J. Steroid Biochem. Molec. Biol. Vol. 39, No. 3, pp. 353–357, 1991
Printed in Great Britain.

0960-0760/91 $3.00 + 0.00

AROMATASE ACTIVITY IN HUMAN ADIPOSE TISSUE STROMAL CELLS; THE EFFECT OF FETAL BOVINE SERUM FRACTIONS ON DEXAMETHASONE-STIMULATED AROMATIZATION

P. LUEPRASITSAKUL and C. LONGCOPE*
Departments of Obstetrics and Gynecology and Medicine, University of Massachusetts Medical School, Worcester, MA 01655, U.S.A.

(Received 20 September 1990; received for publication 29 May 1991)

Summary—Aromatization in human adipose stromal cells is stimulated by dexamethasone, but only in the presence of fetal bovine serum (FBS). To determine whether there was a specific fraction of FBS responsible for this stimulation, FBS was fractionated either by a pressure-driven ultrafiltration membrane or by Sephadex gel filtration techniques. The stimulating factor(s) appeared to be in the FBS fraction of 150,000–300,000 M_w by Sephadex filtration. Conversely, FBS fractions with $< 30,000$ M_w as separated by the former method inhibited the dexamethasone-stimulated aromatization of cultured adipose stromal cells. Bovine serum albumin, which constituted the major portion of FBS, had no discernible effect on the dexamethasone action on the aromatization of these cells.

INTRODUCTION

Subcutaneous adipose tissue has been found to be a major site of estrogen biosynthesis in men and postmenopausal women by converting circulating androstenedione to estrone [1–5]. This peripheral, or extraglandular aromatization, occurs primarily in the stromal cell compartment of the adipose tissue [6]. It also has been demonstrated that the aromatase enzyme complex is stimulated by glucocorticoids, especially dexamethasone, provided the adipose stromal cells in monolayer culture are maintained in a medium that contains fetal bovine serum (FBS) [7–9]. Dexamethasone does not stimulate the aromatase if the cells are maintained in medium alone. To determine whether there is a specific FBS fraction that might be responsible for enhancing the effect of dexamethasone on the aromatization of cultured adipose stromal cells, FBS was fractionated by ultrafiltration and by Sephadex gel chromatography, and the FBS fractions were used to supplement the media before assaying for the aromatization rate in cultures of adipose stromal cells.

*To whom correspondence should be addressed at: Department of Obstetrics and Gynecology, University of Massachusetts Medical School, Worcester, MA. 01655, U.S.A.

MATERIALS

[1β-^{3}H]androstenedione (25.4 Ci/mmol) and tritiated water ^3H_2O (2.79×10^6 dpm/ml) were obtained from New England Nuclear (Boston, MA). The [1β-^{3}H]androstenedione was >97% pure as determined by high pressure liquid chromatography and was used without further purification. Dexamethasone (9α-fluoro-16α-methylprednisolone) was purchased from Sigma Chemical Co. (St Louis, MO). FBS and bovine serum albumin (BSA) were obtained from Gibco Life Technologies Inc. (Grand Island, NY) and Miles Scientific (Naperville, IL), respectively. 44.5 mm Ultra-filtration membranes (PCAC, >1000 M_w; PTGC, >10,000 M_w; and PTTK, >30,000 M_w and PTHK, >100,000 M_w) were obtained from Millipore Corp. (Bedford, MA). Sephadex G-25 (20–80 μ; >5000 M_w), G-50 (20–80 μ; >30,000 M_w), G-100 (40–120 μ; >150,000 M_w) and G-150 (40–120 μ; >300,000 M_w) were purchased from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). Rainbow molecular weight marker (2350–46,000 M_w) and hemocyanin (cross-linked, horseshoe crab) molecular weight marker (70,000–280,000 M_w) were obtained from Amersham Corp. (Arlington Heights, IL) and Sigma, respectively.

FBS fractionations by pressure-driven ultrafiltration membrane filtration

Millipore ultrafiltration membranes with 1000, 10,000, 30,000 and 100,000 M_w cutoffs were rinsed in distilled water before use. The rinsed membranes were then mounted in a stirred ultrafiltration cell, model 8050, obtained from Amicon (Danvers, MA). 1.9 ml Of FBS, diluted to 50 ml with water, was added to the cell and filtered through the ultrafiltration membrane. The filtration rate was increased by nitrogen under pressure, and the amount of pressure depended on the specific M_w cutoff of the membrane: for membranes with cutoff up to 30,000 M_w, we used 50 psi and 2–3 psi for 100,000 M_w cutoff membrane. The initial 50 ml diluted FBS was then pressure-driven through the ultrafiltration membrane as described until the retentate above the membrane was approx. 2 ml. The retentate was diluted to 50 ml with H_2O and the filtration process was repeated until the protein content in the filtrate was zero as measured by Lowry's method [10]. The filtration had to be repeated three times for 1000 and 10,000 M_w cutoff membranes, and four times for 30,000 M_w membrane. The final 2 ml retentate was then transferred to a 15 ml test tube. The membrane was rinsed repeatedly with MEM medium in an attempt to retrieve all the proteins above the membrane and these MEM rinsings were added to the 2 ml retentate in the test tube up to 12.5 ml that made the solution equivalent to a 15% solution of the original FBS. This 15% solution was further filtered through a Costar bottle filter (Cambridge, MA) using a 0.20 μm filter membrane for cold sterilization, and the filtrate stored at $-20°C$ before use. When the 10,000, and 30,000 M_w cutoff membranes were used, the ultrafiltrate was collected, pooled and concentrated using a 1000 M_w membrane to overcome the dilutional effect of the separation method. The concentration ultrafiltrate was similarly processed as described for the retentate by bringing the final 2 ml to 12.5 ml. To obtain the maximal resolution of the 100,000 M_w membrane, low nitrogen pressure of 2–3 psi was selected and the concentration of diluted FBS was kept to $<0.5\%$ throughout the process, but the final retentate and all the ultrafiltrate obtained were processed in the same manner as described for relatively low cutoff membranes. All filtrations were conducted in the cold at 4°C.

FBS fractionations by Sephadex gel filtration

As another approach, group separations of FBS were undertaken by passing 1.9 ml FBS through Sephadex G-type columns. The void volumes for the Sephadex G-25, -50, -100 and -150 in the C 16/40 column used in our laboratory were determined with blue dextran 2000 (2 mg/ml; Pharmacia) and found to be 30, 27, 19 and 19 ml, respectively. The ultimate void and bed volumes obtained were processed as described above using a 1000 M_w membrane to obtain final volumes of 2.0 ml.

FBS fractionations by combined techniques

To obtain the 150,000–300,000 M_w fraction, 1.9 ml FBS was first eluted through a Sephadex G-150 column with a 300,000 M_w exclusion limit. The 70 ml bed volume that held protein constituents smaller than 300,000 M_w was collected and concentrated to 2 ml by passing it through a 30,000 M_w cutoff membrane under 50 psi pressure. This retentate and the membrane rinses (8 ml) were transferred to a 15 ml tube. Aliquots (2 ml) of this fraction containing proteins with $<300,000$ M_w were passed through a Sephadex G-100 column with 150,000 M_W cutoff. The 20.5 ml void volumes from each elution containing the 150,000–300,000 M_w fraction were collected and pooled. This final fraction (about 100 ml) was then reduced to 10 ml by using a stirred ultrafiltration cell with 100,000 M_w cutoff membrane and 2–3 psi pressure. To improve the resolution of this 150,000–300,000 M_w fraction, the 10 ml retentate was diluted to 50 ml using water and the filtration process was repeated ten times before reducing the solution to 2 ml. The 2 ml retentate was pooled with the membrane rinses, 10.5 ml. This 15% solution was stored frozen until used for both the membranes and Sephadex columns. The molecular weight cutoffs were assigned theoretically using the specifications of the manufacturer.

Source, preparation and culture of human adipose stromal cells

We have described all these procedures in detail elsewhere [11]. In brief, abdominal subcutaneous adipose tissue was obtained from women (53–63 years old) at the time of elective surgery for benign conditions. The specimens were minced well and incubated with collagenase type II, 1 mg/ml received from Gibco for 1 h at 37°C. The digested specimen was filtered

through the nylon mesh and centrifuged at 400 *g* for 5 min. The stromal cells were isolated, washed [6] and suspended in minimum essential medium Eagle (modified) (MEM, Flow Labs Inc., McLean, VA) containing penicillin-G (100 U/ml), streptomycin sulfate (100 μg/ml), and 15% FBS. The cells were grown to confluency and were detached from the stock flask with 5 ml of the trypsin-EDTA (0.25%) (Gibco), washed with MEM containing 15% FBS, and plated in 150–225 cm^2 flasks (first passage). Experiments were performed using confluent cells that had undergone at least two passages before plating into 35 mm culture wells.

Incubation of stromal cells with dexamethasone, FBS fractions and radiolabeled steroid

When the stromal cells in 35 mm culture wells became confluent, the media (MEM plus 15% FBS) in the wells were removed and the cells were rinsed twice with MEM to eliminate all traces of 15% FBS before the adipose stromal cells were exposed to dexamethasone (2.5×10^{-7} M) and the various FBS fractions as a 15% solution. The concentration of dexamethasone, 2.5×10^{-7} M, gives maximal stimulation in our hands as well as others [7]. After a 24 h incubation, [1β-^{3}H]androstenedione (150 nM) was added to the media and the incubation continued for another 4 h before assaying for the aromatization rate. In each experiment parallel incubations in the absence of cells were used for blank values.

Aromatase activity assessed by the release of tritiated water from [1β-^{3}H]androstenedione

The aromatization of the [1β-^{3}H]androstenedione was measured using the stereospecific release of tritium to form tritiated water as described [6, 11, 12]. After incubation with [1β-^{3}H]androstenedione (150 nM), the aromatization reaction was terminated by placing the culture cells on ice for 15 min. The medium was then centrifuged at 5000 *g* for 30 min using Centricon-30 microconcentrators (Amicon, W. R. Grace & Co., Danvers, MA). The ultrafiltrate was transferred to tubes containing 5.0 ml chloroform, and the culture wells were rinsed with 10 ml cold normal saline and the rinsings were added to the medium. The stromal cells were then scraped from the well into 1.0 ml saline, homogenized and protein concentration measured [10].

The combined medium and rinses were extracted twice with 5.0 ml chloroform. A 2.0 ml aliquot of the aqueous supernatant was removed and added to an equal volume of Norit-A charcoal (5%) and dextran (0.5%). The mixture was vortexed and incubated at 4°C for 2 h. The tubes were then centrifuged at 1700 *g* for 15 min, and the ^3H_2O in the supernatant measured using a liquid scintillation spectrometer (Packard Instrument Co., Downers Grove, IL).

The aromatase activity was expressed as pmol [1β-^{3}H]androstenedione metabolized per mg cell protein per 4 h after appropriate correction for blanks, dilution and entrapment of ^3H_2O during incubation and processing.

The base-line aromatization, and hence the actual dexamethasone-stimulated activity, varied among the cell cultures. Therefore, the effects of the FBS fractions have been expressed relative to that seen with dexamethasone in the absence of FBS.

The normalized data were analyzed using a one-way analysis of variance and Student Newman–Keuls test for multiple comparisons between means [13].

RESULTS

Serum albumin constitutes the major portion of FBS, and when dexamethasone was incubated with MEM containing 15% human or bovine serum albumin, no increase in aromatase activity over controls was noted (data not shown).

As shown in Table 1, the addition of dexamethasone 2.5×10^{-7} M to the incubation resulted in a marked increase, 40-fold, in the aromatization compared to incubation in FBS alone.

Our initial experiments were done examining low molecular weight fractions of FBS, Table 2, but, as noted, the fraction that resulted in stimulation of aromatase activity in the presence of dexamethasone, 2.5×10^{-7} M, had a M_w >30,000. The ultrafiltrates from the separation of the >10,000 M_w and >30,000 M_w proteins

Table 1. Effect of dexamethasone on the aromatase activity of adipose tissue stromal cells in the presence of 15% FBS

Media	Aromatase activity (pmol/4 h/mg cell protein)
15% FBS	0.042 ± 0.005[a]
15% FBS + dexamethasone (2.5×10^{-7} M)	1.69 ± 0.04[b]

[a]Mean ± SD; [b]difference significant compared to 15% FBS, $P < 0.01$.

resulted in only 20% of the stimulation in the presence of dexamethasone compared to incubations in 15% FBS and dexamethasone. As more low molecular weight substances were excluded there was an increase in aromatization compared to 15% FBS.

We then looked at much greater molecular weight fractions, and, as shown in Table 3, the 5000–150,000 M_w fraction did not permit stimulation with dexamethasone, but the 150,000–300,000 M_w fraction did result in dexamethasone stimulation. The fraction containing molecular weight substances >300,000 did not result in stimulation.

DISCUSSION

We found, as expected, [7, 8, 11], that dexamethasone, in the presence of 15% FBS, resulted in a significant stimulation in the aromatase activity of cultured adipose tissue stromal cells. The substance(s) responsible for this stimulation has a molecular weight between 150,000–300,000 that is larger than most of the growth factors and substances previously tested in this system [14]. When dexamethasone was incubated with growth factors in place of FBS, aromatase activity was not stimulated [5]. FBS, but not growth factors, caused an inhibition of the $(Bu)_2$ cAMP stimulation of adipose tissue stromal cells [8]. Therefore, it would appear that the growth factors so far tested are not the mediators of the dexamethasone stimulation of aromatase activity. However, no studies have been carried out using proteins that fall into the 150,000–300,000 M_w range.

It should be noted that complete purification of this fraction was difficult and serum albumin was present when examined by gel electrophoresis. It is unlikely, however, that the albumin influenced the results since incubation with 15% albumin and dexamethasone did not result in aromatase activity above that formed in control studies.

We also noted a gradual increase in dexamethasone stimulated aromatase as the lower molecular weight fractions were removed. It is possible that this reflects the removal of substances that interfere with the stimulation by dexamethasone. This would include certain growth factors that have been noted to alter aromatization in various systems [14–16]. The dexamethasone stimulation in 15% FBS was inhibited when the FBS was replaced by the 5000–150,000 M_w fraction. However, when FBS was replaced with fractions >1000, 10,000 or 30,000 M_w, some stimulation was noted. The proteins in the 150,000–300,000 M_w range would have been present in the latter incubations and may have negated the inhibition noted from the 5000–150,000 M_w proteins. It should also be noted that our evidence for inhibition was indirect and no reconstitution experiments were done.

We did not attempt to purify proteins of 150,000–300,000 M_w so we cannot say what causes the stimulation. However, it is possible that certain high molecular weight carrier proteins that could modulate the entry of dexamethasone into the cell are necessary for the observed stimulation.

Table 2. Effect of FBS fractions on dexamethasone (2.5×10^{-7} M) stimulation of atomatase activity by cultured adipose tissue stromal cells

Fraction	Stimulation
15% FBS	1.0[a]
>1000 M_w	2.60 ± 0.01[a,b]
>10,000 M_w	2.63 ± 0.01[a]
>30,000 M_w	3.27 ± 0.01[a]

[a]Means are significantly different, $P < 0.01$; [b]mean ± SD. Results expressed in comparison with FBS. Mean of four experiments.

Table 3. The effect of fractions of FBS on stimulation of aromatase by dexamethasone relative to stimulation by 15% FBS

Fraction	Stimulation
15% FBS	1.0[a]
5000–150,000 M_w	0.17 ± 0.01[a,b]
150,000–300,000 M_w	3.45 ± 0.07[a]
>300,000 M_w	0.38 ± 0.01[a]

[a]Difference between means is significant, $P < 0.01$; [b]mean ± SD. Results expressed in comparison with 15% FBS. Mean of four experiments.

Acknowledgements—The authors thank Ms D. Latour for technical assistance and Dr W. Okulicz for his excellent advice and assistance in carrying out many of the experiments. Also, we would like to thank Ms Loretta Lee and the Cell Culture facility for assistance in growing the stromal cells.

REFERENCES

1. MacDonald P. C., Rombaut R. P. and Siiteri P. K.: Plasma precursors of estrogen. I. Extent of conversion of plasma 4-androstenedione to estrone in normal males and nonpregnant normal, castrate and adrenalectomized females. *J. Clin. Endocr. Metab.* **27** (1967) 1103–1111.
2. Siiteri P. K. and MacDonald P. C.: Role of extraglandular estrogen in human endocrinology. In *Handbook of Physiology: Endocrinology* (Edited by R. O. Greep and E. Astwood). Am. Physiol. Soc., Washington, DC, Vol. 2 (1973) pp. 615–629.
3. Grodin J. M., Siiteri P. K. and MacDonald P. C.: Source of estrogen production in postmenopausal women. *J. Clin. Endocr. Metab.* **36** (1973) 207–214.
4. Longcope C.: The significance of steroid production by peripheral tissues. In *Endocrinology of the Ovary* (Edited by R. Scholler) Editions SEPE, Paris (1978) pp. 23–35.

5. Simpson E. R., Merrill J. C., Hollub A. J., Graham-Lorence S. and Mendelson C. R.: Regulation of estrogen biosynthesis by human adipose cells. *Endocrine Rev.* **10** (1989) 136–148.
6. Ackerman G. E., Smith M. E., Mendelson C. R., MacDonald P. C. and Simpson E. R.: Aromatization of androstenedione by human adipose tissue stromal cells in monolayer culture. *J. Clin. Endocr. Metab.* **53** (1981) 412–417.
7. Simpson E. R., Ackerman G. E., Smith M. E. and Mendelson C. R.: Estrogen formation in stromal cells of adipose tissue of women: induction by glucocorticoids. *Proc. Natn. Acad. Sci. U.S.A.* **78** (1981) 5690–5694.
8. Mendelson C. R., Cleland W. H., Smith M. E. and Simpson E. R.: Regulation of aromatase activity of stromal cells derived from human adipose tissue. *Endocrinology* **111** (1982) 1077–1085.
9. Folkerd E. J. and James V. H. T.: The action of dexamethasone and prolactin on aromatase activity in human adipose tissue. *J. Steroid Biochem.* **20** (1984) 679–681.
10. Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J.: Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193** (1951) 265–275.
11. Lueprasitsakul P. and Longcope C.: Aromatase activity of human adipose tissue stromal cells: effects of thyroid hormones and progestogens. *Proc. Soc. Biol. Med.* **194** (1990) 337–341.
12. Orczyk G. P., Mordes J. P. and Longcope C.: Aromatase activity in a rat Leydig cell tumor. *Endocrinology* **120** (1987) 1482–1489.
13. Steel R. G. D. and Torrie J. H.: *Principles and Procedures of Statistics.* McGraw-Hill, New York (1960) pp. 110–111.
14. Lueprasitsakul P., Latour D. and Longcope C.: Aromatase activity in human adipose tissue stromal cells: effect of growth factors. *Steroids* **55** (1990) 540–544.
15. Dorrington J. H., Bendell J. J. and Lobb D. K.: Aromatase activity in granulosa cells: regulation by growth factors. *Steroids* **50** (1987) 411–421.
16. Hsueh A. J. W., Welsh T. H. and Jones P. B. C.: Inhibition of ovarian and testicular steroidogenesis by epidermal growth factor. *Endocrinology* **108** (1981) 2002–2004.