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Inhibitory Effects of Cholesterol Sulfate on Progesterone Production in Human Granulosa-like Tumor Cell Line, KGN

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Abstract. Cholesterol sulfate (CS) is a component of cell membranes that plays a role in stabilizing the cell membrane. We previously reported that CS increased in the endometrium during implantation, suggesting that CS plays an important role in reproduction. It has been reported that CS regulates progesterone and pregnenolone production in the placenta, adrenal glands and ovary. The regulatory mechanisms of steroid hormone production by CS, however, are still unknown. In the present study, we investigated the effect of CS on the expression of progesterone production-related genes in KGN cells, derived from human granulosa-like tumor. KGN cells were cultured with CS (10 μM) or cholesterol (10 μM) in the presence of 8-bromo-cAMP (1 mM). Progesterone levels in the culture media were measured by enzyme linked fluorescent assay at 24 h after treatment of CS and cAMP. Total RNAs were extracted for quantitative real time RT-PCR with specific primer of StAR protein, P450scc, HSD3B2, ferredoxin and ferredoxin reductase. Whole cell lysates were extracted for western blot analysis with antibody for StAR protein. Progesterone concentration in the culture medium increased to 38-fold by treatment of cAMP. CS significantly reduced progesterone concentration by 30% compared with those of cAMP treatment ($p<0.05$), while cholesterol did not change the progesterone concentration. CS treatment down-regulated the expression of StAR mRNA and P450scc mRNA was to 54% and 60%, respectively ($p<0.05$). Western blot analysis revealed that the amount of StAR protein was also reduced by CS treatment. The expression of HSD3B2 mRNA was up-regulated to 3.4-fold by treatment of cAMP. The expression of ferredoxin and ferredoxin reductase mRNA was not affected by CS treatment. These data implied that CS has an inhibitory effect on progesterone production by regulating the expression of StAR and P450scc gene expression.

Key words: Cholesterol sulfate, Progesterone, StAR protein, P450scc, KGN cells

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CHOLESTEROL sulfate(CS), made by sulfonation of cholesterol, is a component of cell membranes and has a stabilizing role, such as protecting erythrocytes from osmotic lysis and regulating sperm capacitation [1]. CS can regulate the activity of selective protein kinase C and modulate the specificity of phosphatidylinositol 3-kinase and thus involved in signal transduction [1]. Patients with recessive x-linked ichthyosis (fish skin disease) accumulate CS in squamous keratinizing epithelia and display abnormal barrier in the skin [2].

We previously reported that CS increased in rabbit endometrium during implantation. Concentration of CS was relatively low in nonpregnant rabbit endometrium, but abruptly increased at day 5 of pregnancy, at the beginning of implantation [3]. During the implantation phase, CS concentration in rabbit endometrium increased to 10 times that in nonpregnant rabbit endometrium [4]. These data indicate that CS plays important roles in reproduction.

Steroidogenesis is one of the essential steps in reproduction. CS stimulates progesterone production in placental trophoblast cells, adrenal cells and ovarian cells [5–8]. There are two different theories about involve-

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ment of CS in adrenal steroid synthesis. First, CS can serve as a substrate for mitochondrial P450side chain cleavage (P450scc), resulting in the formation of pregnenolone sulfate [7]. Second, CS has the ability to inhibit side chain cleavage of free or unconjugated cholesterol in the formation of pregnenolone [9]. Interestingly, this inhibition occurs upon intramitochondrial cholesterol translocation and does not involve the function of P450scc [9, 10]. The effect of CS on steroidogenesis is still controversial, and to date few studies attempted to elucidate the mechanism of the effect of CS on steroidogenesis.

KGN cells were derived from human ovarian granulosa tumor [11]. KGN cells are undifferentiated and maintain physiological characteristics of ovarian cells, including the expression of functional FSH receptor and the expression of aromatase [11, 12]. We used KGN cells as a model of human ovary. To investigate the role of CS in ovarian steroidogenesis, we studied the effect of CS for progesterone production with KGN cells and analyzed the expression of steroid regulating genes in KGN cells to clarify the underlining mechanism.

Materials and Methods

Culture of KGN cells

KGN cells, a human ovarian granulosa-like tumor cell line [11] kindly provided by Prof. Hajime Nawata (Kyushu University), were cultured in DMEM/F12 (phenol red free) supplemented with 10% FBS and antibiotics in a 5% CO_2 atmosphere at 37°C. KGN cells (4 × 105 viable cells) were cultured on 6-well plate for 48 h with DMEM/F12 (phenol red free) supplemented with Charcoal/Dextran treated 10% FBS. After 48 h preincubation, the medium was changed to FBS free DMEM/F12 and then treated with CS (Sigma, St Louis, USA) at a dose of 10 μM or cholesterol (Sigma) at a dose of 10 μM in the presence of 1 mM of 8-bromo-cAMP (Sigma) in DMEM/F12 for an additional 24 h and 48 h. Culture media were collected at the end of the assay and stored at –20°C until use.

Progesterone quantification

Progesterone concentrations in the culture medium were determined using enzyme linked fluorescent assay (Arklay, Kyoto, Japan). Progesterone concentrations were normalized with respect to total cellular protein content as determined by DC protein assay (Bio-Rad, Hercules CA, USA).

Isolation of total RNA and cDNA synthesis

Total RNA was extracted from KGN cells using RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Complementary DNA was synthesized from 500 ng of total cellular RNA using the First Strand cDNA synthesis kit (Toyobo, Osaka, JAPAN).

Quantitative reverse transcription polymerase chain reaction

The amounts of steroidogenic acute regulatory protein (StAR), P450scc, 3beta-hydroxysteroid dehydrogenase type2 (HSD3B2), ferredoxin, ferredoxin reductase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA in KGN cells was quantified by real time polymerase chain reaction (PCR) with the Light Cycler rapid thermal cycler system (Roche Diagnostics, Lewes, UK). It was performed in a 20 μl volume containing 3 μl cDNA, 500 nM primer pairs, 1xSYBR green master mix (Nippon Gene, Tokyo, Japan). Following 10 min of denaturing at 95°C, 35 cycles of amplification were conducted (95 denaturation for 15 sec, 64 annealing for 10 sec, and 72 extension for 10 sec). The primers for the PCR reactions are shown in Table 1. The PCR products were subcloned into pCR2.1-TOPO vector with TOPO TA cloning kit (Invitrogen, Carlsbad CA, USA), and these vectors were used for the standard of quantitative real time PCR. We confirmed the identity of the product by DNA sequencing.

Western blot analysis

KGN cells were scraped into RIPA buffer and allowed to stand on ice for 15 min. After centrifuging at 15,000 rpm for 15 min, the supernatant was removed for western blot analysis. Total cellar protein mixed with 2× sample buffer (containing 0.25 M Tris–HCl, 4% SDS, 10% sucrose, 0.004% bromophenol blue, 10% beta-2 mercaptoethanol), were separated electrophoretically on a 12.5% SDS-polyacrylamide gel. The proteins on the gel were transferred to a PVDF mem-

Table 1. Primers used for the quantitative real-time PCR

Gene	Accession No.	Primer sequences	Fragment size (bp)
StAR	NM_000349	F 5'-GGCATCCTTAGCAACCAAGA-3' R 5'-TCTCCTTGACATTGGGGTTC-3'	200
P450scc	NM_000781	F 5'-GCAACGTGGAGTCGGTTTAT-3' R 5'-TGCAGGACACTGACGAAGTC-3'	268
HSD3B2	NM_000198	F 5'-GCGGCTAACGGGTGGAATCT-3' R 5'-CATTGTTGTTCAGGGCCTC-3'	270
Ferredoxin	NM_004109	F5'-TGGCTTGTTCAAACCTGTCAC-3' R5'-GAGGTCTTGCCCACATCAAT-3'	223
Ferredoxin reductase	NM_004110	F5'-CCTGAGCACCTGGAGAGAAC-3' R5'-CTGGAGACCCAAGAAATCCA-3'	195
GAPDH	NM_002046	F5'-ACCACAGTCCATGCCATCAC-3' R5'-TCCACCACCCTGTTGCTGTA-3'	450

brane, which was then incubated in 5% skim milk–TBS/0.1% Tween20 (TBST) at room temperature (RT) for 1 h to block any nonspecific reaction. The membrane was incubated in 5 μg/ml of anti StAR antibody PA1-560(ABR USA) at RT for 1 h. After three washes in TBST, the membrane was incubated with peroxidase-conjugated anti-rabbit IgG goat IgG (diluted to 1 : 3000) (Santa Cruz Biotechnology, CA, USA) for 1 h at RT. After three washes in TBST the immunocomplexes were visualized using the ECL plus Western Blot system (Amersham Pharmacia Biotech, Buckinghamshire, England).

Statistical Analysis

Statistically significant differences between treatment groups were identified using one way ANOVA test. All statistical tests were conducted using Statview (SAS Institute USA). Data were presented as mean ± S.E., and $P<0.05$ were considered to be statistically significant. Data shown are representative of four or more independent experiments.

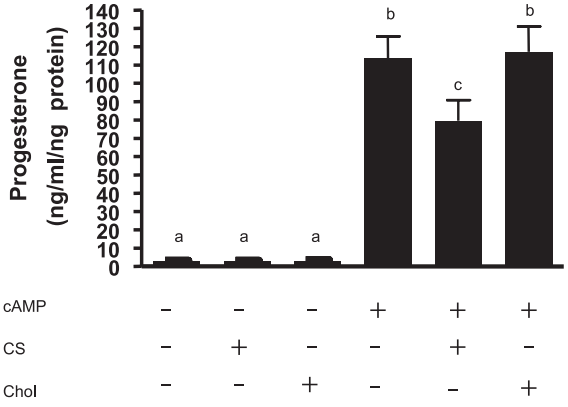

Fig. 1. Effects of CS, cholesterol and cAMP on progesterone production.
KGN cells were cultured with cholesterol sulfate (CS), cholesterol (chol), cAMP, cAMP + CS and cAMP + Cholesterol. Progesterone concentrations in culture media were determined at 24 h. Treatment of CS with cAMP significantly reduced progesterone levels compared with treatment of cAMP. CS suppressed progesterone production by about 70%. On the other hand, Cholesterol with cAMP did not change progesterone levels significantly. Data are represented as means ± SEM for 5 independent experiments. Significant differences are indicated between matching letters ($P<0.05$)

Results

Fig. 1 demonstrated progesterone concentrations in culture medium of KGN cells. Neither CS nor cholesterol changed progesterone concentrations significantly after 24 h without cAMP. Progesterone concentration increased 38-fold compared to control 24 h after cAMP treatment. CS in the presence of cAMP was significantly reduced to 30% of progesterone concentration compared with those with cAMP treatment ($p<0.05$). On the other hand treatment of cholesterol with cAMP did not change progesterone concentrations significantly. We also measured progesterone concentrations at 48 h after treatment. CS in the presence of cAMP was significantly reduced to 38% of progesterone concentration compared with those with cAMP treatment.

Treatment of cAMP significantly up-regulated the expression of StAR mRNA at 24 h (Fig. 2A). Treat-

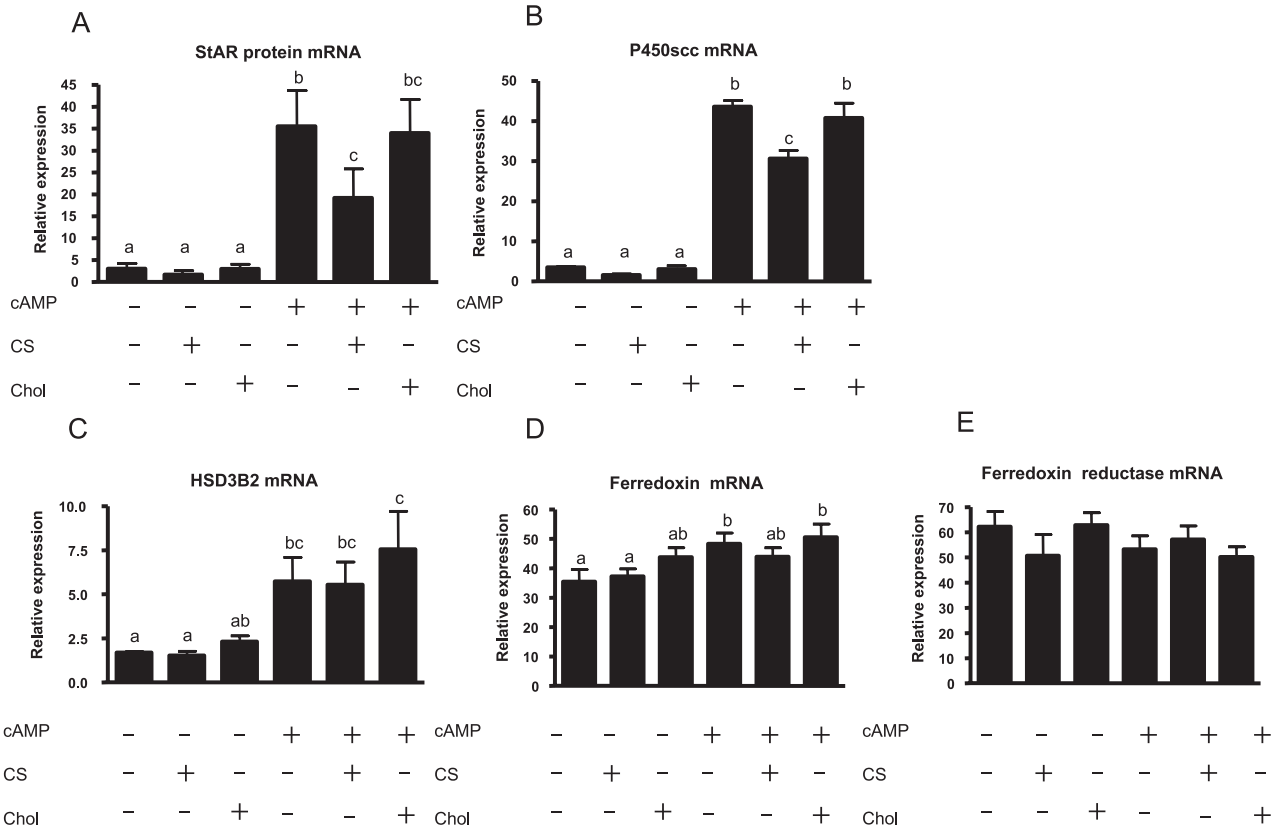

Fig. 2. Effects of CS, cholesterol and cAMP on expression of gene related with steroidogenesis. Total RNA was extracted from KGN cells at 24 h. A: StAR mRNA expression was determined by quantitative real-time RT-PCR. Relative expression was shown. B: Relative expression of P450scc mRNA determined by quantitative real-time RT-PCR. C: Relative expression of HSD3B2 mRNA determined by quantitative real-time RT-PCR. D: Relative expression of ferredoxin mRNA determined by quantitative real-time RT-PCR. E: Relative expression of ferredoxin reductase mRNA determined by quantitative real-time RT-PCR. Data are represented as means ± SEM for 4 independent experiments. Significant differences are indicated between matching letters ($P<0.05$)

ment of CS with cAMP significantly down-regulated the expression of StAR mRNA to 0.54-fold compared to treatment of cAMP. However, treatment of cholesterol with cAMP did not change the expression of StAR mRNA compared to that with the treatment of cAMP.

The expression of P450scc mRNA was up-regulated to 13-fold by cAMP treatment (Fig. 2B). Treatment of CS with cAMP significantly reduced the expression of P450scc mRNA to 0.6-fold compared to treatment of cAMP. But Cholesterol with cAMP treatment did not change the expression of P450scc mRNA compared to the treatment of cAMP.

The expression of HSD3B2 mRNA was significantly up-regulated to 3.4-fold by cAMP treatment (Fig. 2C). Treatment of CS with cAMP or cholesterol with cAMP did not change the expression of HSD3B2 mRNA compared to treatment of cAMP.

The expression of ferredoxin mRNA was up-regulated to 1.4-fold by cAMP treatment (Fig. 2D). Treatment of CS with cAMP or cholesterol with cAMP did not change the expression of ferredoxin mRNA compared to treatment of cAMP.

The expression of ferredoxin reductase mRNA did not change significantly by treatment of CS, cholesterol or cAMP (Fig. 2E).

Effect of CS, cholesterol and cAMP on StAR protein expression by western blot analysis is shown in Fig. 3. Treatment of CS with cAMP suppressed the expression of StAR protein compared to the treatment of cAMP.

Discussion

It was previously reported that CS had two different

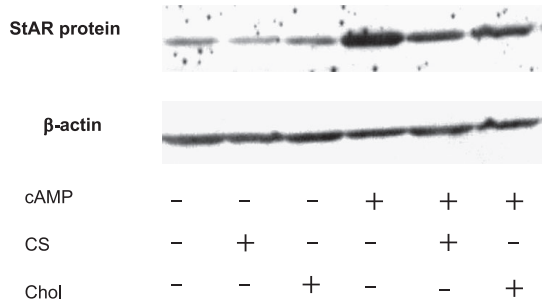

Fig. 3. Western blot analysis of StAR protein in KGN cell protein.
Treatment of CS with cAMP suppressed the expression of StAR protein compared to the treatment of cAMP. The experiments were repeated three times.

effects in steroidogenesis, a stimulatory effect and an inhibitory effect. Tuckey and Tuckey *et al.* reported that CS can serve as a substrate for the P450scc in placenta, adrenal glands and ovary, resulting in the formation of pregnenolone sulfate [5–8]. Lambeth *et al.* and Zu and Lambeth demonstrated that CS inhibits the cholesterol translocation from outer mitochondrial membrane to inner mitochondrial membrane where P450scc is located. CS does not inhibit the function of P450scc [9, 10]. In this study, we demonstrated that CS inhibited progesterone production in the human granulosa-like tumor cell line, KGN cells. Treatment of CS down-regulated the expression of StAR mRNA and P450scc mRNA. Western blot analysis confirmed that the amount of StAR protein was reduced by CS treatment. These data indicate that CS might have an inhibitory effect on ovarian steroidogenesis.

Cyclic AMP serves for the up-regulation of the StAR protein, P450scc and ferredoxin mRNA in the steroidogenic cells and stimulates progesterone production in the steroidogenic cells [13]. The present study showed similar results with those previously reported. StAR protein, one of the rate limiting steps in progesterone production, mediates translocation of cholesterol from the cholesterol-rich outer mitochondrial membrane to the cholesterol-poor inner mitochondrial membrane where the cholesterol side-chain cleavage enzyme is located [14, 15]. Mutations in the StAR gene cause congenital lipoid adrenal hyperplasia leading to impaired production of all steroids [16]. Srivastava *et al.* showed that ethanol reduced the FSH-induced E2 secretion and expression of StAR protein mRNA in rat granulosa cells [17]. Stocco reported that Prostaglandin F2, TGF-β, TNF, GnRH agonist and interleukin1β were negative regulators of StAR protein [18]. CS may be one of the negative regulators of StAR protein.

P450scc, another of the rate limiting enzymes in progesterone production, catalyzes the conversion of cholesterol into pregnenolone at the inner mitochondria membrane. In the present study we demonstrated that CS suppressed progesterone production by down-regulating the expression of StAR mRNA and P450scc mRNA. There are several rate limiting enzymes in progesterone production pathway as in other pathways. Our data showed significant decreases in StAR mRNA and P450scc mRNA by about 54% and 60%, respectively. However, production of progesterone was only 70%. Since the half-life of protein, mRNA and final product are different from each other, we think that it is not necessary that the rate of change in protein, mRNA and final product become congruent with each other.

It has been reported that CS is a ligand of retinoic acid related-orphan receptor alpha (RORalpha), as reported by Kallen *et al.* by inspection of the x-ray crystal structure of the RORalpha ligand binding domain in complex with CS [19, 20]. Kamalakaran reported a database for promoter analysis [21], that allows the user to search the potential target genes containing one or more listed transcription factor binding sites in their upstream elements. According to the database search, there are response elements of RORalpha in the promoter region of StAR protein, P450scc, ferredoxin and HSD3B2. In the present study CS down-regulated the expression of StAR mRNA and P450scc mRNA. It is possible that the inhibitory action of CS may be mediated by RORalpha, but further analysis is required.

We are led to suggestion that CS may function as a physiologic regulator of progesterone production in the ovary. We demonstrated that CS may be involved in suppressing progesterone production by down-regulating the expression of StAR mRNA and P450scc mRNA. CS is present in the rat ovary, in concentrations comparable to the rat adrenal gland [22]. CS concentrations may change, depending on the physiological state of the tissue. Dominguez *et al.* reported that *in vivo* ACTH treatment activated 3β,δ5-steroid sulfatase activity in rat adrenals [23]. CS synthesis in ovary may also change with menstrual cycle. We previously reported that CS increased in rabbit endometrium during implantation [3, 4]. Since the ovary receives vascular flow directly from the uterus, CS synthesized in endometrium may regulate progesterone production in

the ovary.

CS in the endometrium, however, may not directly affect progesterone production in the ovary since our data indicate it is increased during implantation period. Our data also suggest that CS down-regulates local progesterone production, which may apparently affect implantation environment. One possible explanation to this could be that CS may play a role to inhibit excessive progesterone production in the ovary. The precise mechanism in the possible relationship between endometrial CS and ovarian progesterone production has yet to be fully elucidated.

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