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ESTROGEN RECEPTOR IN RAT PANCREATIC ISLETS

MARTA TESONE, GREGORIO D. CHAZENBALK, GUSTAVO BALLEJOS and EDUARDO H. CHARREAU

Laboratorio de Esteroides, Instituto de Biología y Medicina Experimental, Obligado 2490, (1428) Buenos Aires and
Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Argentina

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SUMMARY

The cytosol fraction of pancreatic islets of the female rat was found to contain a specifically binding protein for [^{3}H]-estradiol. This protein was heat sensitive and the [^{3}H]-estradiol binding was eliminated by treatment with protease and sulphydryl-blocking agents. Scatchard analysis of the cytosol binding reaction, measured by charcoal-dextran assay, indicated a single class of estradiol-binding sites having high affinity ($K_d = 2.9 \times 10^{-8}$ M at 0°C). The number of binding sites was calculated to be 29.6 fmol/mg cytosol protein in whole pancreas and 170 fmol/mg cytosol protein in isolated pancreatic islets after collagenase treatment. Competition studies indicated high specificity for the binding reaction, since excess (100-fold) unlabelled estrogens, diethylstilbestrol and the antiestrogen nafoxidine, all significantly reduced the binding of [^{3}H]-estradiol. On the other hand, the nonestrogenic steroids dihydrotestosterone, corticosterone and progesterone had no significant effects on [^{3}H]-estradiol binding. The complex had a sedimentation coefficient of 4–5 S in sucrose density gradient centrifugation in low salt. In streptozotocin-diabetic and in 3-wk pregnant rats a significant decrease in the binding of estradiol to pancreas islet cytosol was found.

INTRODUCTION

For more than 40 yr evidence has indicated that sex hormones influence the development of experimental diabetes.

Though the results obtained by different investigators are somewhat controversial, most of them show a "protecting effect" produced by estrogens on the induction of diabetes by subtotal pancreatectomy [1, 2]. Pharmacological doses of estradiol significantly increased islet secretion above that of untreated control rats, and that was comparable to the augmented islet activity of term, 3-week pregnant rats [3]. This effect indicates that estradiol enhances islet insulin secretion, and also plasma insulin responses to glucose administration during pregnancy [3]. Howell and co-workers [4] could demonstrate in rats treated with estradiol and progesterone that there was an enhanced rate of insulin release by tissue-cultured Langerhans islets in response to stimulation by glucose.

Mammalian pregnancy constitutes a diabetogenic stress. With advancing gestation there is abundant evidence of progressive insulin resistance and endogenous hyperinsulinemia. Also, when pregnancy is accompanied by pancreatic islet hypertrophy, an increased beta/alpha cell ratio can be observed [3, 5, 6]. Earlier studies have suggested the potential roles of placental steroids and placental lactogen in the induction of these events [7].

Steroid hormones are believed to act through a soluble cytoplasmic receptor which, in complex with the steroid, is translocated into the nucleus causing increased transcription of DNA [8–11]. Intracellular receptor proteins for steroid hormones have been looked for and usually found almost exclusively in those tissues and organs which have been recognized to be affected by these steroids [12]. Sandberg *et al.*[13] have reported on the presence of a 3.7–4.2 S estriol–protein complex in the pancreatic tissue of dog, baboon, and man.

In this paper the presence of a specific estrogen-binding protein in the isolated Langerhans islets of the rat has been reported. The concentration of this protein in various physiological states has been determined (streptozotocin-induced diabetes, pregnancy, normal and castrated rats).

MATERIALS AND METHODS

Chemicals. [2,4,6,7-^{3}H]-Estradiol-17 β (SA 109 Ci/mmol) was obtained from New England Nuclear Co. (U.S.A.). The solvents (benzene and ethanol) were evaporated under nitrogen atmosphere at 50°C and the hormones dissolved in ethanol. The purity of the hormones was never less than 98% as tested by thin-layer chromatography. Unlabelled steroids were obtained from Sigma Chemical Co. (U.S.A.).

Animals. Female Wistar rats weighing 150–200 g were used. The animals were maintained on regular Purina diet and water *ad libitum*. Pregnant rats were studied between the 18th and the 20th day of pregnancy.

Diabetes was induced by a single intravenous, 65 mg/kg b.wt. streptozotocin injection (Upjohn Co.,

lot 60140) in 0.01 M citrate buffer, pH 4.5. The effectiveness of the treatment was evaluated by following glucosuria (Test tape, Ely Lilly & Co.) polydypsia and polyuria. Blood glucose was determined at the time of sacrifice by the glucose oxidase method (Glucostat, Boehringer Co.). These animals were used 4 weeks after injection. Blood glucose levels were in the 300–500 mg% range.

All animals were ovariectomized 48 hs before experimentation, except those used as the intact controls and in pregnancy studies. Sacrifice was performed by decapitation.

Isolation of islets. Langerhans islets were isolated by the collagenase technique [14]. The pancreas was removed, minced and carefully washed in Krebs bicarbonate solution (KRBS). The tissue was then placed in freshly prepared collagenase solution (8 mg/3 pancreas) and incubated in a PVC-stoppered bottle using a Dubnoff metabolic shaker at 37°C for 15 min. Collagenase action was interrupted by diluting the mixture with cold buffer solution at the moment in which the islets were sought lying free or almost so, among the fragments of exocrine pancreatic tissue. After decantation, the sediment containing the islets was resuspended in cold KRBS and centrifuged 10 min at 200 *g* for two times, being the supernatants discarded. The islet identification was further made under a dissecting microscope.

Cytosol preparation. Isolated islets were placed in cold 0.05 M Tris-HCl buffer pH 7.4, containing 1.5 mM EDTA and 0.5 mM dithiothreitol (TED buffer). Cytosol fractions were prepared by centrifugation of the homogenates at 105,000 *g* for 60 min at 0°C, and the cytosol protein content was determined by the method of Lowry *et al.*[15].

Incubation procedures. Aliquots of the cytosol fractions were incubated at 0°C for 16 h with varying concentrations of [^{3}H]-estradiol with or without unlabelled steroids. In addition, samples of the cytosol fractions were subjected to (a) heating for 30 min at 20 or 45°C prior to incubation, and (b) treatment with protease (50 μg/ml), DNAse (50 μg/ml), RNAse (50 μg/ml) or N-ethyl-maleamide (NEM) (1 mM) for 3 h at 4°C. The samples were finally analysed as to their binding protein content through the dextran-charcoal assay, as follows.

Charcoal assay. Bound [^{3}H]-estradiol was measured essentially by the method of Korenman and Dukes[16]. Aliquots (0.2 ml) containing 700 up to 1000 μg of protein were placed into two series of centrifuge tubes. To one of them [^{3}H]-estradiol was added at a final concentration of 2×10^{-9} M, while the other contained [^{3}H]-estradiol (2×10^{-9} M) plus unlabelled estradiol (2×10^{-7} M). For Scatchard analysis [17], [^{3}H]-estradiol was added at varying concentrations (0.1 up to 2 nM).

The tubes were incubated for 16 h at 4°C, time enough to achieve 90–95% of the maximal binding. Further, 0.2 ml of a charcoal-Dextran suspension (0.5% w/v activated charcoal plus 0.05% w/v Dextran T70 in TED buffer) was added, the tubes were vortexed and then allowed to stand cold for 15 min. The tubes were routinely centrifuged in order to separate charcoal from cytosol. Aliquots (0.2 ml) of the supernatants were transferred to counting vials and scintillation fluid for radiometric analysis added. Radioactivity was measured in a LS 100 C Beckman liquid scintillation spectrometer. Specific binding of [^{3}H]-estradiol was calculated by subtracting off the non-specific binding observed in the presence of excess nonradioactive estradiol from binding in the presence of [^{3}H]-estradiol alone.

Sucrose density gradient centrifugation. The tritiated 17β-estradiol-receptor complex was formed by incubating the cytosol fraction of pancreatic islets with 1 nM of [^{3}H]-estradiol at 0°C for 3 h. Parallel incubations contained 100 nM unlabelled estradiol in addition to [^{3}H]-estradiol to saturate high affinity binding sites and to allow the assessment of non-specific binding. Samples (300 μl) were layered on 4.6 ml lineal gradients of 5–20% sucrose in TED buffer and were centrifuged for 14 h at 45,000 rev./min in a Beckman L5-50 ultracentrifuge using a SW 56 rotor. Marker proteins were immunoglobulin G (IgG) and bovine serum albumin (BSA). After centrifugation the tubes were fractionated (4 drops per fraction) and collected in counting vials for radioactivity measurements.

RESULTS

Saturation curve of [^{3}H]-estradiol binding to pancreas and to isolated pancreatic islets cytosol

In Fig. 1 the amount of [^{3}H]-estradiol specifically bound by pancreas cytosol is plotted as a function of total [^{3}H]-estradiol concentration. Saturation of receptor is reached at a concentration of 1.6 nM added estradiol. When a Scatchard plot of these data is made (see Fig. 1, *insert*) a straight line is obtained, suggesting a single class of binding sites over the concentration range of steroid used.

The apparent dissociation constant (K_d) has been 2.0×10^{-9} M at 0°C, and the concentration of binding sites was 29.6 fmol of estradiol/mg cytosol protein.

Figure 2 shows the saturation curve of [^{3}H]-estradiol binding to isolated pancreatic islets cytosol. Through Scatchard analysis (Fig. 2, *insert*) a single class of binding sites was also verified, with an apparent K_d of 2.9×10^{-8} M at 0°C and a concentration of binding sites of 170 fmol of estradiol/mg of islet protein.

Specificity of the estradiol receptor

Data in Table 1 show that the estradiol receptor is specific for estrogens. Excess (100-fold) unlabelled estradiol, estrone, estriol, diethylstilbestrol, and the antiestrogen nafoxidine, all significantly reduced the binding of [^{3}H]-estradiol. On the other hand, the nonestrogenic steroids dihydrotestosterone, corticosterone, and progesterone, had no significant effects on [^{3}H]-estradiol binding.

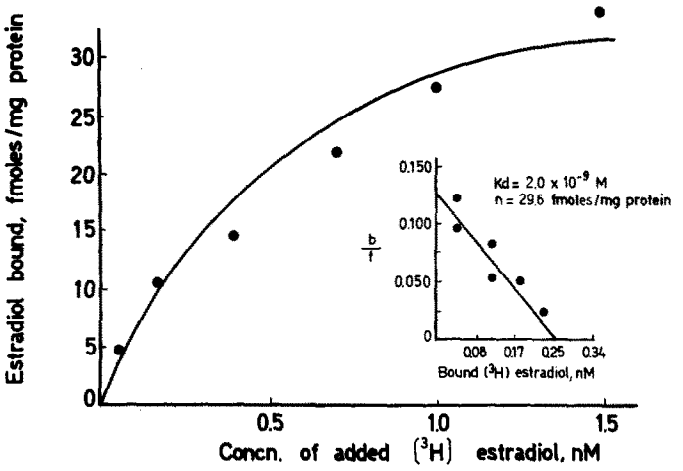

Fig. 1. Saturation curve of [^{3}H]-estradiol binding to whole pancreas cytosol. Aliquots of pancreas cytosol were incubated with various concentrations of [^{3}H]-estradiol for 16 h at 0°C. The amount of specifically bound [^{3}H]-estradiol was determined using a charcoal assay (see Materials and Methods). Each point represents the mean of three determinations. The *insert* presents the Scatchard plot of specifically bound [^{3}H]-estradiol.

Other characteristics of the estradiol receptor

The estradiol–receptor complex was destroyed when pancreatic islets cytosol was treated with protease or NEM (Table 2). No effects on binding were observed after treatment with RNAse or DNAse. After heating of the cytosol at 20°C for 30 min a slight enhancement of binding was detected, whereas the heating at 45°C for the same period caused complete elimination of binding.

Sedimentation pattern of [^{3}H]-estradiol-islet cytosol complexes

The sedimentation profile of the bound radioactivity to isolated pancreatic islets as subjected to sucrose density gradient centrifugation after incubation with [^{3}H]-estradiol is shown in Fig. 4. The complex sedimented in the 4.6 S area of the gradients. In the presence of a 100-fold excess of nonradioactive estradiol this peak is partially displaceable. In uterine cytosol the estrogen receptor has a sedimentation coefficient of 8 S in sucrose density gradients in low salt buffer [8]. This feature points the islet estradiol receptor to have different characteristics from those of the uterine estradiol receptor.

Serum contamination is ruled out since serum from adult rats showed no substantial specific binding to estradiol as evaluated by charcoal-dextran techniques [18].

Effect of streptozotocin diabetes on estradiol binding

The effect of damage of beta cells on estradiol binding to pancreatic islets cytosol preparations was examined. In this condition, the binding of estradiol was significantly reduced when compared to that of control rats (Fig. 3).

Effect of pregnancy on estradiol binding

The influence of changes in hormonal status on the binding of estradiol to pancreas islets cytosol in

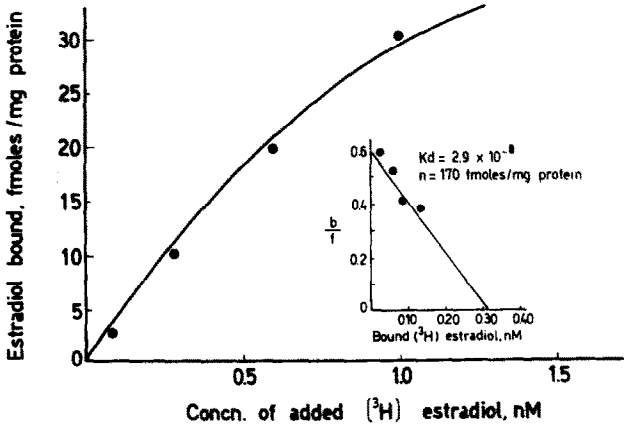

Fig. 2. Saturation curve of [^{3}H]-estradiol binding to isolated pancreatic islets cytosol. The procedure is the same as described in Fig. 1. The *insert* presents the Scatchard plot of specifically bound [^{3}H]-estradiol.

Table 1. Effect of non-labelled steroids on binding of [^{3}H]-estradiol in cytosol of rat isolated pancreatic islets

Steroids in incubation media	Steroid concn	[^{3}H]-estradiol bound (d.p.m./ml)	Binding competed for by other steroids (d.p.m./ml)	% Inhibition of binding by other steroids
[^{3}H]-Estradiol	2×10^{-9} M	139,450	—	—
[^{3}H]-Estradiol and estradiol	2×10^{-9} M 2×10^{-7} M	64,993	74,457	53.4
[^{3}H]-Estradiol and diethylstilbestrol	2×10^{-9} M 2×10^{-7} M	71,225	68,225	48.9
[^{3}H]-Estradiol and nafoxidine	2×10^{-9} M 2×10^{-7} M	86,420	53,030	38.1
[^{3}H]-Estradiol and estrone	2×10^{-9} M 2×10^{-7} M	88,691	50,759	36.4
[^{3}H]-Estradiol and estriol	2×10^{-9} M 2×10^{-7} M	89,806	49,644	35.6
[^{3}H]-Estradiol and dihydrotestosterone	2×10^{-9} M 2×10^{-7} M	129,928	9522	6.8
[^{3}H]-Estradiol and progesterone	2×10^{-9} M 2×10^{-7} M	126,590	12,860	9.0
[^{3}H]-Estradiol and corticosterone	2×10^{-9} M 2×10^{-7} M	133,653	5797	4.2

Pancreatic islet tissue from female rats, 60 days of age, ovariectomized 48 h prior to experiment (incubation protein concn, 1 mg/inc.). Values shown are means of triplicate determinations.

female rats was studied. In the normal rat the specific estradiol binding was less than in castrated rats, but these changes were not significant in our experimental conditions (Fig. 3). Pregnancy significantly decreased the binding of estradiol to pancreas islets cytosol (Fig. 3).

DISCUSSION

The present investigation shows that female rat pancreas has a high-affinity, low-capacity estrogen binding protein. In our conditions, bound [^{3}H]-estradiol was displaced by unlabelled estradiol, estrone, estriol, diethylestilbestrol, and nafoxidine. On the other hand, no significant effect on binding was obtained after addition of dihydrotestosterone, corticosterone or progesterone.

Table 2. Effect of a SH-blocking agent, enzymes, and temperature, on the binding of [^{3}H]-estradiol to pancreatic islets cytosol

Treatment	[^{3}H]-Estradiol bound (% of control)
Control	100
NEM (1 mM)	27
Protease (50 μg/ml)	16
RNAse (50 μg/ml)	96
DNAse (50 μg/ml)	91
20°C 30 min	110
45°C 30 min	0

Aliquots of 0.2 ml of Langerhans islets cytosol fractions, equilibrated with 2 nM of [^{3}H]-estradiol, were treated with NEM, protease, RNAse and DNAse (figures in parenthesis mean final concn's) or heat treatment, and incubated at 4°C for 3 h. The amount of radioactivity bound to islet binding protein was determined by charcoal-dextran and expressed as bound radioactivity as per cent of control. Each value is the mean of duplicate determinations.

The binding protein in pancreas and isolated Langerhans islets described herein exhibits steroid specificity similar to that previously described for the estradiol receptor in rat uterus [8, 19].

The isolated pancreatic islet cytosol has a concentration of estradiol-binding sites approx. five-fold higher than does the whole pancreas cytosol (170 and 29 fmol/mg cytosol protein, respectively), thus reflecting major concentration of receptors in the islets. Nevertheless, a slight contamination of the islet preparation with the surrounding acinous tissue cannot be discarded at all. On the other hand, in the islets of streptozotocin-diabetic rats, where a damage of beta cells is present, the content of binding sites

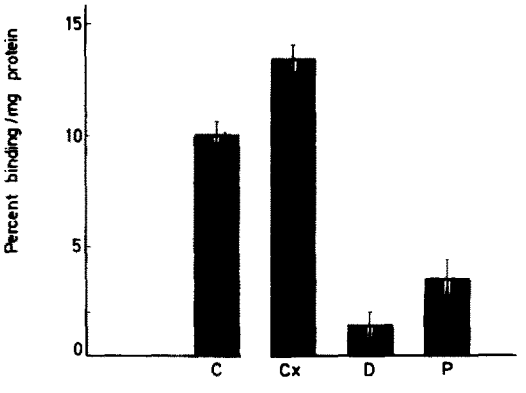

Fig. 3. Effect of castration (C_x), streptozotocin-induced diabetes (D) and pregnancy (P) on the specific binding of [^{3}H]-estradiol by isolated pancreatic islets cytosol as related to intact animals (C). The amount of specifically bound [^{3}H]-estradiol was determined using a charcoal assay (see Materials and Methods). (Means ± SE; $n = 6$ throughout.) The specific binding is significantly lower in D and P ($P < 0.01$ and 0.05 respectively), while C_x gave no significant effect.

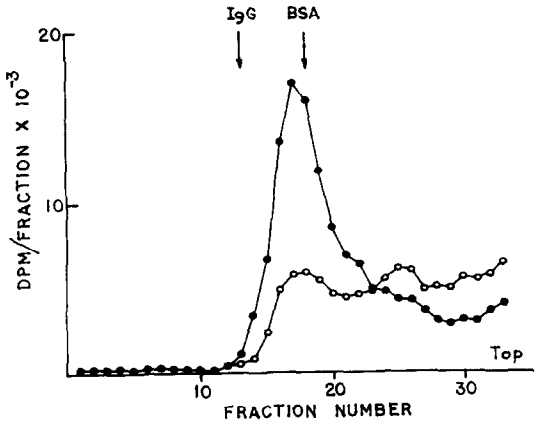

Fig. 4. Sedimentation profile in sucrose gradients of [^{3}H]-estradiol macromolecular complexes in the cytosol of rat pancreatic islets. For details, see Materials and Methods. ●, islet cytosol (3 mg of protein in 0.3 ml) plus 1 nM of [^{3}H]-estradiol; ○, islet cytosol incubated with 1 nM of [^{3}H]-estradiol plus 100 nM non-labelled estradiol. Marker proteins were immunoglobulin G (IgG) and bovine serum albumin (BSA). Each point is the mean of two determinations.

becomes approx. 88% diminished in relation to controls. This suggests that beta cells contain the majority of the binding sites for estrogens describe herein.

The low content of estradiol binding sites present in islets of pregnant rats (Fig. 3) could be an effect of changes in hormonal status. It seems likely that the high concentration of endogenous estrogens interferred with the *in vitro* [^{3}H]-estradiol binding, either by lowering the specific activity of the labelled hormone or by prior occupation of binding sites.

Preliminary experiments in our laboratory have shown a specific binding of [^{3}H]-estradiol to the nuclear fraction of pancreatic islets. Following the technique of Anderson *et al.*[20], the number of nuclear receptors was found to be approx, 11.3 fmol/mg DNA. Further studies are needed in order to optimize the evaluation of the kynetic properties of such binding protein.

The physiological role of the pancreatic estrogen receptor is difficult to evaluate at the present time.

The presence of receptor proteins for estrogens and other steroids in tissues which are not considered as their target organs raises the question of to what extent such proteins could influence the physiological function of these tissues.

Progesterone-induced rises of plasma insulin levels have been observed in both monkey and man after glucose administration [21, 22]. Studies in progress in our laboratory indicate the presence of a progesterone binding protein in the cytosol of isolated pancreatic islets.

Even though no direct evidences exist for the exact cell type in the pancreas for the localization of the estrogen receptor protein, our observations support the suggestion that it could be located preferentially at islet beta cells.

The present findings could support the assumption that some of the effects of estrogens on pancreas might be mediated through their specific interaction with islet cytosol protein.

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