Vol. 98, No. 1, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1981 Pages 197-202

PRELIMINARY CHARACTERIZATION OF THE ESTROGEN BINDING PROTEIN FROM BOVINE AND PORCINE HYPOTHALAMUS

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Received November 16, 1980

SUMMARY: Low salt buffer extracts of fresh bovine or porcine hypothalamus contain a high affinity, low capacity estrogen binding protein (EBP) which is similar to EBP from other estrogen target tissues. It has a 7.8S sedimentation coefficient, an affinity of 6.2×10^9 M^{-1} for ^3H estradiol and a capacity of 5×10^{-15} moles/mg protein. Like rat hypothalamic and calf uterine EBP, bovine and porcine EBP is a high molecular weight protein which chromatographs in the void volume on Sephadex G-200. Utilizing recent advances in EBP purification bovine or porcine hypothalamus could serve as a source for the purification of EBP from neural tissue.

The first direct biochemical evidence that the mechanism of action of estrogen in the hypothalamus is similar to the uterus was presented in 1965 when Eisenfeld and Axelrod (1) demonstrated ^3H estradiol uptake into the hypothalamus. The demonstration of the presence of a specific limited capacity, high affinity, estradiol binding protein (EBP) in rat hypothalamic cytosol (2) with properties similar to uterine EBP further supported a similar mechanism of action in the two tissues. Uterine EBP had previously been shown to be essential for estrogen action in the uterus (3). The presence of rat hypothalamic EBP has been confirmed by others (4,5) and has also been found in the hypothalamus of mouse (6), guinea pig (7), and hamster (8).

Hypothalamic EBP has not been as well characterized as the EBP from uterus, which has been purified to homogeneity (9). The task of purification of EBP from neural tissue is made more difficult because the concentration of EBP in hypothalamus is lower than in uterus and the amount of neural tissue containing EBP (predominantly hypothalamus and amygdala) is smaller than the uterus.

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0006-291X/81/010197-06$01.00/0

In this communication a preliminary characterization of EBP from bovine and porcine hypothalamus is presented. Bovine and porcine hypothalamic EBP is similar to EBP from rat hypothalamus and rat and calf uterus and could provide a potential source for the purification of EBP from a neural tissue.

MATERIALS AND METHODS:

Fresh and frozen porcine hypothalami and fresh bovine hypothalami were obtained from a local slaughterhouse in St. Louis. The tissue was homogenized in three volumes of cold 10 mM Tris, 1 mM EDTA, 10 mM dithiothreitol pH 7.5 (TED) spun 10,000 x g for 10 minutes. The supernatant was then spun 105,000 x g for 1 hour and the supernatant (cytosol) decanted. The cytosol protein concentration was determined by the method of Lowry (10) with bovine serum albumin as the standard.

The binding assay was performed in 10 x 75 mm glass tubes (Kimble), utilizing TED buffer, 1 ml of cytosol, and increasing concentration of ^{3}H estradiol (83 Ci/mmole, Amersham Corp.). The final volume of the assay was 2.2 ml. After 18 hours at 4°C, 0.5 ml of 7.5 mg/ml protamine sulfate (Sigma) was added and the tubes were vortexed and spun 2000 x g for 10 minutes. The pellet was rinsed three times with cold TED and the counts in the pellet were extracted with 1 ml absolute ethanol and counted in Scintiverse (Fisher).

The ^{3}H estradiol binding data were evaluated by the method of Scatchard (11) utilizing the following equation:

$$\frac{r}{(A)} = Kn - Kr$$

where r is the number of moles of estradiol bound per mg of cytosol protein, (A) is the concentration of free estradiol expressed as nanomolar, n is the maximum number of binding sites per mg of cytosol protein and K is the affinity constant of estradiol binding.

RESULTS:

In Figure 1, the results of Sephadex G-25 filtration of fresh porcine hypothalamic cytosol labeled with ^{3}H estradiol is shown. A peak of radioactivity elutes in the void volume of the column with the same elution as blue dextran 200. The second broader peak is unbound ^{3}H estradiol. This column can be used for the binding assay, though the number of samples that can be processed is limited. When either fresh porcine or fresh bovine hypothalamic cytosol is mixed with ^{3}H estradiol and filtered through Sephadex G-200 in TED a major peak of radioactivity also emerges in the void volume, indicating that EBP is a protein with a molecular weight greater than about 200,000. Calf uterine EBP (12) and rat hypothalamic EBP (13) both display the same behavior on Sephadex G-200 in the absence of chaotropic agents or

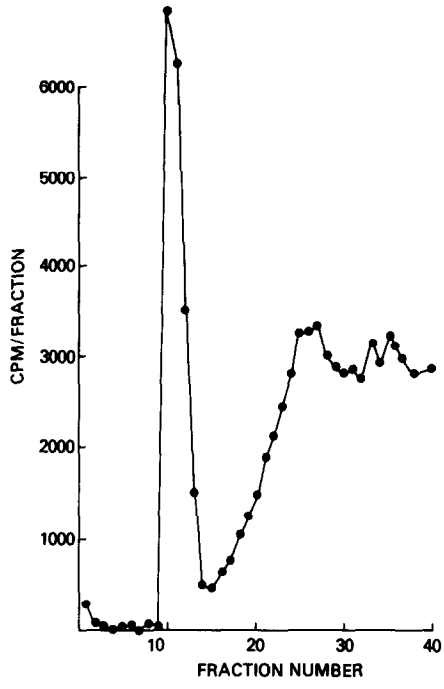

Figure 1: Sephadex G-25 column chromatography (.7 x 20 cm) of porcine hypothalamic cytosol in TED buffer with 1μCi ^{3}H estradiol. The peak of radioactivity elutes in the void volume in the same position as dextran blue 200. One milliliter fractions were collected.

heparin. In the presence of heparin calf uterine EBP displays a molecular weight of about 70,000 on G-200 (9).

The high molecular weight EBP seen on G-200 in fresh hypothalamus is not present when frozen or freeze-dried hypothalamus is used as the source of the cytosol (data not shown).

A "nuclear" form of EBP is also present in bovine and porcine hypothalamus. When the pellet from the first low speed centrifugation is washed three times with TED and then rehomogenized in 0.6 M KCl in TED, additional EBP is extracted which binds ^{3}H estradiol and elutes in the void volume of G-200 columns (data not shown).

In Figure 2, the results of sucrose density centrifugation of fresh bovine low salt hypothalamic cytosol labeled with ^{3}H estradiol is shown. There is a peak of radioactivity with an "S" value of 7.8 relative to

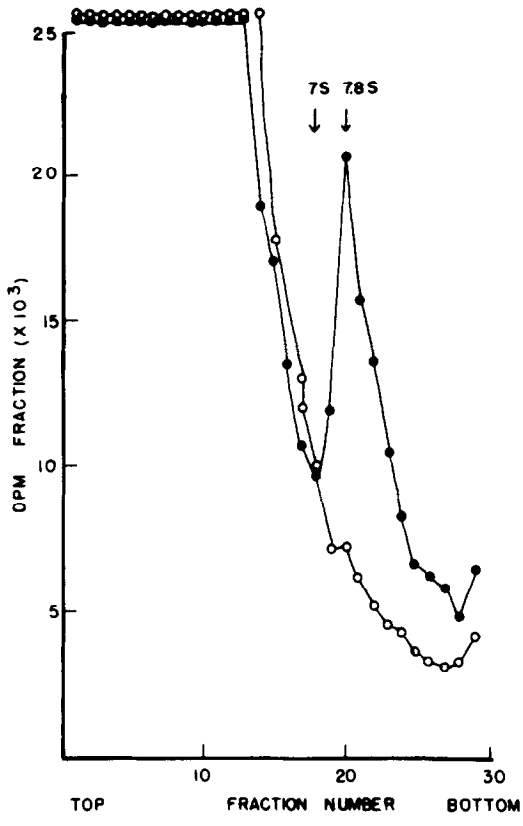

Figure 2: Sucrose density centrifugation of bovine hypothalamic cytosol on a 5 to 25% linear sucrose density gradient in TED, run 10 hours at 60,000 rpm in a Ti 60 SW rotor. Closed circles: cytosol containing 3.6 mg protein and 20 nM ^{3}H estradiol; open circles: same as closed circle with 2μM diethylstilbestrol. The fractions contained 100 microliters of the gradient and were collected from the top by piercing the bottom of the tube and pumping in a 40% sucrose solution from the bottom.

γ-globulin and bovine serum albumin. This peak is absent from a similar sample when 2 μM (100 fold excess) diethylstilbestrol (a potent synthetic estrogen) is present. This indicates that the peak is specific for estradiol and has a relatively high affinity for estradiol. This S value is similar to the 8 S reported for rat hypothalamic EBP (13), the 8.6 S reported for the calf uterine EBP (12), but lower than the 9.5 S reported for rat uterine EBP (14).

In Figure 3, the Scatchard plot of the binding of ^{3}H estradiol to fresh low salt bovine hypothalamic cytosol is shown. There appears to be two sets

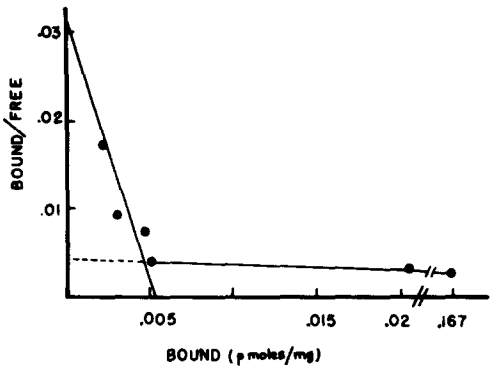

Figure 3: Scatchard plot of the binding of ^{3}H estradiol to fresh porcine hypothalamic cytosol.

of non-interacting binding sites, one set with high affinity (6.2×10^{9} M^{-1}) and low capacity (5×10^{-15} moles/mg protein) and another set with low affinity and high capacity. The affinity for estradiol of the bovine hypothalamic cytosol is similar to rat hypothalamus rat uterus 1.4×10^{9} M^{-1} (15) and calf uterus $1.5\text{-}3.5 \times 10^{9}$ M^{-1}. However, the estradiol binding capacity is lower in bovine hypothalamus than in rat hypothalamus 5×10^{-15} moles/mg protein and 2×10^{-14} moles/mg protein, respectively. The reason for this is not known, though the distribution of EBP in bovine, porcine, and rat hypothalamus may be different, and considerable tissue which does not contain EBP may be included in the bovine and porcine dissection.

DISCUSSION:

Bovine and porcine hypothalamic EBP is similar in sedimentation coefficient, chromatographic behavior and estradiol binding affinity to EBP for rat hypothalamus, rat uterus, and calf uterus. If fresh bovine or porcine hypothalamus could be obtained in large quantity it could be a good potential source for further characterization of EBP from neural tissue. Utilizing the recent advances in EBP purification technique (9) it may be possible to purify neural EBP and study its properties.

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