Cholesterol sulphate affects production of steroid hormones by reducing steroidogenic acute regulatory protein level in adrenocortical cells

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Abstract
Steroidogenic acute regulatory (StAR) protein plays a crucial role in the intramitochondrial movement of cholesterol, where P450 side chain cleavage enzyme resides. Cholesterol sulphate (CS), which is present ubiquitously in mammalian tissues, is not only a precursor of sulphated adrenal steroids but also an inhibitor of cholesterol biosynthesis. This study was designed to examine the biological roles of CS in steroidogenesis in adrenocortical cells. Human adrenocortical carcinoma H295R cells were cultured with various amounts of CS. To evaluate steroid hormone synthesis, pregnenolone production in cells was assayed. The amount of pregnenolone produced by H295R cells in culture medium, to which over 50 μg/ml CS was added, was significantly (P<0.05) decreased compared with that produced by control cells. Western blot analysis was performed to determine StAR protein level using whole cell extracts from cells. StAR protein level decreased when the concentration of CS in the medium was 50 μg/ml, whereas the level of glyceraldelyde-3-phosphate dehydrogenase did not change. To examine the mechanism by which CS gene expression is controlled, we performed RT-PCR and measured promoter activity in cells transfected with pGL3 StAR reporter constructs. StAR mRNA level and promoter activity were decreased in cells. The decrease in StAR protein level is a result of the low StAR gene expression level. In conclusion, CS affects the production of steroid hormones by reducing StAR protein level in adrenocortical cells.


Introduction
Steroid hormone biosynthesis from cholesterol requires several enzymatic steps. The first step of steroid hormone synthesis is the conversion from cholesterol to pregnenolone by cytochrome P450 side chain cleavage enzyme (P450scc). Since the intermembrane space of mitochondria is hydrophilic, a transport system is needed for the entry of cholesterol into the inner membrane, where P450scc resides. Steroidogenic acute regulatory (StAR) protein, which mediates the rate-limiting step in the synthesis of steroid hormones, plays a key role in the intramitochondrial movement of cholesterol (Stocco & Clark 1996, Strauss et al. 1999, Christenson & Strauss 2000).

Cholesterol exists in serum either bound to lipoprotein (LDL or HDL) or as cholesterol sulphate (CS). Steroid sulphatase (STS) is a membrane-bound protein in the endoplasmic reticulum, which catalyzes desulphation of CS and 3β-hydroxy-steroid sulphates, such as progesterone sulphate, dehydroepiandrosterone sulphate and estrone sulphate (Willemsen et al. 1988, Stein et al. 1989). STS hydrolyzes CS to provide free cholesterol in cells. STS is present ubiquitously in tissues, including steroid hormone-producing cells, ovary, testis, adrenal gland and placenta (Martel et al. 1994). It has been shown in previous studies that STS increases StAR protein level and stimulates steroid production in cells (Sugawara & Fujimoto 2004). Cholesterol including oxysterols is thought to enhance StAR protein translation to increase StAR protein function (Sugawara & Fujimoto 2004). CS has been reported to be an inhibitor of steroidogenesis in isolated rat adrenal mitochondria by inhibiting cholesterol movement in the mitochondria (Lambeth et al. 1987). STS has been suggested to play an important physiological role in steroidogenic cells, but the effect of CS on steroidogenesis remains unclear (Clemens et al. 2000).

Production of steroid hormones is rapidly increased by the stimulation of trophic hormones adrenocorticotropic, lutetinizing hormone and follicle-stimulating hormone (ACTH, LH and FSH). When trophic hormones bind to their cognate receptors, increase in intracellular cAMP levels results in activation of protein kinase A (PKA). This pathway involves a mechanism that increases transcription of genes encoding the enzymes of steroid biosynthesis (Stocco & Clark 1996). The promoter activity of human StAR gene is increased by cAMP stimulation (Sugawara et al. 1997, Sandhoff et al. 1998, Clark & Combs 1999). This study was
designated to examine the effects of CS on basal and cAMP-stimulated steroidogenesis in adrenocortical cells.

Materials and Methods

Cell culture

Human adrenocortical carcinoma H295R cells were grown in Dulbecco’s modified Eagle’s medium (DMEM)/F12 containing 2% ULTROSER G (BioSepra, Cergy-Pontoise, France) and 1% ITS Premix (Becton Dickinson and Co., Franklin Lakes, NJ, USA). Cultures of sub-confluent cells were plated so that 35 mm tissue culture dishes received equal numbers of cells. The culture medium was replaced with serum-free medium, and various concentrations of CS (0–200 μg/ml) were added to the medium. The cells were allowed to incubate for 48 h. Some cells were treated with Br-cAMP in the culture medium. At the end of the treatment period, the media were collected for hormonal assays. In some dishes, 22R-hydroxycholesterol (22-OH cholesterol; 5 μg/ml), which is a more soluble pregnenolone precursor and an intermediate in the cholesterol side chain cleavage reaction, was added to the culture medium.

Pregnenolone ELISA

Pregnenolone was measured in cell culture supernatant by ELISA using anti-pregnenolone polyclonal serum provided by Dr Yamazaki of the University of Hiroshima, Higashi-Hiroshima, Japan. ELISA was performed by the ‘sandwich’ method. Briefly, Immuno 96 MicroWell Plates (Nunc A/S, Kamstrupvej, Denmark) were coated with goat anti-rabbit IgG antibody (CHEMICON Inc., Temecula, CA, USA; 70 μg/ml, 100 μl/well) in 50 mM NaHCO3 buffer (pH 9.6) overnight at 4°C. Plates were washed thrice with PBS and blocked with 2% (w/v) skim milk for 1 h at 37°C. After the plates had been washed thrice with PBS, anti-pregnenolone serum (1:1000; 100 μl/well) in PBS was added to the wells and incubated for 2 h at 37°C. The plates were washed five times with PBS containing 0.05% Tween 20. Duplicate 50 μl samples or standards were incubated with 1 μg/ml pregnenolone-3-succinate-horseradish peroxidase in 50 μl PBS per well and incubated for 2 h at 37°C, followed by washing five times with PBS with 0.05% Tween 20. One hundred microlitres of peroxidase substrate solution (one ml PBS per well and incubated for 2 h at 37°C, followed by washing five times with PBS with 0.05% Tween 20. One hundred microlitres of peroxidase substrate solution (one tablet of 2,2′-azino-di-[3-ethylbenzthiazolone sulphonate (6') (ABTS); Roche) in 5 ml buffer containing ABTS (Roche) was added to the wells, and the reaction was allowed to proceed at room temperature for 30 min. The absorbance was measured at 405 nm. The limit of assay sensitivity was 0.5 ng/ml pregnenolone. The intra- and interassay coefficients of variation were 9-2% and 11% respectively.

Western blot analysis

Extracts of H295R cells were harvested with RIPA buffer (50 mM Tris–HCl, 1% Nonidet P-40, 0-1% deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0-1 mM phenylmethylsulphonyl fluoride and 1X proteinase inhibitor) after transfection. Ten micrograms of cell extract were subjected to 12% SDS-PAGE. After electrophoresis, the gels were transferred to polyvinylidene difluoride (PVDF) membranes for immunodetection with anti-StAR rabbit serum, anti-STS serum, anti-cytochrome C IgG, anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) IgG and anti-P450scc serum. Anti-STS serum was prepared by immunization of rabbits with human STS, as described previously (Sugawara et al. 1994). Dr Jerome F Strauss III of the Virginia Commonwealth University, Richmond, USA, generously provided human anti-StAR serum. Human anti-P450scc was a gift from Dr Toshihiro Tajima of the Hokkaido University School of Medicine, Sapporo, Japan. Anti-cytochrome C IgG was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), and anti-GAPDH IgG was from American Research Products Inc., (Belmont, MA, USA). The signal was detected by chemiluminescence using ECL or ECL Plus Western Blotting Detection Reagents (Amersham Pharmacia Biotech). The relative abundance of StAR protein levels was quantified using NIH Image 1.55 (Omhendorf Research Inc., Ottawa, IL, USA), normalized against levels of GAPDH protein levels and expressed as a percentage of the control value.

Transfection and luciferase assays

pGL2-Basic was purchased from Promega Corp. A luciferase reporter gene for the 1-3 kb human StAR promoter was used as the pGL2-1-3 kb StAR vector as described previously (Sugawara et al. 2001). A β-galactosidase expression vector (pCH110; Amersham Pharmacia Biotech) was used for normalization of luciferase data. National Cancer Institute (NCI)-H295R cells at 80–60% confluence were transfected with 0.5 μg pGL2 plasmid and 0.5 μg pCH110 using 4 μl Lipofectamine 2000 (Roche Molecular Biochemicals) per 1 μg DNA. pGL2-1-3 kb StAR plasmids and pCH110 were transfected into NCI-H295R cells. After 3 h, the culture medium was replaced with serum-free medium, and CS (100 μg/ml) was added to the medium. Some cells were treated with 8-Br-cAMP (1 mM) during the final 24 h of culture. Cells were harvested after a 48-h culture period, and the cell lysate was subjected to a luciferase assay. Luciferase assays were performed using a Luciferase Assay System (Promega). Each treatment group contained triplicate cultures, and each experiment was repeated thrice.

Reverse transcription PCR (RT-PCR)

Total RNA was isolated from H295R cells. cDNA synthesis was carried out at 37°C for 60 min using 150 pmol oligo dT as a primer, 1 μg total RNA and 200 units of SuperScript II Reverse Transcriptase (Life Technologies Inc./BRL). Twenty microlitres of reaction mixture for reverse transcriptase contained 50 mM Tris–HCl (pH 8-3), 75 mM KCl, 3 mM MgCl2, 20 mM...
dithiothreitol and 0.5 mM each of dATP, dCTP, dGTP and dTTP. Then, PCRs were carried out with 1 μl reverse transcription reaction product using the following oligonucleotide primers designed for amplification of StAR: sense, 5′-GCAGCAGACCGCGCCGACGACG-3′; antisense, 5′-CTCTTGGTGTGGCTAAGGAT-3′. Fifty microlitres of the PCR mixture contained 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 0.2 mM dNTPs and 10 pmol each of the primers. The reaction was subjected to 22 cycles of denaturing at 94 °C for 45 s, annealing at 55 °C for 45 s and extension at 72 °C for 1 min. RT-PCR for GAPDH (25 cycles) as a control was performed using the following primers for GAPDH: sense, 5′-TGCCGTCTAGAAAAACCTGC-3′; antisense, 5′-ACCCTGGTGCTGTAGCAGCAA-3′. The relative abundance of StAR mRNA expression was quantified using NIH Image 1.55 f (Ohlendorf Research, Inc., Ottawa, IL, USA), normalized against levels of GAPDH mRNA expression and expressed as a percentage of the control value.

**Data analysis**

Values are presented as means ± S.E.M. Significance in difference between experimental values was determined using Student’s unpaired t test, and one-way ANOVA was used to test differences in repeated measures across experiments. Significant results from ANOVA were further analyzed by Tukey’s post hoc test. P<0.05 was considered significant.

**Results**

To examine the effect of CS on the production of steroid hormones by cells, we assayed pregnenolone production, which is the first step of steroid hormone synthesis. When 50 μg/ml CS was added to the culture medium, the production of pregnenolone by H295R cells was significantly reduced 24-1% compared with that without the addition of CS. Pregnenolone concentration in the medium containing over 50 μg/ml CS was significantly decreased compared with that in the medium containing no CS. When 22-OH cholesterol was added to the culture medium, the production of pregnenolone increased, even in the culture media containing CS, indicating that pregnenolone synthesis in the presence of 22-OH cholesterol was not affected by CS treatment (Fig. 1).

We examined the time course of pregnenolone production in order to predict the mechanism by which CS affects steroidogenesis. We measured pregnenolone concentrations of medium samples that were collected at 24, 48 and 72 h after the addition of CS to the culture medium of H295R cells. Although pregnenolone concentration in the medium without and with CS (2 and 10 μg/ml) increased with the elapse of time, pregnenolone concentration reached a plateau at 48 h after the addition of 50 μg/ml CS to the culture medium (Fig. 2A). When 1 mM Br-cAMP was added to the culture medium, pregnenolone production in the cells increased, and the rate of increase in pregnenolone production with 50 μg/ml CS was significantly different from that without CS or with 2 or 10 μg/ml CS. At 48 h after the addition of CS to the culture medium, pregnenolone production with 50 μg/ml CS was significantly decreased from that without CS or with 2 or 10 μg/ml CS (Fig. 2B). Thus, the effect of CS on basal pregnenolone production appears in the early hours after the addition of CS to the culture medium.

To determine the effect of CS on StAR protein level, we performed western blot analysis of extracts from H295R cells. The StAR protein levels decreased with 50 μg/ml CS and the rate of decrease in pregnenolone production with 50 μg/ml CS was significantly decreased from that without CS or with 2 or 10 μg/ml CS (Fig. 3A). When 50 μg/ml CS was added to the culture medium, the level of mature StAR protein (30 kDa) was significantly decreased compared with that with the addition of CS. When the medium contained over 50 μg/ml CS, the level of mature StAR protein (30 kDa) was significantly decreased compared with that in the medium containing no CS. The level of pre-StAR protein (37 kDa) was also significantly decreased with that in the medium without CS when CS concentration in the culture medium was over 100 μg/ml (Fig. 3B).

STS is an endoplasmic reticulum protein, and its substrate is CS. Cytochrome C is present in the mitochondria matrix and is associated with apoptosis. P450scc is a steroidogenic enzyme and it cleaves the side chain of cholesterol and synthesizes pregnenolone from cholesterol. To determine whether the effect of CS on StAR protein level is specific, and whether the decrease in steroid hormone production depends on StAR protein, we performed western blot analysis using StAR, STS, cytochrome C and GAPDH antibody. Although the StAR protein level clearly decreased, the protein levels of
STS, cytochrome C and GAPDH did not change when H295R cells were cultured in the medium containing 200 μg/ml CS (Fig. 4A). The protein level of P450scc increased in response to Br-cAMP stimulation. P450scc protein level did not change in the presence of CS, and the protein level increased in response to Br-cAMP stimulation (Fig. 4B). CS had no effect on P450scc protein level, and the effect of CS is specific to StAR protein level.

To clarify the effect of CS on steroidogenesis, RT-PCR was performed to examine the effect of CS on StAR gene expression. The basal StAR gene expression level in H295R cells in the medium containing 50 μg/ml CS was decreased. Although StAR gene expression level increased with Br-cAMP stimulation, StAR gene expression level in H295R cells also decreased in the culture medium containing CS (Fig. 5A). When the medium contained 50 μg/ml CS, the basal StAR gene expression level was significantly decreased compared with that in the medium containing no CS. The cAMP-stimulated StAR gene expression level was also significantly decreased compared with that in the medium without CS, when CS concentration in the culture medium was 100 μg/ml. CS had an effect on both the basal StAR gene expression and the cAMP-stimulated StAR gene expression (Fig. 5B).

To confirm that CS has an effect on StAR gene expression, we analyzed StAR promoter activity of H295R cells with

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**Figure 2** Time courses of pregnenolone production in basal and cAMP-stimulated conditions in the presence of CS. Cultures of subconfluent NCI-H295R cells were plated so that 35 mm culture dishes received equal numbers of cells. The next day the culture medium was replaced with serum-free medium, and CS (0–50 μg/ml) was added to the medium. Cells were incubated without (A) and 8-Br-cAMP (1 mM) (B). Culture medium samples were collected at various times and kept at –80 °C until assay of pregnenolone concentration. Values presented are the means ± S.E.M. of pregnenolone production from three separate experiments. a–dGroups with different letters are significantly different (P<0.05) from each other.

**Figure 3** Effect of CS on StAR protein level in cells. NCI-H295R cells were plated onto 35 mm culture dishes. The next day the culture medium was replaced with serum-free medium, and CS (0–200 μg/ml) was added to the medium. After 24 h, extracts were harvested for western blot analysis. Ten micrograms of cell extract were then subjected to 12% SDS-PAGE. After electrophoresis, the gels were transferred to PVDF membranes for immunodetection with anti-StAR serum and anti-GAPDH IgG. The signal was detected by chemiluminescence using ECL western blotting detection reagents. (B) The abundance of StAR protein was quantified by densitometric scanning. StAR pre-protein (37 kDa) and mature protein (30 kDa) abundance ratios are expressed relative to the amount of GAPDH protein in cells. Values are expressed as percentages of the control. Results for StAR protein levels are presented as means ± S.E.M. from three separate experiments. a–dGroups with different letters are significantly different (P<0.01) from each other.
CS in the culture medium. Although the StAR promoter activity is stimulated by Br-cAMP regardless of the presence of CS, the basal and cAMP-stimulated StAR promoter activity levels were significantly decreased when the culture medium contained 50 ng/ml CS (Fig. 6A). Thus, CS affects the StAR promoter activity. To determine whether the effect of CS on StAR gene expression is specific, we assayed the StAR promoter activity of cells in a culture medium to which 25-hydroxycholesterol (25-OHC) and lipoprotein-containing cholesterol had been added. Although the StAR promoter activity level did not decrease in cells in the medium containing 25-OHC and lipoprotein, the promoter activity level of StAR was significantly decreased compared with that of the control in both basal and cAMP stimulation conditions (Fig. 6B). CS has a specific effect on StAR promoter activity, and the decrease in StAR gene expression level is a result of the decrease in StAR gene promoter activity. CS affects the StAR gene expression by controlling StAR promoter activity. The decrease in pregnenolone production results from the decreased StAR protein level, and the decrease in StAR protein level results from the control of StAR gene expression.

Figure 4 Specific effect on StAR protein level in the presence of CS. NCI-H295R cells were cultured with serum-free medium containing CS (0–200 μg/ml). After 48 h, cell extracts were collected for western blot analysis. Immunodetection was performed with anti-StAR serum, anti-STS serum, anti-cytochrome C IgG, anti-GAPDH IgG (A) and P450scC serum (B). Some cells were treated with Br-cAMP (1 mM and 2 mM) during the final 24 h of culture. The experiment was repeated thrice.
Discussion

CS is a substrate of STS and is desulphated into cholesterol. Synthesis of cholesterol from CS is different from *de novo* synthesis of cholesterol in the endoplasmic reticulum, and the synthesized cholesterol is redistributed to all parts of the cell (Schorderet *et al.* 1988). Overexpression of STS results in an increase in StAR protein level and an increase in the production of steroid hormones (Sugawara & Fujimoto 2004). StAR mRNA levels were quantified by densitometric scanning. Results were normalized for the amount of GAPDH, expressed as a percentage of the control. The values for StAR mRNA levels are presented as means ± S.E.M. from three separate experiments. a–d Groups with different letters are significantly different (P < 0.05) from each other.

**Figures 5 and 6**

**Figure 5** StAR gene expression in NCI-H295R cells in culture medium with CS. RT-PCR was performed using mRNA extracted from NCI-H295R cells. NCI-H295R cells were cultured with serum-free medium containing CS (0–200 μg/ml) for 48 h. Cells were treated with 8-Br-cAMP (1 mM) during the final 24 h of culture or not treated with 8-Br-cAMP. The sizes of PCR products were 347 bp (StAR) and 231 bp (GAPDH). (B) The StAR mRNA expression levels were quantified by densitometric scanning. Results were normalized for the amount of GAPDH, expressed as a percentage of the control. The values for StAR mRNA levels are presented as means ± S.E.M. from three separate experiments. a–d Groups with different letters are significantly different (P < 0.05) from each other.

**Figure 6** StAR promoter activity in NCI-H295R cells. (A) pGL2-1.3 kb StAR plasmids and pCH110 were transfected into NCI-H295R cells. Some cells were treated with 8-Br-cAMP (1 mM). The cell lysate was subjected to a luciferase assay. The results are presented as means ± S.E.M. from three experiments. (a–d) Groups with different letters are significantly different (P < 0.05) from each other. (B) The human 1.3 kb StAR promoter and pCH110 were transfected into NCI-H295R cells. After 3 h, the culture medium was replaced with serum-free medium, and CS (50 μg/ml), 25-hydroxycholesterol (50 μg/ml) and lipoprotein (containing 50 μg/ml cholesterol) were added to the medium. Some cells were treated with 8-Br-cAMP (1 mM) during the final 24 h of culture. Cells were harvested after a 48-h culture period, and the cell lysate was subjected to a luciferase assay. The results are presented as means ± S.E.M. from three experiments. **Significantly different from basal promoter activity of control. ***,**, Significantly different from promoter activity of control with 1 mM 8-Br-cAMP. ***,**, P < 0.01.
STS, SCC and GAPDH, did not change. The results showing that the production of pregnenolone was not reduced by CS treatment in the presence of 22-OH cholesterol, a membrane-permeable form of cholesterol, support the specific effects of CS to StAR protein. The specific effect of CS on the StAR gene is a result of an effect on the StAR gene promoter activity in cells. Therefore, the decrease in pregnenolone production results from the decreased StAR protein level, and the decrease in StAR protein results from the control of StAR gene expression.

Eukaryotic cells have membranes composed of phospholipids and proteins. Cholesterol and CS are also important components of cell membranes. CS has been shown to protect red blood cells against osmotic lysis and function as a membrane-stabilizing agent at physiological concentrations. The effect of CS on cell membranes requires an amphipathic property linked to the sulphate group at the 3 position of the sterol ring and the sterol side chain (Strott & Higashi 2003). On the contrary, a high concentration of CS added to a culture mixture of STS, GAPDH, cytochrome C and P450(20) were not changed by the addition of CS to the culture medium, StAR protein synthesis was affected by CS. StAR protein is synthesized in the cytosol and is imported into the mitochondria matrix (Reed et al. 2005). StAR protein could not undergo its proper protein processing, resulting in its degradation in the cell, because the mitochondrial membrane may have been affected by CS and resulted in impairment of StAR protein import to the mitochondria matrix. This also seems to be one of causes of the decrease in StAR protein level.

Sterol sulphate can easily be transported from serum to cells through cell membranes by the organic anion transporting polypeptide-B (Reed et al. 2005). Nuclear receptors are ligand-dependent transcription factors that play important roles in many biological functions (Mangelsdorf et al. 1995). Recently, ligands of retinoic acid receptor-related orphan receptor α (RORα), a previously reported orphan receptor, have been found. Cholesterol and cholesterol derivatives have been reported to be natural ligands (Kallen et al. 2002, Bitsch et al. 2003). CS has also been suggested from X-ray determination of its crystal structure to be a ligand of RORα (Kallen et al. 2004). The 5′ upper regions of human StAR gene have been reported to contain several response elements of transcription factors (Strauss et al. 1999). In the present study, even when CS was added to the culture medium, the promoter activity of StAR was decreased. CS has been shown not only to inhibit cholesterol esterification and HMG-CoA reductase activity and to modulate intracellular cholesterol level (Williams et al. 1985), but also to regulate promoter activity of the StAR gene. RORα has been shown to be associated with regulation of plasma cholesterol levels via apolipoprotein (Vu-De et al. 1997, Raspe et al. 2001), and its expression is present in several organs, including the testis (Steimnay et al. 1998, Boukhitouche et al. 2004). CS is not only a precursor of steroid hormones but also seems to be associated with tissue-specific signal transduction and differentiation.

CS decreases synthesis of steroid hormones by reducing StAR protein level. Further study is needed to clarify the important roles of CS in steroidogenic cells.

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