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EFFECTS OF ESTROGEN ON VERY LOW DENSITY LIPOPROTEINS (VLDL) SYNTHESIS IN AVIAN LIVER SLICES *IN VITRO*: LACK OF CORRELATION WITH NUCLEAR ESTROGEN RECEPTORS

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SUMMARY

Very low density lipoproteins (VLDL) synthesis in liver slices maintained in organ culture was stimulated by the administration of estradiol-17β (E_2) *in vitro*. However, the effects of the hormone were inconsistent and in only 25% of our experiments was significant stimulation observed. To study whether the failure of induction was due to the complete disappearance of estrogen receptor in the liver slices *in vitro*, we have measured the level of nuclear estrogen receptors. Irrespective of whether induction of VLDL synthesis was observed, estrogen receptor was consistently demonstrated in the liver slices maintained in culture, with an apparent K_D of 6×10^{-9} M. These findings suggest that the failure of consistent induction of VLDL synthesis in liver slices *in vitro* by estrogen is due to defects in hormone action subsequent to the nuclear binding of the hormone.

INTRODUCTION

Hypertriglyceridemia is a well known complication of oral contraceptive therapy. The elevated level of plasma triglycerides is associated with an increased amount of very low density lipoproteins (VLDL). Estrogen is thought to be the agent in the contraceptives which is responsible for the hyperlipidemia [1–3]. In experimental animals, the hormone induces the same changes in plasma VLDL [4–6]. In the chicken, estrogen stimulates VLDL synthesis by increasing the amount of mRNA for one of the VLDL proteins suggesting that gene transcription is a major site of action of estrogen [6].

In a number of experimental systems, estrogen action is thought to be initiated by the binding of the hormone to specific cytoplasmic receptors. The hormone–receptor complex is then translocated into the cell nucleus where, in an unidentified fashion, specific gene transcription is induced [7, 8].

In the present communication, we have developed a system in which liver slices were kept in organ culture for up to 2 weeks. Using this system, we have attempted to correlate specific nuclear estrogen receptors with the induction of VLDL synthesis *in vitro*.

EXPERIMENTAL

Animals. Three week old white Leghorn cockerels (0.2 kg) were used in the study.

Preparation of VLDL antisera. Antisera against VLDL were prepared by injection of purified VLDL into rabbits as previously described [6]. The γ-globulin fraction which was purified by ammonium sulfate precipitation (50%) was used in all experiments.

Organ culture systems. Liver slices (5–10 mg) were taken from the experimental animals and placed in sterile medium 199 (Grand Island Biological Co., Grand Island, NY, U.S.A.), containing 1 U/ml of penicillin and 1 mg/ml of streptomycin. About 15 mg of tissue were placed in each well of a Linbro FB-16-24-TC culture dish and covered with 1 ml of the culture medium. Cultures were incubated at 41°C in air on a rocking table; the medium was changed daily. Estradiol-17β was added to 1 ml cultures in 5 μl of propylene glycol; control cultures received the carrier only.

In vitro *labeling of VLDL.* The liver slices were rinsed with 2–3 ml medium 199 without amino acids. They were then incubated with 25 μCi of L-4,5-[^{3}H]-lysine monohydrochloride (S.A. 19 Ci/mmol, Amersham–Searle Corp., Arlington Heights, IL, U.S.A.) in 1 ml of medium 199 without amino acids. Incubation was carried out for 2 h at 41°C on the rocking table in air. With these incubation conditions, incorporation of L-[^{3}H]-lysine into immunoprecipitable and trichloroacetic acid precipitable material was linear for at least 2 h. After incubation, excess (0.5 mmol) unlabeled L-lysine was added and the tissues were weighed and homogenized in the incubation medium by six strokes in a glass–Teflon homogenizer. The homogenate was then centrifuged at 105,000 *g* for 60 min at 4°C. The supernatant fluid was used for determination of radioactive VLDL and TCA-precipitable radioactivity.

Quantitation of in vitro *synthesized VLDL.* Quantitation of the *in vitro* synthesized VLDL was performed on the 105,000 *g* supernatant fraction by immunoprecipitation with a specific anti-VLDL serum as previously described [6]. This method was found to give results similar to those obtained by ultracentrifugal flotation [6]. TCA-precipitable radioactivity was determined on aliquots from the same samples.

Determination of nuclear binding sites by the [^{3}H]-estradiol-exchange assay [9]. Liver explants (from 8 wells, total weight 100 mg) from three week old cockerels were incubated in culture medium for six days as described above. On the 7th day, non-labeled estradiol-17β was added to a final concentration of 7.2×10^{-7} M. After incubation overnight (18 h) in the estradiol-17β, the medium was removed and the explants washed with ice cold saline. The tissue was then homogenized in a Teflon–glass homogenizer in TE-buffer (0.01 M Tris–HCl–0.0015 M EDTA, pH 7.9) at 0°C (one explant per ml of buffer), and the homogenate centrifuged for 20 min at 800 *g*. The crude nuclear pellet was washed three times with TE-buffer by thorough vortexing followed by centrifugation at 800 *g* for 10 min. The washed nuclear pellet was gently homogenized in TE-buffer in a Dounce glass–glass homogenizer at a ratio of one explant/ml of buffer.

A slightly modified version of the [^{3}H]-estradiol exchange assay described by Anderson, Clark and Peck [9] was used to measure the nuclear content of estradiol receptors. Portions (250 μl) of the nuclear suspension were dispensed in two series of tubes A and B. Series A contained various concentrations of [^{3}H]-estradiol (0.4–12 nM) in 50 μl of TE-buffer, and series B an equal amount of [^{3}H]-estradiol plus a 100-fold excess of non-labeled diethylstilbestrol (DES). The tubes were vortexed thoroughly and incubated at 0–2°C for 24 h. After the incubation the nuclear pellets were washed three times with TE-buffer and then extracted with 1.0 ml of absolute ethanol at 30°C for 30 min. The ethanol extract was added to 5 ml of scintillation liquid (Permablend® in toluene) and the radioactivity determined. The amount of specifically bound hormone was determined by subtracting the non-specific binding from the total binding (Series A minus Series B).

Table 1. Rate of protein synthesis in liver slices maintained in culture*

Days after sacrifice	TCA-precipitable c.p.m. $\times 10^{-6}$/gm
0	25.0
1	7.2
3	9.4
6	5.0
8	3.2
14	3.2

* Rate of protein synthesis is determined by the rate of incorporation of [^{3}H]-L-lysine into TCA-precipitable material in 2 h *in vitro* as described in Experimental. Each value represents the mean of duplicate cultures.

RESULTS

Estrogenic induction of VLDL synthesis in vitro

Liver tissue maintained in culture media *in vitro* remains viable for at least 2 weeks as judged by the rate of incorporation of [^{3}H]-lysine into TCA precipitable protein (Table 1). There is an initial 70% drop in rate of this incorporation rate within 24 h after incubation. Thereafter, the rate remains stable at about 20–30% of the initial rate for at least 1 week and at about half that rate for another week.

Attempts to stimulate VLDL synthesis *in vitro* by addition of estradiol have met with partial success. In the experiment depicted in Table 2, the chicks were treated with 1 mg estradiol-17β 5 days before sacrifice. This pretreatment of the experimental animals was found to facilitate the response of the liver slices to the hormone *in vitro*. A 5-day waiting period was decided on since the VLDL biosynthetic response was over in 3 days [6]. The liver slices were maintained in culture for an additional 6 days before incubation overnight (18 h) with 7.2×10^{-7} M estradiol-17β. As shown in Table 2, there was a 3-fold stimulation of VLDL synthesis following the overnight exposure to the hormone. However, even in this instance, the stimulation was significant only to the 10% level. Furthermore, despite various hormonal manipulations (e.g. repeated pretreatments with estradiol-17β, longer incubations with the hormone, or even higher doses of the hormone) the results in Table 2 were repeatable with a success rate of only 25% (16 experiments were performed in all). In the other experiments, no difference was noted between control and E_2-treated cultures.

Table 2. Stimulation of VLDL synthesis in liver slices by E_2 *in vitro**

Culture	VLDL $\frac{\text{c.p.m.} \times 10^{-3}}{\text{gm tissue}}$	TCA precipitable $\frac{\text{c.p.m.} \times 10^{-6}}{\text{gm tissue}}$
Control ($n = 3$)	7.5 ± 1.6	1.37 ± 0.17
E_2-treated ($n = 3$)	23.0 ± 7.8	1.94 ± 0.26

* A two-week-old cockerel was treated with DES 2.5 mg subcutaneously 5 days before sacrifice. Liver slices were maintained in culture for 6 days before exposure to estradiol-17β (7.2×10^{-7} M) for 18 h as described in Experimental.

Results as expressed as $\bar{X} \pm$ S.E.M.

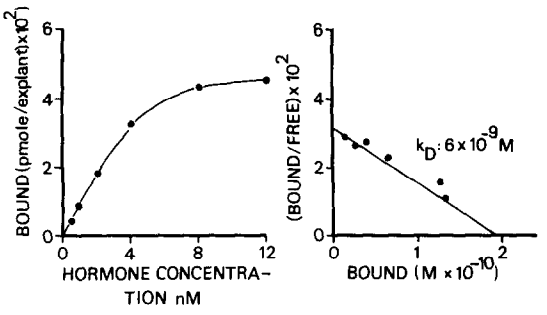

Fig. 1. (Left) Saturation curve of specific E_2-binding sites in nuclei isolated from the liver slices in organ culture. Experimental details are described in Experimental. (Right) Scatchard analysis of the binding data.

Binding of estradiol-17β to isolated nuclei in liver explants in vitro

Nuclear preparations from explants of male chicken liver were incubated with increasing concentrations of [^{3}H]-estradiol or [^{3}H]-estradiol plus a 100-fold excess of non-labeled competitor. Figure 1 shows the saturation curve obtained and a Scatchard analysis [10] of the binding data. A high affinity, low capacity binding site for estradiol was identified with an apparent K_D of approximately 6×10^{-9} M existing in a concentration of 0.07 pmol per explant (100 mg tissue). The results are consistently repeatable, irrespective of the VLDL response of the particular cultures examined. The prior administration (5 days) of estradiol to the cockerel before isolation of the liver did not result in any significant difference in the K_D or concentration of nuclear estradiol receptors.

DISCUSSION

According to our present understanding of steroid hormone action, the hormone appears to be bound to specific cytoplasmic receptors which are then translocated to the cell nucleus where the hormone–receptor complexes exert their effects on specific gene transcription. In our laboratory, we have found that estrogen regulates VLDL synthesis in the chicken liver at a transcriptional level [6]. Preliminary studies have also suggested the presence of nuclear estradiol receptors in the chick liver *in vitro* ([11–13] and Snow *et al.*, manuscript in preparation). Because of the technical difficulties in working with whole animals, we have attempted to study the induction process in liver slices maintained in organ culture. The synthesis of VLDL by rat hepatocytes in suspension has also been observed by other workers [14]. However, to our knowledge, the regulation of VLDL synthesis in organ culture has not been reported. While we observed stimulation of VLDL synthesis by estradiol *in vitro* in selected experiments, the response was not consistently demonstrable in other experiments carried out in an identical fashion. When all the experiments are taken together the degree of stimulation of VLDL synthesis by estradiol (E_2) is not significant. This observation is in contrast to the effect of E_2 in frog liver cells in culture where vitellogenin synthesis was induced by *in vitro* treatment with the hormone [15, 16].

One possibility for the failure of liver cells to respond to estrogen *in vitro* was the disappearance of the specific receptors to the hormone. We have therefore measured the specific nuclear binding sites for estradiol in the same cultures. We consistently found approximately the same amount of specific nuclear sites in all cultures irrespective of the presence or absence of a VLDL response to E_2. The concentration of nuclear receptors is also quite similar to that observed in liver cell nuclei isolated from newly sacrificed animals (Snow *et al.*, manuscript in preparation). The defect in those cultures which fail to respond to the hormone must then occur subsequent to the nuclear binding. This report also constitutes an initial demonstration of the existence of specific nuclear E_2 receptors in liver slices maintained in organ culture *in vitro*.

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