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EFFECT OF SERUM AND 12-*O*-TETRADECANOYL-PHORBOL-13-ACETATE ON FSH-STIMULATED CONVERSION OF 4-ANDROSTENE-3,17-DIONE TO OESTROGENS IN CELL AND ORGAN CULTURES OF SUCKLING MOUSE OVARIES

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Summary—The effect of serum and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) on the FSH-stimulated oestrogen production was studied in both cell and organ cultures. Ovaries were removed from (WB × C57BL/6)F_1 mice at 9-days of age, and the conversion of 4-androstene-3,17-dione to oestrogens was stimulated by the addition of FSH *in vitro*. Either 10% serum (fetal calf, mouse, rat and horse) or 0.1 μM TPA markedly inhibited the FSH-stimulated oestrogen production by dispersed and cultured ovarian cells. In contrast, neither serum nor TPA influenced the oestrogen production in the organ culture. This suggests that the presence of tissue architecture may prevent the inhibitory effect of serum and TPA on the FSH-stimulated oestrogen production.

INTRODUCTION

The administration of FSH increases the aromatase activity, progesterone production and number of LH receptors in ovaries of immature rats [1–5]. These *in vivo* actions of FSH are reproducible *in vitro* when dispersed rat granulosa cells are cultured in serum-free medium [6, 7]. In contrast, the actions of FSH are not observed if serum is added to the cell culture [6, 8, 9]. Welsh *et al.* [10] have recently reported a similar suppressive effect of 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), a potent tumor promotor in two step carcinogenesis, on the FSH-stimulated conversion of 4-androstene-3,17-dione to oestrogens in cultured rat granulosa cells. Although TPA is not a natural constituent of the body, serum is the ubiquitous constituent. Therefore, the physiological meaning of the above-mentioned effect of serum is difficult to interpret. There is a possibility that the presence of tissue architecture may inhibit the effect of serum on FSH-actions. Since we recently developed methods of cell and organ cultures of suckling mouse ovaries, we compared the effect of serum and TPA on the ovarian cell culture with their effect on the ovarian organ culture, in which the tissue architecture was maintained. We found that the addition of serum and TPA suppressed the FSH-stimulated conversion of 4-androstene-3,17-dione to oestrogens in the cell culture but did not in the organ culture.

EXPERIMENTAL

Animals and serum

Female (WB × C57BL/6)F_1 mice were raised in our laboratory. Mice were killed at 9-days of age. Ovaries were removed aseptically under the dissection microscope and were used for *in vitro* studies. Sera of mouse and rat were obtained by decapitation of female (WB × C57BL/6)F_1 mice and female Sprague–Dawley rats (Awazu Laboratory for Experimental Animals, Osaka, Japan) at 3-months of age. Fetal calf and horse sera were purchased from Irvine Scientific, Santa Ana, CA, U.S.A. and from Pel-Freez Biologicals, Rogers, AK, U.S.A., respectively. All sera were stored at −70°C.

Chemicals

Chemicals were obtained from the following sources: [2,4,6,7-^{3}H]oestradiol-17β (Sp. act. 115 Ci/mmol), [methyl-^{3}H]thymidine (Sp. act., 88.8 Ci/mmol, 1 mCi/ml water) and Protosol (tissue solubilizer) from New England Nuclear Corp., Boston, MA, U.S.A.; Medium 199 (Hanks' salt) from Grand Island Biological Co., Grand Island, NY, U.S.A.; collagenase (type II, 144 units/mg) from Worthington Biochemical Corp., Freehold, NJ, U.S.A.; deoxyribonuclease I (from bovine pancreas, 2680 Kuritz units/mg), porcine FSH, penicillin G, bovine serum albumin, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), trypsin (from porcine pancreas, 1045 BEE units/mg) and non-radioactive steroids from Sigma Chemical Co., St Louis, MO, U.S.A.; streptomicin sulfate from Banyu

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Pharmaceutical Co., Tokyo, Japan; TPA from Consolidated Midland Corp., Brewster, NY, U.S.A. Crystalline porcine zinc insulin was a gift from Eli Lilly and Co., Indianapolis, IN, U.S.A. Other reagents were of analytical grade. The contamination of LH in porcine FSH is less than 0.03 Armour units/a unit FSH.

Medium

Medium 199 was supplemented with penicillin G (35 μg/ml) and streptomycin sulfate (50 μg/ml). Hormones, TPA and serum were added to this medium. Hormones and TPA were stocked at the following concentrations at -20°C; insulin, 5 mg/ml in 0.005 N HCl; 4-androstene-3,17-dione, 3.5 μmol/ml in absolute ethanol; FSH, 50 units/ml in 0.45% (w/v) NaCl; TPA, 0.1 μmol/ml in absolute ethanol.

Cell culture

Ovarian cells were dispersed according to the method described by Magoffin *et al.* [11]. Ten to twenty ovaries of mice at 9 days of age, freed from adjacent tissues, were incubated in 4 ml of Medium 199 (pH 7.4) containing Hepes (25 mM), collagenase (4 mg/ml), deoxyribonuclease (10 μg/ml) and bovine serum albumin (10 mg/ml) for 80 min at 37°C. Ovaries were flushed through Pasteur pipets at 20 min intervals. Dispersed cells were centrifuged at 250 *g* for 10 min and washed twice with Medium 199. The final pellet was resuspended in a known volume of Medium 199 so that 0.5 ml of Medium 199 contained dispersed cells from two ovaries. The viability of the cells was 60–70% when estimated by the trypan blue dye exclusion test. The cell suspension (0.5 ml) was put into 35 × 10 mm tissue culture dish (Corning, NY, U.S.A.); the medium (0.5 ml) containing hormones and serum (or TPA) was added. The cells were cultured in a humidified incubator at 37°C containing 99.2% air and 0.8% CO_2.

Organ culture

Ovaries of mice at 9 days of age were cultured according to the method used for the mammary gland explant culture [12]. Two ovaries were put on a piece of siliconized lens paper, which was floated on 2 ml of medium in a 35 × 10 mm tissue culture dish. The culture conditions were the same as used for the cell culture.

Oestrogen production from 4-androstene-3,17-dione

Dispersed ovarian cells from two ovaries or two intact ovaries were cultured in Medium 199 containing 4-androstene-3,17-dione (0.35 μM) and insulin (5 μg/ml) or these hormones plus FSH (0.5 units/ml) in the presence or absence of 10% (v/v) serum or TPA (0.1 μM). After the culture, medium was collected and stored at -20°C until the assay of oestrogens. Amount of oestrogens in the medium was measured by radioimmunoassay using [^{3}H]oestradiol-17β and the antibody raised against estrone-17-carboxymethyloxime–bovine serum albumin. The amount of oestrogens was calculated, using oestradiol-17β as a standard. The antibody cross-reacted with oestradiol-17β (73%) and estriol (68%) but did not with non-oestrogenic steroids such as testosterone, 4-androstene-3,17-dione, progesterone, 3α-hydroxy-5-pregnen-20-one, 17-hydroxy-4-pregnene-3,20-dione, cortisol and corticosterone. Since the antibody bound to oestradiol-17β, oestrone and oestriol with similar dissociation constants (1.6×10^{-9} M, 1.1×10^{-9} M, and 1.8×10^{-9} M for oestradiol-17β, oestrone and oestriol, respectively) and since the amount of oestrogens was measured without chromatographic separation, the values represent the amount of total oestrogens formed. The intra- and inter-assay coefficients of variation were calculated according to Rodbard [13]. For this purpose, we used the quality control sample, which was prepared by adding a known amount of oestradiol-17β (2 ng) to the culture medium (1 ml) containing insulin (5 μg/ml), 4-androstene-3,17-dione (0.35 μM) and FSH (0.5 units/ml). The intra- and inter-assay coefficients of variation which were obtained from ten assays, were 8.8 and 12.2%, respectively.

DNA synthesis

The extent of DNA synthesis was determined by the incorporation of [^{3}H]thymidine into fractions insoluble to trichroloacetic acid (TCA). Either dispersed cells from two ovaries or two intact ovaries were cultured for 48 h in Medium 199 containing [^{3}H]thymidine (0.5 μCi/ml), 4-androstene-3,17-dione (0.35 μM), insulin (5 μg/ml) and FSH (0.5 units/ml) in the presence or absence of TPA (0.1 μM). The DNA synthesis of ovaries in the organ culture was determined as described previously [14]. The DNA synthesis of dispersed and cultured ovarian cells was determined as follows. At the end of the culture, cells were harvested by incubating with 1 ml of Hanks' balanced salt solution (Ca^{2+}, Mg^{2+}-free) containing 0.05% (w/v) trypsin and 0.025% EDTA for 30 min. Cells were then treated successively with ice-cold 10 and 5% TCA for 20 min each, and were washed twice with absolute ethanol. The residues were air-dried, digested in 0.4 ml of Protosol and counted for radioactivity as described previously [14].

RESULTS

The effect of fetal calf serum on the oestrogen production from 4-androstene-3,17-dione was examined in both cell and organ cultures of suckling mouse ovaries. The culture medium was collected at 24, 48 and 72 h after the initiation of cultures. Without FSH, only little amount of oestrogens was produced in both culture systems during 72 h (Figs 1A and 1B). FSH markedly stimulated the oestrogen production in the absence of serum in both cell and organ cultures (Figs 1A and 1B). In the cell culture, the addition of fetal calf serum reduced the FSH-

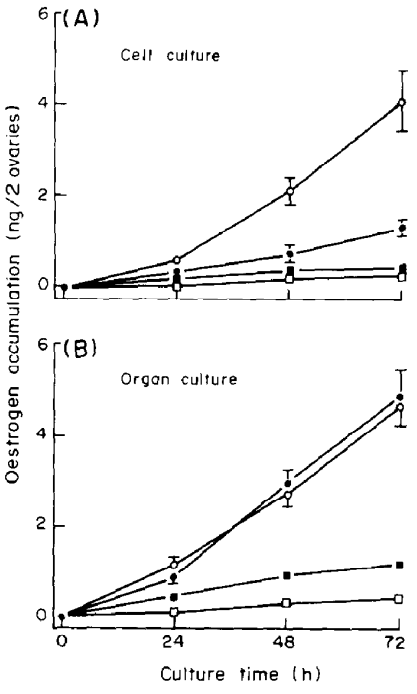

Fig. 1. Amount of oestrogen accumulated in the medium at various times after the initiation of the cell cultures (A) or the organ culture (B). Dispersed cells from two ovaries or two intact ovaries were cultured for the indicated time in medium containing insulin and 4-androstene-3,17-dione, supplemented with none (□), 10% fetal calf serum (■), FSH (○) or FSH plus 10% fetal calf serum (●). Each point, Mean ± SE (standard error of the mean) of 4 separate determinations.

stimulated oestrogen accumulation by about 80% at 48 and 72 h of culture (Fig. 1A). In contrast, the addition of fetal calf serum had no effect on the stimulatory action of FSH in the organ culture.

In the next experiment, we examined whether the effect was specific for fetal calf serum. Sera of the mouse, rat and horse were tested. As shown in Table 1, these sera also inhibited the stimulatory action of FSH in the cell culture but not in the organ culture.

Tables 2 and 3 show the effect of TPA on the FSH-stimulated oestrogen production from 4-androstene-3,17-dione in both cell and organ cultures, respectively. TPA, at the concentration of 0.1 μM, diminished the FSH-stimulated oestrogen production to the level that was observed in the absence of FSH in the cell culture (Table 2). In contrast with the case of the cell culture, the addition of the same concentration of TPA did not decrease the FSH-stimulated oestrogen production in the organ culture (Table 3).

Since TPA stimulated proliferation of various types of cultured cells [15, 16], The effect of TPA on the DNA synthesis of ovarian cells was investigated in both cell and organ cultures. Addition of TPA (0.1 μM) did not influence the incorporation of [^{3}H]thymidine into DNA in both culture conditions (Table 4).

DISCUSSION

Sera of the fetal calf, mouse, rat and horse inhibited the stimulatory effect of FSH on the

Table 1. Effect of sera of various animals on the oestrogen production from 4-androstene-3,17-dione in cell and organ cultures of the suckling mouse ovaries

Presence of FSH	Serum	Oestrogen production (ng/2 ovaries/48 h)* Cell culture	Organ culture
No	None	0.2 ± 0.04 (4)	0.4 ± 0.06 (9)
	Fetal calf	0.3 ± 0.03 (4)	0.9 ± 0.05 (4)
	Mouse	0.3 ± 0.01 (3)	0.3 ± 0.08 (4)
	Rat	0.3 ± 0.06 (3)	0.5 ± 0.14 (4)
	Horse	0.3 ± 0.03 (3)	0.5 ± 0.03 (4)
Yes	None	2.8 ± 0.60 (4)†	2.2 ± 0.1 (6)†
	Fetal calf	0.7 + 0.08 (4)†‡	2.9 ± 0.5 (4)†
	Mouse	0.3 ± 0.01 (3)‡	2.2 ± 0.1 (6)†
	Rat	0.3 ± 0.06 (3)‡	2.1 ± 0.3 (4)†
	Horse	0.7 ± 0.20 (3)‡	2.2 ± 0.4 (4)†

*Mean ± SE, number of separate determinations is shown in parethesis.
†Addition of FSH had significant effect ($P < 0.05$ by t-test).
‡Addition of serum had significant effect ($P < 0.05$ by t-test).

Table 2. Effect of TPA (0.1 μM) on the oestrogen production from 4-androstene-3,17-dione in the cell culture of the suckling mouse ovaries

Presence of FSH	Presence of TPA	Oestrogen production (ng/2 ovaries)* in 0–48 h	in 48–96 h
No	No	0.8 ± 0.3 (4)	0.7 ± 0.2 (4)
Yes	No	4.0 ± 1.0 (9)†	2.4 ± 0.5 (9)†
Yes	Yes	1.2 ± 0.2 (4)‡	1.0 ± 0.1 (4)‡

*Mean ± SE, number of independent determinations is shown in parenthesis.
†Addition of FSH had significant effect. ($P < 0.01$ by t-test).
‡Addition of TPA had significant effect. ($P < 0.01$ by t-test).

Table 3. Effect of TPA (0.1 μM) on the oestrogen production from 4-androstene-3,17-dione in the organ culture of the suckling mouse ovaries

Presence of FSH	Presence of TPA	Oestrogen production (ng/2 ovaries)* in 0–48 h	in 48–96 h
No	No	0.4 ± 0.1 (3)	0.4 ± 0.1 (3)
Yes	No	2.8 ± 0.2 (10)†	2.8 ± 0.5 (3)†
Yes	Yes	2.8 ± 0.2 (11)‡	3.4 ± 0.7 (4)‡

*Mean ± SE, number of independent determinations is shown in parenthesis.
†Addition of FSH had significant effect. ($P < 0.01$ by t-test).
‡Addition of TPA had no significant effect. ($P > 0.2$ by t-test).

oestrogen production in the cell culture of suckling mouse ovaries. Although cell separation was not done in the present experiment, only granulosa cells are known to respond to FSH [1, 17–19]. Therefore, the present result is in agreement with the results of Erickson *et al.* [8, 9] that sera inhibit the FSH action in the culture of isolated rat granulosa cells.

Serum inhibited the stimulatory effect of FSH on the oestrogen production in the cell culture but not in the organ culture. This is consistent with the result of Nimrod *et al.* [20] that LH receptors are induced by FSH in fragments of rat ovaries but not in the isolated rat granulosa cells in the presence of 5% rat serum. Nimrod *et al.* [20] speculated that the induction of LH receptors requires the participation of ovarian cells other than granulosa cells. The present result does not necessarily support the speculation, since the addition of sera did abolish the stimulatory action of FSH on granulosa cells in the cell culture which also contained ovarian cells other than granulosa cells.

The effect of TPA on the FSH-stimulated oestrogen production is similar to that of sera. TPA inhibited the FSH-stimulated conversion of 4-androstene-3,17-dione to oestrogens in the cell culture but did not in the organ culture. Since TPA did not influence the DNA synthesis of dispersed and cultured ovarian cells, the effect of TPA on the FSH-stimulated conversion of 4-androstene-3,17-dione is not attributable to its toxicity.

There are two possibilities that may explain the difference between the cell and organ cultures. (1) Serum factor(s) or TPA may not penetrate into ovarian tissues. Thus, granulosa cells in the organ culture are not exposed to the serum factor(s) or TPA (2) Only isolated granulosa cells may respond to the serum factor(s) or TPA. These two possibilities are not mutually exclusive, and further studies are necessary to clarify these mechanisms.

Table 4. Effect of TPA (0.1 μM) on the DNA synthesis of ovarian cells in organ and cell cultures

Culture system	Presence of TPA	DNA synthesis* (cpm/2 ovaries)
Organ culture	No	32200 ± 2800 (11)
	Yes	34800 ± 3400 (10)
Cell culture	No	83300 ± 7000 (4)
	Yes	87400 ± 14400 (5)

*Mean ± SE, number of independent determinations is shown in parenthesis.

The present results suggest that effects of biological factors or chemicals demonstrated in cell culture systems may not occur in intact tissues of animals which are administrated with these substances. Comparison between cell and organ cultures is a promising method to predict actions of various substances in the body at the tissue level.

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