Effects of β -Carotene and Vitamin A on Progesterone Production by Cultured Bovine Granulosa Cells

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ABSTRACT. The effects of β -carotene and vitamin A on progesterone production by cultured granulosa cells of cows were studied. The granulosa cells were isolated from medium-sized follicles (8–10 mm) by the needle aspiration. When granulosa cells were treated with 50 μ g/dl of β -carotene for 4 days, progesterone production was increased slightly. In contrast, treatment with the same amount of vitamin A was associated with a significant ($P < 0.01$) increase in progesterone production of the cells. Stimulation of progesterone production by vitamin A was noted in dose-dependent manners. Proliferation of granulosa cells was suppressed by vitamin A treatments, and some morphological changes were observed in cultures grown in media with vitamin A. These results suggest that β -carotene, mediated by vitamin A may stimulate steroidogenesis of granulosa cells.—**KEY WORDS:** β -carotene, dairy cattle, ovary, progesterone.

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The role of vitamin A in livestock has been reported from many different aspects [1, 16]. The role of β -carotene, a provitamin A in plant materials, however, is less clear. Recently, German researchers [13, 15] reported that supplementation with β -carotene has been associated with improved reproductive functions such as greater intensity of estrus and increase in conception rates. We have previously observed that the levels of plasma β -carotene are significantly lower in cows with ovarian cysts than those in cows without ovarian cysts, when they are raised on a feed with low β -carotene contents [8]. Because a relatively high concentration of β -carotene is in bovine corpus luteum [6, 11], β -carotene may influence production of ovarian steroids. Juneja *et al.* [12] found decreased progesterone production in ovaries of rats deficient of vitamin A. Thus no far studies have related β -carotene in the corpus luteum and steroids production of cows.

The purpose of this study is to examine the effects of β -carotene and vitamin A on

progesterone production by cultured granulosa cells of cows.

MATERIALS AND METHODS

Culture conditions: Granulosa cells were isolated from ovaries of Holstein cows by the method of Channing and Ledwitz-Rigby [3]. Medium-sized follicles (8–10 mm) were aspirated aseptically with an 18-gauge needle, and the granulosa cells recovered by low speed centrifugation. Approximately 2×10^5 viable cells, as determined by the exclusion of trypan blue, were plated in 35 mm plastic dishes with 2 ml culture fluid consisting of Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) buffered with bicarbonate, 0.1 mg streptomycin/ml, 100 IU penicillin G/ml, 10% (vol/vol) fetal calf serum (M. A. Bioproducts, Walkersville, MD), and 100 μ M cholesterol. Cholesterol and vitamins were dissolved in ethanol and diluted in medium as the ethanol concentration did not exceed 0.1%. Dishes were placed in a water-jacketed

Fig. 1. The effects of β -carotene and vitamin A on progesterone production by bovine granulosa cells. Bovine granulosa cells plated at 10^5 cells/ml were maintained in the presence of control solvent (○), β -carotene (50 μ g/dl, ▲), or vitamin A (50 μ g/dl, ●). Data are the mean $\pm$ SEM ($n=4$), expressed as nanograms of progesterone per ml/48 hr. * $P < 0.01$ vs. control cultures.

incubator at 37°C in the atmosphere of 95% air-5% CO₂. The culture medium was changed at intervals of 48 hrs. The spent medium was centrifuged at 2,000 $\times$ g and then stored at -20°C until analyzed for progesterone content. In separate experiments, granulosa cells were harvested from cultures by trypsinization (0.25% trypsin for 20 min at 23°C) for the enumeration of cell density. Photomicrographs were taken in certain experiments in which cells were grown on glass cover slips (15 $\times$ 15 mm) placed in dishes. Those cover slips were removed 4 days after plating, fixed in ethanol, and stained with Giemsa. Cholesterol, retinol, and β -carotene (Sigma, St. Louis, MO) were used in this study.

Progesterone assay: Progesterone was determined in media of cultured cells during 2 days of accumulation. The steroid was measured by radioimmunoassay as previously described [7]. Results of the assay

Fig. 2. The effect of vitamin A on progesterone production by bovine granulosa cells. Bovine granulosa cells plated at 10^5 cells/ml were maintained in the presence of indicated doses of vitamin A. Data are the mean $\pm$ SEM ($n=4$), expressed as nanograms of progesterone per ml/48 hr.

were analyzed for significance by Student's t test.

RESULTS

β -Carotene and vitamin A were added separately to cultures of granulosa cells (Fig. 1). After 4 days of culture, the progesterone secretion by cells cultured with β -carotene increased slightly above that by the control cells. In contrast, the addition of vitamin A to the cells resulted in a 2-fold increase in progesterone production during a 4 days period. The effects of vitamin A upon steroid production by cells were dose dependent (Fig. 2).

The effect of vitamin A on granulosa cell growth was examined. Proliferation of granulosa cells was suppressed by vitamin A (Fig. 3), but the increased steroidogenic activity was associated with morphological changes in the cells (Fig. 4). While secreting relatively small amounts of progesterone in

control dishes, the cells were oblong in configuration or spindle shaped, and refractile cytoplasmic inclusions were sparse. Concomitant with the enhancement of progesterone secretion in dishes supplemented with vitamin A, the cells became polygonal in shape, and the number of cytoplasmic inclusions increased.

DISCUSSION

It has been reported that β -carotene itself

Fig. 3. The effect of vitamin A on the growth rate of bovine granulosa cells. Cultures of bovine granulosa cells were maintained in the presence (●) or absence (○) of vitamin A (50 $\mu\text{g}/\text{dl}$). Data are expressed as the mean $\pm$ SEM ($n=4$). * $P<0.05$, ** $P<0.01$.

plays a specific role on the reproduction in cattle apart from that of vitamin A [13, 15], while more recent studies [2, 14] report a conflicting role of β -carotene. In the present studies, we observed stimulatory effects of vitamin A on progesterone production by the bovine granulosa cells. But β -carotene had small effects on the steroid production by the cells. These findings suggest that the stimulation of progesterone production by β -carotene may be mediated by vitamin A.

As with the cells cultured with vitamin A, increased progesterone production occurred in association with the suppressed cell proliferation and the morphological changes. These findings favor the notion that the proliferative activity and the steroidogenic responsiveness in granulosa cells are inversely regulated [4]. We infer that the action of vitamin A on the bovine granulosa cells is related to the cell differentiation. In this regard, Jetten [10] has been reported that vitamin A inhibited rat mammary cell proliferation and altered the cell differentiation. Furthermore, Fontana *et al.* [5] reported that the induction of leukemic cell differentiation by vitamin A was associated with the elevation of the cyclic adenosine 3':5'-monophosphate dependent protein kinase (cAMP-dPK) activities. We have

Fig. 4. The effect of vitamin A on the morphology of bovine granulosa cells in culture. Bovine granulosa cells were cultured in the presence or absence of vitamin A (50 $\mu\text{g}/\text{dl}$). Photomicrographs were taken on day 4 of culture. The steroid production of these cells is shown in Fig. 1. (A) Photomicrograph of cells cultured in the absence of vitamin A, revealing elongated shape and few cytoplasmic inclusions. $\times 1000$. (B) Cells cultured in the presence of vitamin A, revealing polygonal shape with accumulation of refractile inclusions. $\times 1000$.

previously shown that the biosynthesis of progesterone in rat luteal cell mitochondria was directly regulated by cAMP-dPK [9]. In conjunction with the present work, it is postulated that possible mechanisms of action of vitamin A on the bovine granulosa cells include inhibition of cell proliferation, altered cell differentiation, enhanced cAMP-dPK activities, and increased progesterone production.

The present studies may be applicable to our previous finding that low levels of plasma β -carotene is associated with a high incidence of ovarian cysts in cows [7]. The delay of luteinization of granulosa cells might be occurred in the cows with low level of plasma β -carotene, since enough amount of vitamin A was not provided.

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要 約

牛培養卵胞顆粒膜細胞のプロジェステロン産生に対する β -カロチンおよびビタミン A の影響：稲葉俊夫・免山央時¹⁾・清水亮佑¹⁾・小野忠義²⁾・森 純一（大阪府立大学農学部家畜臨床繁殖学教室，¹⁾家畜外科学教室，²⁾大阪府農林技術センター）—— β -カロチンおよびビタミン A が乳牛の卵胞顆粒膜細胞からのプロジェステロン産生におよぼす影響について検討した。顆粒膜細胞は中等大（直径 8～10 mm）の卵胞から注射器を用いて吸引・分離した。 β -カロチン 50 $\mu\text{g}/\text{dl}$ を 4 日間添加することにより，顆粒膜細胞からのプロジェステロン産生量はわずかに上昇したが，同量のビタミン A 添加によって有意に上昇した ($P<0.01$)。ビタミン A 添加によるプロジェステロン産生量の増加は，添加量に比例したが，一方添加によって顆粒膜細胞の増殖は抑制され，形態的变化が観察された。これらの成績から， β -カロチンはビタミン A を介して顆粒膜細胞のステロイド合成を促進することが示唆された。