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The Role of Cholesterol Esterification in Ovarian Steroidogenesis: Studies in Cultured Swine Granulosa Cells Using a Novel Inhibitor of Acyl Coenzyme A:Cholesterol Acyltransferase*

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ABSTRACT. We have used a novel competitive inhibitor of acyl coenzyme A:cholesterol acyltransferase (ACAT), Sandoz compound 58-035 [3-(decyldimethyl-silyl)*N*-[2-(4-methyl-phenyl)1-phenylethyl propanamide], to assess the importance of the cholesterol esterification reaction in ovarian steroidogenesis. Compound 58-035 markedly (≥96%) inhibited ACAT activity of swine ovarian microsomes in a dose-dependent (0.1–3.5 μg/ml) fashion. In addition, treatment of cultured granulosa cells with this fatty acylamide effectively (≥98%) suppressed hormonally stimulated cholesterol esterification, as assessed by the incorporation of [^{3}H]oleic acid into cholesteryl ester. Accordingly, we used this inhibitor to test the role of cholesterol esterification in ovarian cells.

In cultures with limited or no serum supplementation, long term (2- to 6-day) treatment of granulosa cells with compound 58-035 significantly increased basal progesterone production and amplified by 2- to 10-fold the stimulatory actions of trophic hormones, such as estradiol, FSH, estradiol combined with FSH, or insulin. The amplifying effect of ACAT inhibition on hormone-stimulated progesterone production could be mimicked by providing exogenous cholesterol substrate in the form of low density lipoprotein (LDL). Cotreatment with compound 58-035 and LDL resulted in no further augmentation of steroidogenesis. In contrast to the facilitative effects of compound 58-035 in longer term cultures, this ACAT inhibitor did not alter progesterone biosynthesis acutely (2–20 h) in swine or hamster ovarian cells.

These observations suggest that there is an obligatory partitioning of some sterol into the ester pool in granulosa cells. In times of diminished availability of cholesterol, inhibition of the esterification pathway can make additional cholesterol available for use in steroid hormone biosynthesis. Thus, in the intact Graafian follicle, where LDL cholesterol delivery to granulosa cells and intracellular cholesteryl ester stores are limited, regulation of the ACAT reaction may significantly modulate rates of progesterone biosynthesis.

The present results indicate that the use of a selective inhibitor of cholesterol esterification can permit one to probe the functional significance of the esterification reaction in steroidogenic cells. (*Endocrinology* **116:** 25–30, 1985)

STEROIDOGENIC cells can accumulate cholesterol in the form of sterol esters. This pool of cholesterol is believed to provide a storage form of precursors for steroid hormone biosynthesis (1–3). Thus, hormonal regulation of the enzyme responsible for the formation of sterol esters, acyl coenzyme A:cholesterol acyltransferase (ACAT), may provide an important mechanism for controlling rates of steroidogenesis in gonadal tissues by modulating the availability of substrate. To date, this hypothesis has been difficult to test directly in intact cells, in part because of the lack of availability of suitable enzymic inhibitors of the acyltransferase reaction. In the present work, we have been able to probe the functional importance of the ACAT reaction in ovarian progesterone biosynthesis by using a novel inhibitor of ACAT activity, Sandoz compound 58-035 [3-(decyldimethyl-silyl)*N*[2-(4-methylphenyl)1-phenylethyl propanamide] (4).

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Materials and Methods

Hamster luteal cells were prepared as reported previously (5). Swine granulosa cells were isolated from 1- to 5-mm follicles of immature ovaries, as previously described (6). Monolayer

cultures were established in bicarbonate buffered Eagle's Minimum Essential Medium in the presence of the indicated hormone(s), and/or ACAT inhibitor (Sandoz 58-035) and 1% or 3% (vol/vol) fetal bovine serum. Unless noted otherwise, tissue culture medium was replenished at 48-h intervals. Progesterone was assayed in extracts of medium or cells combined with medium by RIA, as validated previously (7). Total cellular protein was determined by the method of Bradford (8).

The incorporation of [^{3}H]oleic acid into cellular cholesteryl ester was assessed by presenting monolayer cultures of granulosa cells with [^{3}H]oleic acid (5 μCi/ml) complexed with albumin at a molar ratio of fatty acid to albumin of 1:1. The final concentration of added oleic acid in these experiments was 0.1 mM. At indicated times after the addition of labeled fatty acid, cultures were harvested by mechanical scraping, and the subsequent lipid extract was subjected to TLC, as previously described (9). The activity of ACAT in microsomes of immature porcine ovaries was determined as previously reported (10).

All hormones were added at maximally effective concentrations at the outset of culture, using 1 μg/ml 17β-estradiol, 200 ng/ml FSH, and 1 μg/ml highly purified porcine insulin. Fresh media containing hormone(s) were added at each culture change and before the incorporation studies.

Statistical analyses employed analysis of variance to ascertain significance among multiple means, with Duncan's multiple range test to assess individually significant effects (11). Untransformed data are presented as the mean ± SEM.

To assess the generality and reproducibility of our inferences, all experiments were performed at least twice with independent pools of granulosa cells collected from 200–280 pig ovaries.

Materials

[9,10-N-^{3}H]Oleic acid (5.7 Ci/mmol) was purchased from Amersham Corp. (Arlington Heights, IL). [1,2-N-^{3}H]Progesterone was obtained from New England Nuclear Corp. (Boston, MA). 17β-Estradiol was purchased from Sigma Chemical Co. (St. Louis, MO). Tissue culture medium was obtained from Grand Island Biological Co. (Grand Island, NY). Ovine FSH (NIH FSH-S13; FSH potency equal to 15 NIH FSH-S1 units/mg; LH potency equal to 0.05 NIH LH-S1 units/mg) was provided by the Hormone Distribution Office, National Pituitary Agency, NIAMDD, NIH (Bethesda, MD).

Results

In porcine ovarian microsomes, compound 58-035 inhibited ACAT activity in a dose-dependent fashion over the concentration range 0.1–3.5 μg/ml (Fig. 1). Basal ACAT activity was 270 pmol cholesteryl oleate formed/mg protein·min, which was inhibited by 96% at a concentration of 3.5 μg/ml compound 58-035.

In cultured granulosa cells, the incorporation of [^{3}H]oleic acid into cholesteryl ester in the presence of 3% serum was shown to increase linearly over 2–24 h (Fig. 2). Time-dependent [^{3}H]oleic acid incorporation into cholesteryl ester was significantly ($P < 0.01$) increased in the presence of insulin (Fig. 2A), or estradiol (Fig. 2B). Moreover, treatment of cultured granulosa cells with the ACAT inhibitor (3 μg/ml) for 48 h slightly (~20%) depressed basal rates of cholesterol esterification and markedly (>98%) inhibited the increased rates of oleic acid incorporation stimulated by insulin (Fig. 3A) or estrogen (Fig. 3B).

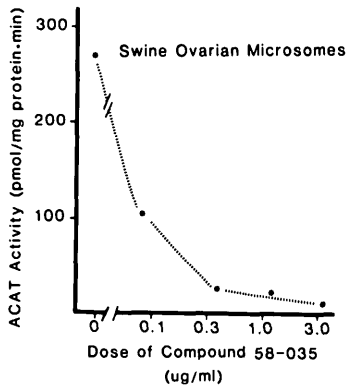

FIG. 1. Compound 58-035 inhibits ACAT activity in swine ovarian microsomes in a dose-dependent fashion. Pig ovarian microsomes (175 μg protein/tube) were incubated in the absence or presence of the indicated doses of the ACAT inhibitor, compound 58-035. At an inhibitor concentration of 3.5 μg/ml, the activity of ACAT was inhibited by 96%.

To assess the functional effects of inhibiting ACAT activity on progesterone biosynthesis, monolayer cultures of granulosa cells were established in 1% fetal calf serum in the presence or absence of 17β-estradiol and/or compound 58-035 at a dose of 1 or 3 μg/ml (Fig. 4). Cells and medium were then harvested at various intervals for the subsequent assay of progesterone. The ACAT inhibitor did not significantly alter basal progesterone production acutely (20 h) and did not antagonize the suppressive effect of estradiol at this time. However, after 4 and 6 days of culture, the ACAT inhibitor significantly increased basal and estrogen-stimulated progesterone production ($P < 0.05$, treatment effect). In particular, compound 58-035 (3 μg/ml) amplified the stimulatory action of estradiol by approximately 4-fold on day 4 ($P < 0.01$).

In further experiments, the enhancement of estrogen action was confirmed, and the capacity of this ACAT inhibitor (3 μg/ml) to augment the stimulatory effect of FSH was demonstrated (Fig. 5). Moreover, inhibition of acyltransferase activity also amplified the synergism between estradiol and FSH in these cultures ($P < 0.01$).

When granulosa cells were treated with insulin for 2 or 4 days, concomitant administration of compound 58-035 (3 μg/ml) significantly augmented insulin's effects. This facilitative action of the ACAT inhibitor was particularly prominent on day 4 ($P < 0.01$) and was observed in cultures maintained in the absence of serum or in the presence of limited (3%) serum (Fig. 6).

When granulosa cells were cultured in serum-free me-

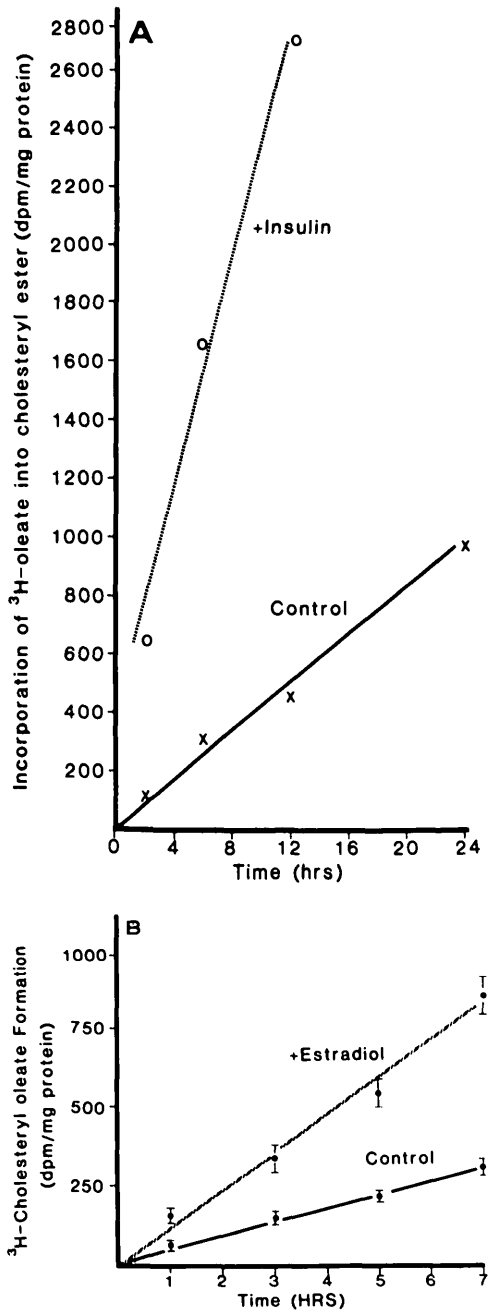

FIG. 2. Time-dependent incorporation of [^{3}H]oleic acid into cholesteryl esters in cultured pig granulosa cells. Swine ovarian cells were cultured in the presence or absence of a maximally effective dose of insulin (A) or estradiol (B) for 48 h, and then exposed to [^{3}H]oleic acid for the intervals shown. The subsequent cellular extracts were analyzed for cholesteryl ester content by TLC. Data are means from duplicate (insulin) or triplicate (estradiol) cultures at each time point. These results were confirmed in two independent experiments.

dium in the presence of a maximally effective dose of human low density lipoprotein (LDL; 300 μg/ml), the stimulatory effect of LDL was approximately equal to that of the ACAT inhibitor alone (Fig. 7). In contrast, cotreatment with LDL combined with compound 58-035 (3 μg/ml) did not augment progesterone production further compared with LDL alone or compound 58-035 alone.

In contrast to the stimulatory effects of compound 58-035 under longer term culture conditions, acutely (2–20 h), this inhibitor was devoid of effect in swine granulosa (Fig. 4) or hamster luteal cells. In particular, in hamster luteal cells, basal and LH-stimulated progesterone production per 2 h were, respectively, 0.99 ± 0.04 and 7.1 ± 0.39 ng/10^4 cells. Neither basal nor LH-stimulated progesterone production over these 2 h was significantly influenced by various concentrations of the ACAT inhibitor (1, 2.5, 5, or 10 μg/ml). For example, at 10 μg/ml compound 58-035, basal and LH-stimulated progesterone production were, respectively, 0.86 ± 0.09 and 6.9 ± 0.53 ng/10^4 cells·2 h (P = NS *vs.* corresponding control cultures).

Discussion

In gonadal cells, the substrate cholesterol for steroid hormone synthesis can be derived by *de novo* biosynthesis from acetyl coenzyme A or from blood-borne lipoprotein (1–3, 12–14). Steroidogenic cells also accumulate stores of cholesteryl ester, which can be used as hormone precursor. To date, it has been difficult to directly test the functional relationship of sterol ester stores to the steroidogenic pathway. We have been able to approach this question using a representative of a novel class of fatty acylamides that act as competitive inhibitors of ACAT (4), the enzyme responsible for the esterification of free cholesterol.

Our observations indicate that the ACAT inhibitor, compound 58-035, can effectively suppress acyltransferase activity in ovarian microsomes and intact granulosa cells. This ACAT inhibitor was devoid of evident toxicity and promoted a significant increase in basal progesterone production after 4 or 6 days of culture. Moreover, in the presence of compound 58-035, there was a marked amplification of the stimulatory actions of estradiol, FSH, estradiol combined with FSH, and insulin on total progesterone production. This ability of an inhibitor of cholesterol esterification to augment basal and stimulated rates of steroid hormone biosynthesis suggests that regulating the entry of cholesterol into ester storage pools under some conditions can significantly modulate effective rates of steroidogenesis in ovarian cells.

We observed that compound 58-035 significantly enhanced basal and hormonally stimulated progesterone production by granulosa cells cultured for 2–6 days with a limited extracellular source of cholesterol (*e.g.* serum-free medium or medium supplemented with only 3% bovine serum). These conditions restrict the availability of LDL-carried cholesterol and, therefore, mimic the *in*

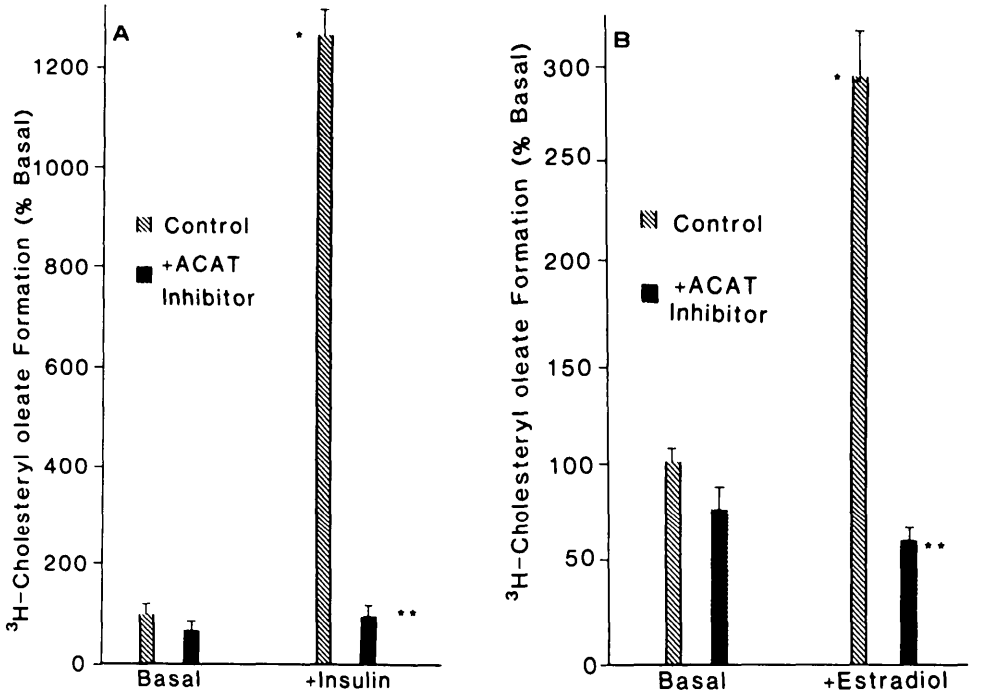

FIG. 3. Ability of the ACAT inhibitor, compound 58-035 (3 μg/ml) to inhibit [^{3}H]oleic acid incorporation into cholesteryl esters in cell cultures. Pig granulosa cells were cultured in 3% serum for 48 h in the presence of the various indicated hormones, and cholesteryl ester formation was subsequently assessed over 18 h. Data are the mean ± SEM (n = 3 separate cultures), expressed as the percent increase above basal incorporation in the absence of inhibitor (mean, 830 dpm/mg protein·18 h). *, $P < 0.01$ *vs.* basal; **, $P < 0.01$ *vs.* control.

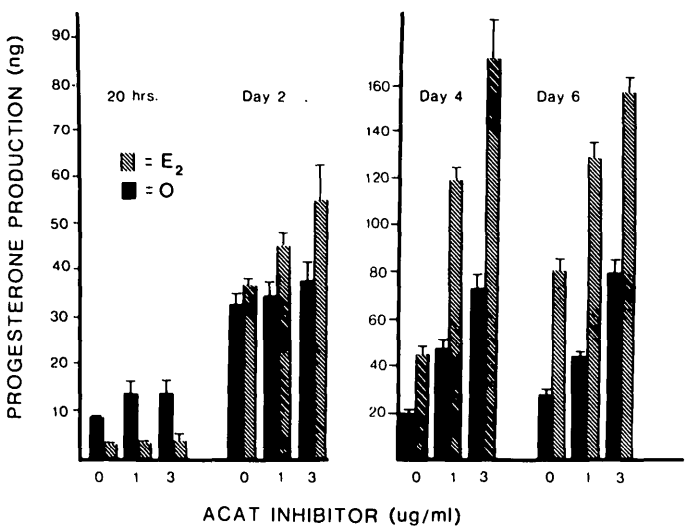

FIG. 4. Swine granulosa cells were maintained in monolayer culture supplemented with 1% serum for varying intervals, as shown, in the absence (0) or presence of estradiol (E_2), with or without increasing doses of the ACAT inhibitor, compound 58-035. Cultures were harvested for subsequent assay of total progesterone content. Data are the mean ± SEM (n = 4 separate cultures) for this typical experiment. These inferences were corroborated in two independent experiments.

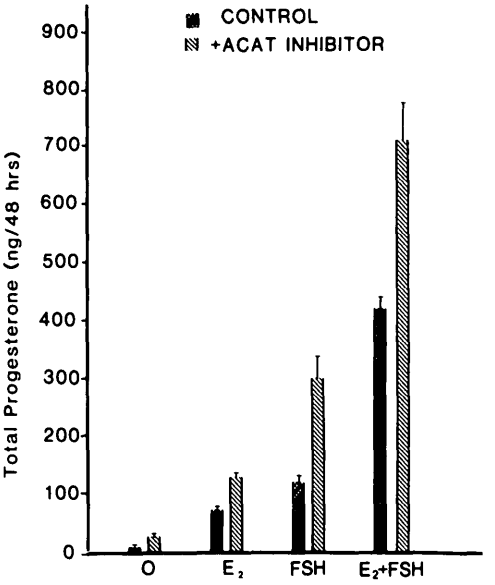

FIG. 5. Granulosa cells were cultured for 4 days in the presence of 1% serum and in the absence (0) or presence of estradiol (E_2), FSH, or both, with or without the ACAT inhibitor, compound 58-035 (3 μg/ml). Total progesterone produced is expressed as nanograms per 48 h (mean ± SEM; n = 4 separate cultures).

vivo milieu of swine and human granulosa cells, which are exposed to LDL-deficient antral follicular fluid before ovulation (14–16). In contrast, when LDL was supplied exogenously, progesterone production increased to a degree similar to that attained after treatment with compound 58-035. In addition, when LDL was provided in combination with compound 58-035, inhibition of ACAT activity did not further increase progesterone production. Moreover, in short term (2-h) incubations of hamster luteal cells that are replete with sterol ester stores, inhibition of ACAT activity did not significantly influence basal or LH-stimulated progesterone production. Thus, significant cellular responses are demonstrated in LDL-deficient cultures after longer term, but not acute, treatment with the ACAT inhibitor. This suggests that granulosa cells can mobilize endogenous sterol esters adequately under short term conditions until these stores become depleted. At such times of diminished availability

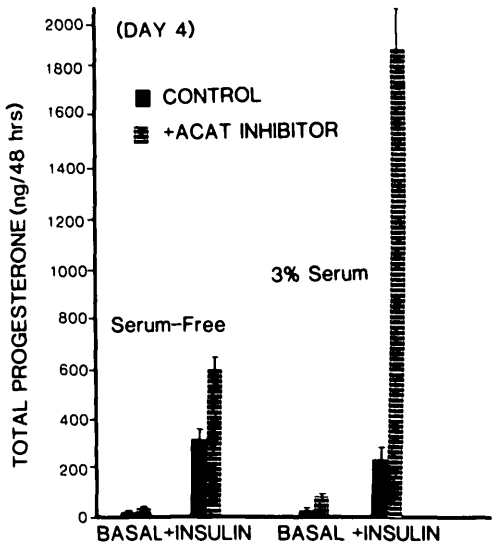

FIG. 6. Swine granulosa cells were cultured for 4 days in the presence or absence of 3% serum under basal conditions or with a maximally stimulating concentration of insulin. Cultures were treated with control solvent or compound 58-035 (3 μg/ml). Data are the mean ± SEM (n = 4 separate cultures), expressed as nanograms of total progesterone per 48 h/3 × 10^6 cells. The ability of the ACAT inhibitor to amplify insulin's stimulatory action was confirmed in two other independent experiments.

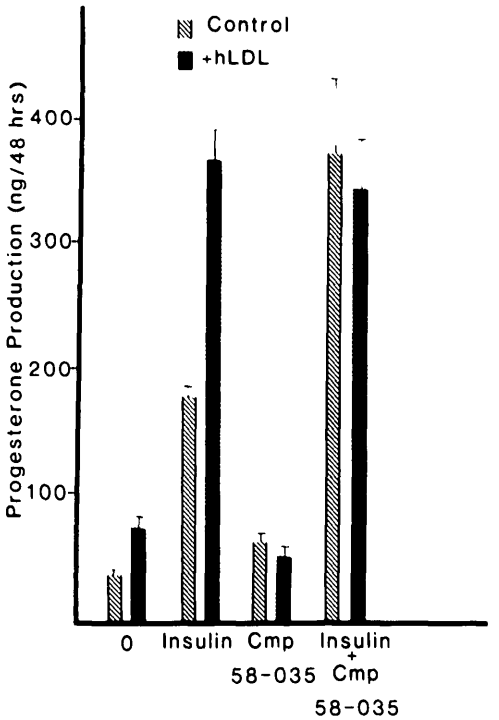

FIG. 7. Provision of exogenous cholesterol substrate in the form of LDL mimics the effect of the ACAT inhibitor, compound 58-035. Swine granulosa cells were cultured for 2 days in serum-free medium in the presence of a maximally stimulating dose of insulin, with or without the addition of human LDL (hLDL; 300 μg/ml) or the ACAT inhibitor, compound 58-035 (3 μg/ml). Data are the mean ± SEM (n = 4 separate cultures).

of cholesterol, reduced activity of the esterification pathway can then make significant additional quantities of cholesterol available for use in steroid hormone biosynthesis. Thus, we speculate that regulation of ACAT activity may significantly modulate effective rates of progesterone biosynthesis *in vivo* in granulosa cells residing in relatively cholesterol-depleted antral fluid.

In conclusion, our findings indicate that there is an obligatory contribution of cholesterol to the ester pool of ovarian cells. The degree of partitioning of cholesterol into this ester pool can materially influence the rate of progesterone production under certain conditions. Thus, particularly in times of diminished availability of cholesterol, endocrine regulation of rates of sterol ester formation may significantly modulate effective rates of steroidogenesis in the ovary.

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