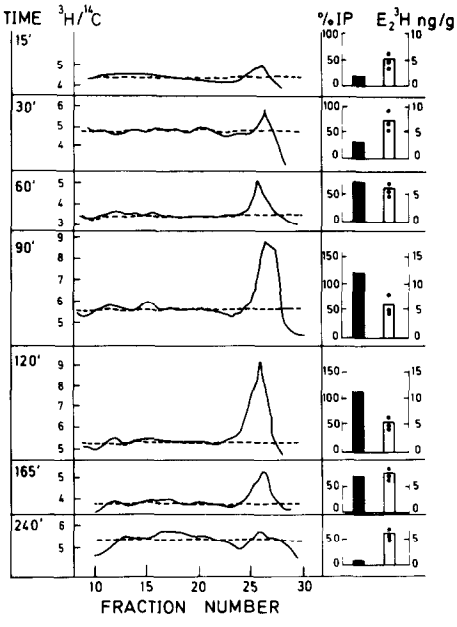

Fig. 3. Time sequence of I.P. response of ovariectomized rat uteri in course of $E_2{}^3H$ infusion at a rate of 5.4 μg/h (8 min) + 67.5 ng/h. Left panel: $^3H/^{14}C$ ratio on the cellogel strip slices after varying times of infusion. (See methods for details). Right panel: filled columns indicate % I.P. increase at varying times calculated from the isotope ratio (see Fig. 1). Empty columns indicate mean uterine $E_2{}^3H$ content in ng/g wet weight at corresponding times for the three individual animals in each experiment (black dots).

* The word "impact" in this and the following paper [12] is used in its original meaning of "collision": a forcible striking together that sets the struck body in motion (Webster).

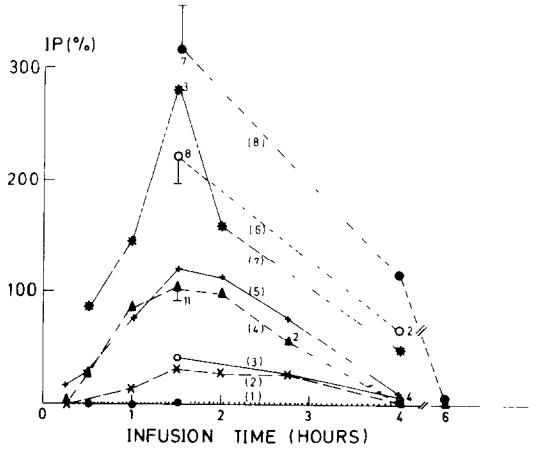

Fig. 4. Time course of I.P. response of ovariectomized rat uteri from 15 min to 4 h, at varying infusion rates. Short term (8 min)–high rates (μg/h) and long term–low rates (ng/h) are respectively: (1): 0.5 + 1.25; (2): 1.6 + 8.0; (3): 2.0 + 13; (4): 5.4 + 67.5; (5): 5.4 + 67.5; (6): 10.8 + 500; (7): 48 + 1,500; (8): 96 + 1,500. I.P. values are the means ± S.E.M. of the number of experiments indicated as unbracketed figures.

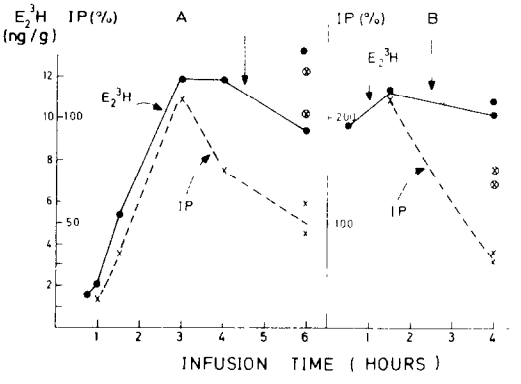

Fig. 6. Time sequence of I.P. response in two parallel experiments where similar tissue levels of $E_2{}^3H$ were attained, either after continuous infusion of 500 ng/h of $E_2{}^3H$ from time zero (A), or after short term-high rate infusion (10.8 μg/h for 8 min) followed by long term-low rate infusion at 500 ng/h (B). Vertical arrows show the time when an additional short term-high rate infusion of $E_2{}^3H$ (96 μg/h for 8 min) was given. . · complementary rise in I.P. synthesis ( ) or $E_2{}^3H$ level at 90 minutes after the second stimulation with estradiol. $E_2{}^3H$ levels are the means of 3 animals.

attained after 30 min, thanks to the high rate-short term infusion preceding the long term–low rate infusion. The maximal I.P. synthesis was obtained after 3 h in experiment A and, as expected, after 90 min in experiment B. In both instances, the I.P. synthesis decreased once the maximal level had been reached. It should, however, be stressed that the maximal level attained was twice as high in experiment B as compared to A, despite a similar maximal tissue level of $E_2{}^3H$. Although the cumulative response in A and B cannot possibly be compared, since the I.P. response in experiment A was more spread in time than in experiment B, it can be concluded that for a similar tissue level of $E_2{}^3H$, a higher maximal I.P. response was induced when the degree of impact was realized within a short time.

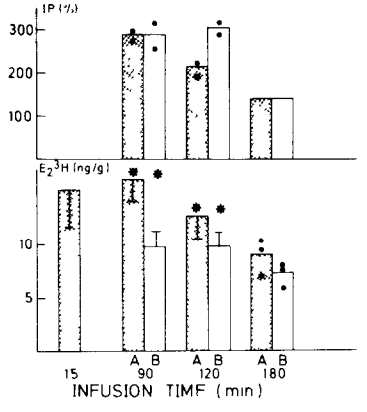

Fig. 5. I.P. responses at varying time intervals after continuous (A) or interrupted (B) $E_2{}^3H$ infusion of ovariectomized rats. Group B: high rate infusion: 96 μg/h for 1.5 min. Low rate infusion: 500 ng/h interrupted after 13.5 min. Group A: high rate infusion: 96 μg/h for 1.5 min. Low rate infusion: 500 ng/h up to the indicated times. Upper panel. % increase in I.P. synthesis. (Mean of number of experiments indicated by the dots). Lower panel: uterine $E_2{}^3H$ level (mean ± S.D. of six animals) in ng/g wet weight. (The 15 min column shows the tissue concentration which is common to both groups).
(*) $P < 0.005$ between A and B (Student's "t" test).

The decrease in I.P. synthesis in both groups on the other hand, did not correspond to the exhaustion of the capacity of response of the uterine tissue (I.P. synthesizing potentiality) [12]. Indeed, as shown also on Fig. 6, a second impact with a high dose of estradiol, when the I.P. response was decreasing, was able to elicit a secondary rise in I.P. response although not to the same extent as the first maximal response (a more quantitative discussion of this point appears in the next paper [12]).

DISCUSSION

Uterine growth is stimulated by estrogens, provided the hormone remains bound to the tissue for several hours [13–16]. However, initiation of early manifestations of estrogen action does not require a prolonged presence of the hormone, and it can be obtained not only with estradiol-17β, but also with less active estrogens such as estriol which is less firmly bound within the target tissue [13–17]. The stimulation of I.P. synthesis appears to be an early and transient phenomenon [7]. Nevertheless, its apparent close relationship with the hormone receptor complex binding to the nucleus [5, 18] makes it a valuable model to study some characteristics of the cell response to a specific stimulus.

I.P. synthesis starts with a lag period of about 40 min [2], although the I.P.-synthesizing capacity increases promptly after the impact of estrogen with the cell [5, 7]. Our results show that I.P. synthesis

was measurable already after 15 min of infusion. This early time however, should be corrected by taking into consideration the 1 h *in vitro* incubation phase in the presence of leucine, during which I.P. synthesis occurs.

The maximal I.P. synthesis takes place after $1\frac{1}{2}$ h of infusion, a data which also fits with the reported values, taking into account the same correction factor [4, 7]. The decrease in I.P. synthesis after 2 h corresponds to a decrease in I.P. synthesizing capacity, as discussed by De Angelo *et al.*[7]. In De Angelo's experiments, as well as in others, estradiol was administered as a single injection, which involved a non steady state of the hormone at the tissue level. In our work, we have maintained the tissue level constant throughout the time period. Nevertheless, I.P. synthesis decreased after $1\frac{1}{2}$ h of infusion, suggesting that the I.P. response is conditioned by the degree of the initial "impact" of the hormone with the cell. The maintenance of the hormone in the tissue at this same "impact" level, will neither result in further I.P. synthesis, nor in the maintenance of the initial synthesis, even in the presence of a residual I.P. synthesizing potentiality as shown on Fig. 6.

In long term infusion experiments, by sequential tritium labeled and unlabeled estradiol, we have observed that the hormone was in a constant equilibrium between the several subcellular fractions, as a result of a continuous flow of hormone through them [8, 19, 20]. It thus appears that these hormonal movements will be without effect on the I.P. synthesis, as long as they remain below the initial "impact" level.

After a first estradiol stimulation, a refractory period of up to 40 h has been described [6] during which time no further I.P. synthesis can be obtained. A similar or even longer refractory period follows the administration of anti-estrogens [21]. During that refractory period however, estradiol can still be taken up by the cell nuclei and bound in a form similar to the estradiol receptor complex [21]. In the present work, it is pointed out that this refractory period involves only that part of the I.P. synthesizing potentiality which has been used up in the first stimulation. The presence of residual I.P. synthesizing potentiality would not be affected by the hormonal exchange taking place below the hormonal level realized at the first "impact". When the hormonal level in the tissue, hence the degree of the "impact", was slowly increased as shown on Fig. 6, the I.P. response was delayed and spread over a longer period of time: the maximal I.P. response was lower although the tissue level reached eventually the same value as when the tissue content was increased rapidly. In both instances, however, a subsequent decrease in I.P. response occurred despite the existence of a residual I.P. synthesizing potentiality. This observation again stresses the importance of the degree of "impact", the nature of which will be discussed further in the next paper [12].

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