The potential function of steroid sulphatase activity in steroid production and steroidogenic acute regulatory protein expression

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The first step in the biosynthesis of steroid hormones is conversion of cholesterol into pregnenolone. StAR (steroidogenic acute regulatory) protein plays a crucial role in the intra-mitochondrial movement of cholesterol. STS (steroid sulphatase), which is present ubiquitously in mammalian tissues, including the placenta, adrenal gland, testis and ovary, desulphates a number of 3β-hydroxysteroid sulphates, including cholesterol sulphate. The present study was designed to examine the effect of STS on StAR protein synthesis and steroidogenesis in cells. Steroidogenic activities of COS-1 cells that had been co-transfected with a vector for the cholesterol P450scc (cytochrome P450 side-chain-cleavage enzyme) system, named F2, a StAR expression vector (pStAR), and an STS expression vector (pSTS) were assayed. Whole-cell extracts were subjected to SDS/PAGE and then to Western blot analysis. pSTS co-expressed in COS-1 cells with F2 and pStAR increased pregnenolone synthesis 2-fold compared with that of co-expression with F2 and pStAR. Western blot analysis using COS-1 cells that had been co-transfected with pSTS, F2 and pStAR revealed that StAR protein levels increased, whereas STS and P450scc protein levels did not change. The amount of StAR protein translation products increased when pSTS was added to an *in vitro* transcription–translation reaction mixture. Pulse–chase experiments demonstrated that the 37 kDa StAR pre-protein disappeared significantly ($P < 0.01$) more slowly in COS-1 cells that had been transfected with pSTS than in COS-1 cells that had not been transfected with pSTS. The increase in StAR protein level is not a result of an increase in StAR gene expression, but is a result of both an increase in translation and a longer half-life of the 37 kDa pre-StAR protein. In conclusion, STS increases StAR protein expression level and stimulates steroid production.

Key words: cholesterol sulphate (CS), MLN64, steroid hormone, steroidogenic acute regulatory protein (StAR protein), steroid sulphatase (STS).

INTRODUCTION

Steroid hormone biosynthesis from cholesterol requires several enzymic steps. The first step of synthesis is the conversion from cholesterol into pregnenolone by P450scc (cytochrome P450 side-chain-cleavage enzyme). Free cholesterol is transported to the outer mitochondrial membrane by cytoskeletal components, such as microtubules, microfilaments and intermediate filaments [1]. The intra-membrane space of mitochondria is hydrophilic, and a transport system is needed for entry of cholesterol into the inner membrane, where P450scc resides. StAR (steroidogenic acute regulatory) protein plays a key role in the intra-mitochondrial movement of cholesterol [1]. After StAR gene cloning had been performed, the limiting step was thought to be StAR protein [2,3]. It has been proven that the StAR gene is a candidate gene for congenital lipoid adrenal hyperplasia, and it has been clarified that StAR protein is the true rate-limiting step in the synthesis of steroid hormones [3]. StAR gene expression has been reported not only in steroid-hormone-producing cells, ovary, testis and adrenal gland, but also in the brain [3]. StAR protein is derived from a 37 kDa pre-protein that is synthesized in the cytoplasm and imported into mitochondria, where it is processed into the 30 kDa mature protein [1].

STS (steroid sulphatase) is a membrane-binding protein in the endoplasmic reticulum, which desulphates a number of 3β-hydroxysteroid sulphates [4]. The natural substrates of STS are several sterol sulphates, such as CS (cholesterol sulphate), pregnenolone sulphate, dehydroepiandrosterone sulphate and oestrone sulphate [5]. Cholesterol, due to its strong hydrophobic property, exists in serum either bound to lipoprotein [LDL (low-density lipoprotein) or HDL (high-density lipoprotein)] or as CS. CS can, by virtue of its amphipathic nature, act as a detergent and form micelles in serum. STS is present ubiquitously in tissues, including steroid-hormone-producing cells: ovary, testis, adrenal gland and placenta [6]. The StAR gene is not expressed in the placenta, although many steroid hormones are produced in the placenta to maintain pregnancy [7]. The MLN64 gene, which has a StAR homologue domain, is expressed in the placenta instead of StAR gene expression [8,9]. STS produces free cholesterol, which the StAR protein transports into the mitochondria of steroid-hormone-producing cells. STS has been suggested to play important physiological and pathophysiological roles in steroid-hormone-producing cells, including those in the ovary, but the effect of STS on steroidogenesis remains unclear [10]. The present study was designed to examine the effect of STS on StAR protein levels and steroidogenesis in cells.

MATERIALS AND METHODS

Plasmid constructs

The human StAR cDNA encoding the full length of StAR protein (pStAR) was cloned into pSV · SPORT 1 (Life Technologies/BRL, Washington, DC, U.S.A.) as described previously [7]. The STS

Abbreviations used: CS, cholesterol sulphate; DHEAS, 7-dehydroepiandrosterone sulphate; DMEM, Dulbecco's modified Eagle's medium; FCS, foetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HDL, high-density lipoprotein; LDL, low-density lipoprotein; P450scc, cytochrome P450 side-chain-cleavage enzyme; SREBP, sterol-element-binding protein; StAR, steroidogenic acute regulatory; START, StAR-related lipid transfer; STS, steroid sulphatase; TNT®, transcription/translation.

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expression vector (pSTS) was prepared by inserting an *Eco*RI fragment from human STS cDNA into pSV · SPORT 1 [11]. The human P450scc–adrenodoxin reductase–adrenodoxin fusion enzymes of the cytochrome P450scc system, called the F2 system, were kindly provided by Dr Walter L. Miller (Department of Pediatrics, University of California, San Francisco, CA, U.S.A.) [12]. The human MLN64 cDNA expression vector in pCMV5 (pMLN64) was generously provided by Dr Hidemichi Watari (Department of Obstetrics and Gynecology, University of Pennsylvania, Philadelphia, PA, U.S.A.) [13].

Cell culture

Monkey kidney COS-1 cells were obtained from RIKEN Cell Bank (Tsukuba, Japan). The COS-1 cells were cultured in DMEM (Dulbecco's minimum essential medium) supplemented with 10 % FCS (foetal calf serum) and 50 μg/ml gentamycin, and maintained at 37 °C in an atmosphere containing 5 % CO_2. Cultures of sub-confluent COS-1 cells were plated so that 35-mm-diameter tissue-culture dishes received equal numbers of cells.

Transfection

COS-1 cells were transfected with F2, pSTS and pStAR or pMLN64 using FuGENE 6 (Roche Molecular Biochemicals, Mannheim, Germany) as described previously [11]. COS-1 cells were cultured in a medium containing 10 % charcoal-stripped FCS (Sigma-Aldrich, St. Louis, MO, U.S.A.) in some experiments. The cells were allowed to incubate for 48 h. Some dishes were treated with CS during the final 24 h of culture. At the end of the treatment period, the medium was collected for evaluation of steroidogenic activity. Cells were harvested for assay of STS enzyme activity and Western blotting. The cells were washed twice with PBS, and cell extracts were prepared in 200 μl of lysis buffer [10 mM Tris/HCl, 1 % Triton X-100 and 1 × proteinase inhibitor (Roche Molecular Biochemicals)]. Protein concentration was determined using a BCA (bicinchoninic acid) protein assay kit (Pierce, Rockford, IL, U.S.A.). Each experiment was performed on three separate occasions with different cell preparations to confirm the generality of the findings.

Immunoassay of pregnenolone

The steroidogenic activities of COS-1 cells that had been co-transfected with the P450scc system and various kinds of expression vectors were evaluated. Each experiment included triplicate cultures for each treatment group. At 48 h after transfection, the medium was collected for RIA of pregnenolone as described previously [7].

STS enzyme activity

STS activity was assayed by the method described previously [11]. The reaction mixture, which contained 20 mM Tris/HCl (pH 8.0), 50 000 d.p.m. of ^{3}H-labelled DHEAS (7-dehydroepiandrosterone sulphate) (16 Ci/mmol; New England Nuclear, Boston, MA, U.S.A.), 10 nmol of unlabelled DHEAS, 1 % Triton X-100 and 25 μl of an enzyme source, was incubated at 37 °C for l h. At the end of incubation, desulphated DHES was extracted by the addition of 1 ml of benzene. Then 0.6 ml of benzene was added to 3 ml of a scintillation cocktail, and radioactivity was measured by a liquid-scintillation counter. One unit of activity was defined as the amount of enzyme that catalyses the hydrolysis of 1 pmol of DHEAS/h per mg.

Western blot analysis

COS-1 cell extracts were harvested for Western blot analysis after transfection. Cell extract (10 μg) was then subjected to SDS/12 % PAGE [14]. After electrophoresis, the gels were transferred on to nitrocellulose membranes for immunodetection with rabbit anti-StAR serum, anti-STS serum and anti-P450scc serum. Anti-STS serum was prepared by immunization of rabbits with human STS, as described previously [15]. Dr Jerome F. Strauss III (Department of Obstetrics and Gynecology, University of Pennsylvania, Philadelphia, PA, U.S.A.) generously provided human anti-StAR serum. Human anti-P450scc was a gift from Dr Toshihiro Tajima of the Department of Pediatrics, Hokkaido University School of Medicine, Sapporo, Japan. Rabbit polyclonal anti-StAR, anti-STS and anti-P450scc were used as primary antibodies at dilutions of 1:1000, 1:1000 and 1:1000 respectively. After incubation for 1 h at 25 °C, the blot was incubated for a further 60 min at 25 °C with horseradish-peroxidase-conjugated affinity pure goat anti-rabbit antibody (Roche Molecular Biochemicals) at a dilution of 1:10 000. The signal was detected by chemiluminescence using ECL® (enhanced chemiluminescence) Western blotting detection reagents (Amersham Biosciences, Uppsala, Sweden). The membrane was then exposed to Fuji RX-U film (Fuji, Tokyo, Japan). Luminescence detection was quantified using Kodak Digital Science EDAS290 (Eastman Kodak, Rochester NY, U.S.A.).

Northern blot analysis

The COS-1 cells were cultured for 1 day after being subcultured and were then transfected with various amounts of F2, pStAR or pSTS using FuGENE 6. The protocols for the preparation, culture and isolation of total RNA from COS-1 cells were described in detail previously [11]. For Northern blot analysis, 30 μg of total RNA from each dish was separated by electrophoresis and transferred on to a nylon membrane (Biodyne, ICN, Glen Cove, NY, U.S.A.). Detection of the signal was performed in accordance with the standard protocol for the nucleic acid detection kit (Roche Molecular Biochemicals). Northern blots were probed with human StAR cDNA. Each blot was stripped and hybridized with a probe specific for mouse β-actin cDNA.

Preparation of a cDNA probe

The human StAR cDNA in pSport-1 was linearized with *Sal*I. The mouse β-actin cDNA was prepared with a RT (reverse transcriptase)-PCR primer set (Toyobo, Tokyo, Japan) by using PCR. Mouse β-actin was subcloned into the pCR2 vector using a TA cloning kit (Invitrogen, Carlsbad, CA, U.S.A.). The mouse β-actin cDNA was linearized with *Hin*dIII. Digoxigenin-labelled human StAR and mouse β-actin RNA probes were produced by *in vitro* transcription with T7 RNA polymerase and an RNA labelling kit (Roche Molecular Biochemicals).

In vitro translation

StAR and STS proteins were synthesized *in vitro* using an Sp6 RNA polymerase-based TNT® (transcription/translation)-coupled reticulocyte lysate system (Promega, Madison, WI, U.S.A.) according to the manufacturer's manual. F2 system (500 ng) and various amounts of the pSTS (0.1 or 0.5 μg) or pStAR (0.1 μg) expression vector were mixed with *in vitro* translation reaction mixtures. The translation reactions were performed with Sp6 TNT® RNA polymerase for 90 min of incubation at 30 °C. To 10 μl of *in vitro* translated products, 10 μl

of 2× SDS sample buffer was added, before heating for 5 min. Samples were then subjected to SDS/(12 %) PAGE and Western blot analysis.

Pulse–chase experiments

COS-1 cells were transfected with pStAR (0.1 μg) and pSTS (0.5 μg) 1 day before metabolic labelling. COS-1 cells were incubated with methionine-free DMEM containing CS (25 μg/ml) for 15 min and then labelled with [^{35}S]methionine (0.4 mCi/ml) for 30 min. After labelling, the radioactive medium was replaced with DMEM containing 4 mM methionine for the indicated time. Cells were washed with PBS and scraped into 400 μl of 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 PMSF and 1× proteinase inhibitor). Aliquots of protein from cell extracts were pre-cleared with 20 μl of Protein G–Sepharose (Amersham Biosciences) for 30 min at 4 °C. After centrifugation at 200 ***g*** for 1 min, supernatants were incubated with 1 μl of anti-StAR serum or anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase; American Research Products, Belmont, MA, U.S.A.) for 3 h at 4 °C and then overnight on a rocking platform with 20 μl of Protein G–Sepharose at 4 °C. Immunocomplexes were washed four times by resuspension in 500 μl of RIPA buffer and collected by centrifugation. Pellets were resuspended in 10 μl of 2× SDS sample buffer and were then subjected to SDS/PAGE. Gels were dried and exposed to Fuji RX-U film. Quantitative analysis of StAR protein levels was achieved using the Kodak Digital Science Electrophoresis Documentation and Analysis System EDAS290.

RESULTS

Effect of STS on steroid hormone production

To examine the effect of STS on steroid hormone production, we transfected the human STS expression vector with the human P450scc system and/or the human StAR expression vector into COS-1 cells, which do not express endogenous P450scc, StAR or STS protein, or produce steroid hormones [16]. To evaluate steroid-hormone-producing activity, we assayed pregnenolone production, which is the first step of steroid hormone synthesis. After transfection with F2 (0.5 μg/plate), COS-1 cells produced pregnenolone (8.4 $\pm$ 1.3 ng/dish). After co-transfection with F2 (0.5 μg/plate) and pStAR (0.1 μg/plate), the amount of pregnenolone produced by COS-1 cells increased (62 $\pm$ 13 ng/dish). After co-transfection with F2 (0.5 μg/plate), pStAR (0.1 μg/plate) and pSTS (0.5 μg/plate), the amount of pregnenolone produced by COS-1 cells (123 $\pm$ 21 ng/dish) was significantly ($P < 0.05$) greater than that produced by COS-1 cells that had been co-transfected with F2 and pStAR (Figure 1). However, the amount of pregnenolone produced by COS-1 cells that had been co-transfected with F2 and pSTS was not greater than that produced by COS-1 cells that had been transfected only with F2. To determine the dose-dependent effect of STS, various amounts of pSTS were transfected into COS-1 cells with F2 (0.5 μg/plate) and pStAR (0.1 μg/plate). The amounts of pregnenolone produced by COS-1 cells that had been transfected with pSTS and the amounts produced by COS-1 cells not transfected with pSTS were compared (Figure 2A). STS enzyme activity levels (Figure 2B) and levels of STS protein (Figure 2C) in COS-1 cells that had been transfected increased with increase in the amount of pSTS. The amounts of pregnenolone produced by COS-1 cells increased 1.3-, 2.0- and 4.2-fold when the cells were transfected with 0.1 μg, 1 μg and 5 μg of pSTS respectively.

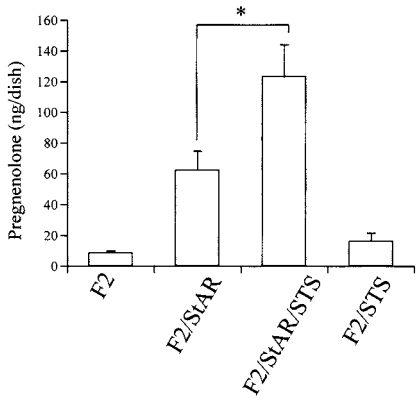

Figure 1 Effects of STS on pregnenolone production by COS-1 cells transfected with pStAR

COS-1 cells were co-transfected with the human cytochrome P450scc system, called F2, and pStAR and pSTS expression plasmids with FuGENE 6. The media were collected 48 h after transfection, and the amounts of pregnenolone were determined by RIAs. The results of three separate experiments are presented. Results are means $\pm$ S.E.M. of pregnenolone concentration in the medium. *$P < 0.05$ for pregnenolone production compared with that by cells co-transfected with F2/StAR and that by cells co-transfected with F2/StAR/STS.

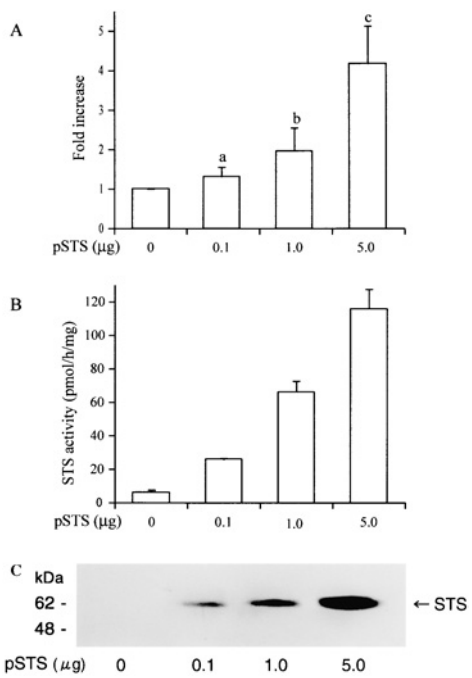

Figure 2 Dose-dependent effect of STS on pregnenolone production

(**A**) Stimulation of pregnenolone production with increasing amounts of pSTS expression plasmid. COS-1 cells were co-transfected with the F2 cytochrome P450scc system, StAR and increasing amounts of pSTS expression vectors. Results are the means $\pm$ S.E.M. of fold induction of pregnenolone production, expressed as fold of pregnenolone production with an empty vector instead of the pSTS expression vector. Significant difference ($P < 0.05$) in fold increase for a compared with b, and b compared with c. (**B**) STS activity was assayed, and (**C**) Western blot analysis was performed with COS-1 cell extracts using anti-STS. The results of three separate experiments in which each treatment group contained three replicate cultures are presented.

Thus the amount of pregnenolone produced increased significantly with an increase in the level of STS enzyme activity. To determine the effect of STS on the F2 steroid-producing system, CS (25 μg/ml), which is a natural substance of STS,

Table 1 Increases in pregnenolone production by cells incubated with CS

CS (25 μg/ml) was added to the culture medium. Results are means ± S.E.M. of pregnenolone production for three separate experiments. Fold induction is expressed as the fold increase in pregnenolone production with CS. *, † $P < 0.05$.

Treatment	Pregnenolone (ng/dish)	Fold induction
F2	17.6 ± 2.8	
+ CS	13.7 ± 2.7	0.75 ± 0.06*
+ pStAR	75.0 ± 8.2	
+ CS	77.8 ± 2.1	0.98 ± 0.14†
+ pSTS	22.0 ± 3.8	
+ CS	29.6 ± 3.5	1.50 ± 0.24*
+ pStAR	110 ± 11	
+ CS	146 ± 13	1.34 ± 0.03†

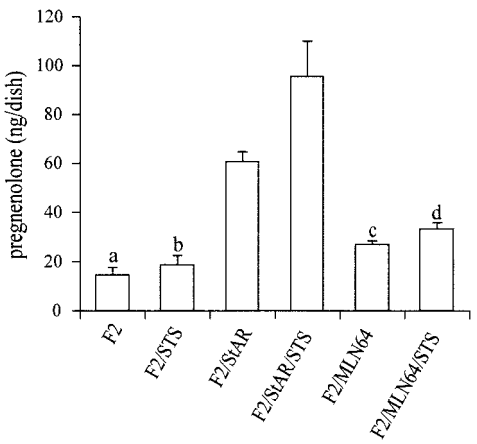

Figure 3 The StAR homologue MLN64 stimulates steroidogenesis with STS

COS-1 cells were co-transfected with F2 and STS expression plasmids, including the StAR homologue MLN64 expression plasmid. The results of separate experiments in which each treatment group contained triplicate cultures are presented as means ± S.E.M. of pregnenolone concentrations in the medium. Significant difference ($P < 0.05$) in pregnenolone production for a compared with c, and c compared with d. Means were not significantly different for a compared with b.

was added to the culture medium (Table 1). The rates of pregnenolone production by COS-1 cells with either F2 or F2 + pStAR did not differ significantly when CS was present in the medium. STS is necessary for CS to elicit its effect on the production of steroid hormones. Indeed, the increase in rate of pregnenolone production by COS-1 cells with CS present in the medium was significantly ($P < 0.05$) different in the presence of STS (F2 + pSTS or F2 + pStAR + pSTS) from in the absence of STS (F2 or F2 + pStAR).

StAR homologue MLN64 stimulates steroidogenesis with STS

MLN64, which is a homologue of the StAR gene, stimulates activity in cells to produce pregnenolone [8]. The amount of pregnenolone produced by COS-1 cells into which both F2 and MLN64 had been introduced was significantly (Figure 3, a compared with c; $P < 0.05$) greater than that by COS-1 cells into which only F2 had been introduced. A comparison of the amounts of pregnenolone produced by COS-1 cells into which F2 and MLN64 had been introduced and COS-1 cells into which only F2 had been introduced showed that the rate of increase in the production of pregnenolone was 187%. The amount of pregnenolone produced by COS-1 cells into which F2, MLN64 and pSTS had been transfected was significantly (Figure 3, b compared with d, $P < 0.05$) greater than that produced by COS-1 cells into which only F2 and pSTS had been transfected. The amount of pregnenolone produced by COS-1 cells into which F2, MLN64 and STS expression vector had been introduced was significantly (Figure 3, c compared with d, $P < 0.05$) greater than that produced by COS-1 cells into which only F2 and MLN64 had been introduced. To determine the dose-dependent effect of STS on the production of the steroid hormone by MLN64, 0.1 μg and 1 μg of pSTS were introduced into COS-1 cells, and the rates of increase in pregnenolone production were compared. The amounts of pregnenolone produced by COS-1 cells that had been co-transfected with F2, with F2 and pStAR, and with F2 and pMLN64 increased 0.9-, 2.2- and 1.5-fold respectively. The rate of increase in the amount of pregnenolone produced by COS-1 cells into which F2 and pMLN64 had been introduced was significantly ($P < 0.01$) greater than that of pregnenolone produced by COS-1 cells into which only F2 had been introduced (Table 2). Thus the effect of STS is elicited when StAR protein or a StAR protein homologue exists in cells.

Table 2 Effects of STS on the production of pregnenolone

Results are means ± S.E.M. of fold increase in pregnenolone production for three separate experiments, expressed as relative pregnenolone production with 1 μg of pSTS vector to that with 0.1 μg of pSTS vector introduced into COS-1 cells. *, † $P < 0.01$.

Treatment	Fold increase in pregnenolone production [pSTS (1 μg)/pSTS (0.1 μg)]
F2 + pSTS	0.90 ± 0.03*†
F2 + pStAR + pSTS	2.2 ± 0.31*
F2 + pMLN64 + pSTS	1.53 ± 0.13†

Effect of STS on StAR protein expression

To examine the effect of STS on StAR protein levels, we performed Western blot analysis of extracts from cells into which various kinds of plasmid had been transfected. CS was added to some of the COS-1 cell-culture medium. The cytochrome P450 expression level in COS-1 cells was the same regardless of the kind of plasmid that had been co-transfected into COS-1 cells (Figure 4A). The levels of both pre-StAR protein (37 kDa) and mature StAR protein (30 kDa) in COS-1 cells that had been transfected with pSTS were significantly higher than those in COS-1 cells that had not been transfected with pSTS. When CS was added to the culture medium, the level of StAR protein increased significantly (Figure 4B). The amount of pregnenolone produced by COS-1 cells into which F2, pSTS and pStAR had been transfected was dependent on the level of StAR protein. To examine the dose-dependent effect of CS, increasing amounts of CS were added to a culture medium containing 10% charcoal-stripped FCS. pStAR (0.1 μg/ml) was co-transfected into COS-1 cells with or without pSTS (0.1 μg/ml). The increase in the level of StAR protein depended on the amount of cholesterol sulphate in the culture medium. The pre-protein levels increased in COS-1 cells transfected with pSTS (Figure 4C). To determine the effect of STS on StAR protein-expression processes, we examined the efficiency of plasmid expression using *in vitro* transcription/translation. Translation products were subjected to SDS/PAGE and then to Western blot analysis. The amounts of StAR translation products increased when pSTS was added to the

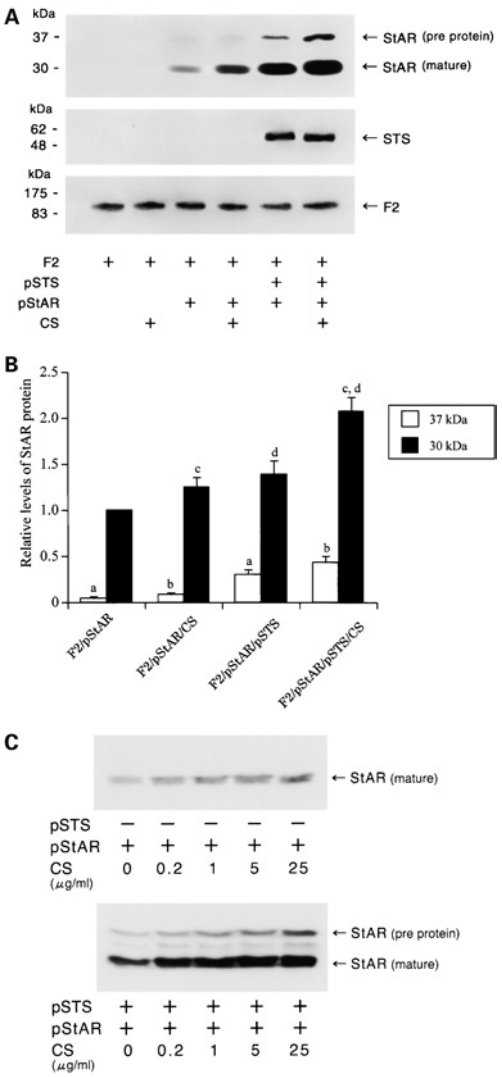

Figure 4 Effects of STS and CS on StAR protein expression

(**A**) COS-1 cells were transfected with the indicated plasmids. CS was added to some of the culture media. The cells were collected from each dish by scraping. The cell extracts were subjected to SDS/12 % PAGE, and then Western blot analysis was performed using anti-StAR, anti-STS and anti-P450scc antibodies. (**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 the 30-kDa StAR protein in COS-1 cells transfected with F2 and pStAR expression plasmid; the amount of the 30 kDa StAR protein in those cells being set to 1.0. Results are means ± S.E.M. of three separate experiments. Bars with different letters are significantly different (a, b and c, $P < 0.01$; d, $P < 0.05$). (**C**) COS-1 cells were transfected with pStAR and pSTS containing 10 % charcoal-stripped FCS. pStAR (0.1 μg/ml) was co-transfected into COS-1 cells with or without pSTS (0.1 μg/ml). Culture dishes were treated by adding increasing amounts of CS to the culture medium, and Western blotting was performed using anti-StAR antibody.

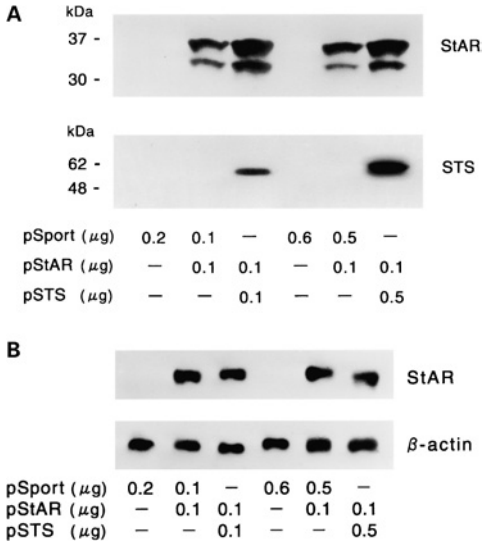

Figure 5 STS has no effect on expression of the StAR gene

(**A**) StAR protein and STS were synthesized using *in vitro* translation. The translation was performed using a TNT®-coupled reticulocyte lysate system. F2 (0.5 μg), pStAR (0.1 μg) and pSTS (0.1 or 0.5 μg) were incubated with Sp6 TNT® RNA polymerase. The translated products were subjected to SDS/PAGE and then to Western blot analysis using anti-StAR and anti-STS sera. (**B**) COS-1 cells were transfected with F2 (0.5 μg/plate), pStAR (0.1 μg/plate) and pSTS (0.1/plate or 0.5 μg/plate) using FuGENE 6. RNA was extracted from COS-1 cells after 24 h of co-transfection treatment. Total RNA (30 μg) was subjected to Northern blot analysis, and blots were probed with human StAR and mouse β-actin RNA probes. Each experiment was replicated on three separate occasions.

in vitro transcription/translation reaction mixture. Furthermore, there was no apparent pSTS concentration-dependent increase (Figure 5A). To examine pStAR expression, we performed Northern blot analysis using total RNA from COS-1 cells that had been transfected with various kinds of plasmid. StAR mRNA levels were unaltered in COS-1 cells that had been transfected with plasmid expression vectors. There was no change in the mRNA expression of StAR in COS-1 cells into which pSTS had been introduced (Figure 5B). The amount of StAR protein in COS-1 cells that had been transfected with pStAR, pSTS and F2 increased without any change in StAR mRNA expression. These observations suggest that STS has a translational effect on StAR protein level.

The half-life of StAR protein was increased by STS protein

We performed pulse–chase experiments to determine whether the increase in StAR protein levels is related to the increase in the life-span of StAR protein. COS-1 cells were transfected with F2 and StAR and/or STS expression plasmids and then metabolically labelled with [^{35}S]methionine. After the pulse period (zero time), there was a significant increase in the amount of pre-StAR 37 kDa protein in COS-1 cells that had been transfected with pSTS compared with that in COS-1 cells that had not been transfected with pSTS. The results of pulse–chase experiments showed that the pre-StAR 37 kDa protein disappeared faster from COS-1 cells that had been transfected with pStAR alone than from COS-1 cells that had been transfected with pSTS. The mature StAR protein (30 kDa) accumulated much more in COS-1 cells than had not been transfected with pSTS than in COS-1 cells that had been transfected with pSTS (Figures 6A and 6B). The half-life of GAPDH in COS-1 cells that had been transfected with pSTS did not change (Figures 6C and 6D). The half-life of the 37 kDa StAR protein without pSTS was 16 ± 1.7 min (mean ± S.E.M.), significantly ($P < 0.01$) shorter than that with pSTS (31 ± 3.6 min). The amount of mature StAR protein (30 kDa) without pSTS was significantly different from that of the protein with pSTS at 60 min ($P < 0.05$) (Figure 6E). The increase in the amount of StAR protein is not a result of an increase in the level of StAR gene expression, but is due to a post-transcriptional mechanism by which both the translation and half-life of the 37 kDa StAR protein are increased.

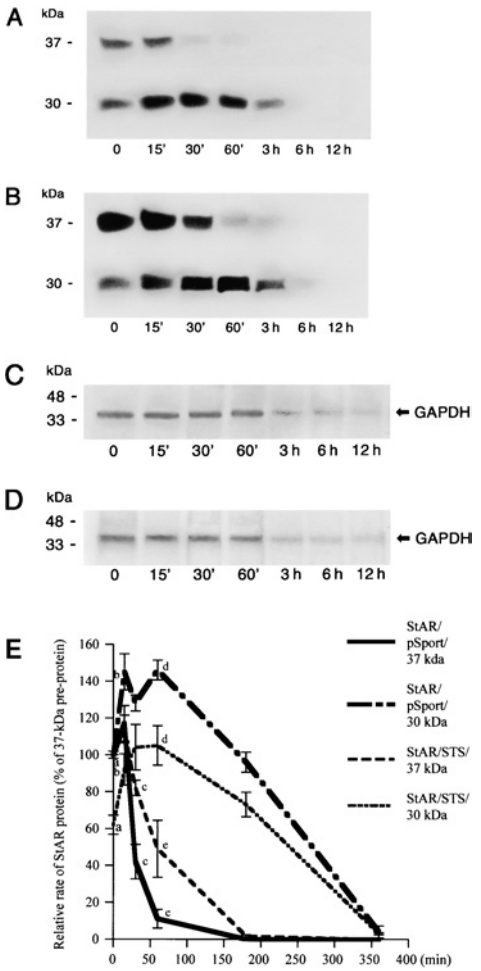

Figure 6 Results of pulse–chase experiments carried out using COS-1 cells transfected with pStAR and pSTS expression plasmids

COS-1 cells were transfected with pStAR (0.1 μg/plate) and pSTS (0.5 μg/plate) using FuGENE 6. After transfection, COS-1 cells were pulse-labelled with [^{35}S]methionine for 30 min and then chased with an excess of unlabelled methionine followed by immunoprecipitations [anti-StAR (**A**, **B**) and anti-GAPDH (**C**, **D**) antibodies] of the proteins and SDS/PAGE. Autoradiograms with pStAR and pSPORT transfection (**A**, **C**) and with pStAR and pSTS transfection (**B**, **D**) are shown. (**E**) The abundance of StAR proteins was plotted by quantified densitometric scanning. StAR pre-protein (37 kDa) and mature protein (30 kDa) abundance ratios are expressed as percentages of 37 kDa pre-StAR protein. Results are means $\pm$ S.E.M. for three separate experiments. Bars with different letters are significantly different (a and c, $P < 0.01$; b, d and e, $P < 0.05$).

DISCUSSION

In steroidogenic cells, steroid hormones are synthesized from cholesterol supplied from LDL, and the cholesterol synthesized *de novo* from acetate by the acetyl-CoA enzyme in the endoplasmic reticulum is less important [17,18]. It has been shown that STS hydrolyses CS to provide free cholesterol in cells in the endoplasmic reticulum [19]. It has also been shown that cholesterol from LDL and CS is involved in independent growth of CHO (Chinese-hamster ovary) cells [20]. The transport pathway of cholesterol that is desulphated by STS may be different from that of LDL origin. The amount of pregnenolone produced by COS-1 cells transfected with F2 alone was reduced by the presence of CS in the culture medium. Our results are consistent with results of previous studies showing that CS is an inhibitor of steroidogenesis in isolated rat adrenal mitochondria by inhibiting cholesterol movement in the mitochondria [21,22]. The increase in the rate of pregnenolone production by COS-1 cells in the culture medium containing CS in the presence of STS was significantly greater than that by COS-1 cells in the absence of STS. The effect of the addition of CS to the culture medium is associated with STS. CS is important for the production of steroid hormones in cells.

An increase in the synthesis of the steroid hormone by STS was observed in COS-1 cells that had been transfected not only with StAR, but also with MLN64, which is a homologue of the StAR protein at the C-terminal domain and resides in late endosomes and lysosomes [23,24]. StAR homology domains are known as START (StAR-related lipid transfer) domains [25]. Placenta contains a 27 kDa form of MLN64 that is proteolytically cleaved and includes START domains [26]. Cholesterol combines with MLN64 and StAR proteins with START domains, resulting in a change in the structure of the StAR protein [23,27]. Changes in the structure of the StAR protein on interaction with cholesterol-rich membranes [28] and a StAR-protein-induced alteration in membrane lipid organization have been reported [29]. When pSTS and pStAR expression plasmids are co-transfected into COS-1 cells, the abundance of free cholesterol may change the ability of StAR protein to transport cholesterol into the inner mitochondrial membrane. R2C cells, a rat Leydig tumour cell line, constitutively express high levels of StAR protein [30]. StAR protein functions in R2C cells, which are rich in free cholesterol because of the high levels of scavenger receptor type B class 1 and hormone-sensitive lipase, to maintain high levels of free cholesterol in the mitochondria. The higher free cholesterol content in the mitochondria of R2C cells is available for steroidogenesis [31].

Oxysterols 25-hydroxycholesterol and 27-hydroxycholesterol elevate StAR protein levels through post-transcriptional actions without changing the StAR mRNA levels either by enhancing translation or by increasing protein life-span [32]. It has been reported that the actions of StAR protein are not specific to steroidogenesis, but extend to other mitochondrial cholesterol-metabolizing enzymes in cells. The expression of StAR protein in COS-1 cells that had been co-transfected with cholesterol 27-hydroxylase and adrenodoxin resulted in a 6-fold increase in formation of 3β-hydroxy-5-cholestenoic acid [33]. Since it is thought that STS desulphates CS to produce free cholesterol in cells, StAR protein could also increase the levels of oxysterols in cells in the presence of STS. Cholesterol, including oxysterols, is thought to enhance StAR translation to increase StAR protein function.

Following synthesis, the 37 kDa StAR pre-protein is imported into mitochondria with subsequent cleavage of the mitochondria-targeting sequence, yielding a 30 kDa mature StAR protein located in the matrix. Since StAR protein acts outside the outer mitochondrial membrane, only the newly synthesized 37 kDa StAR protein is functional and facilitates the transport of cholesterol from the outer mitochondrial membrane into the inner mitochondrial membrane without other protein components [11,34–36], and StAR activity is regulated by its speed of mitochondrial import [37]. Although the half-life of StAR pre-protein in rat adrenal cortex cells has been reported to be 3–4 min [38], COS-1 cells process the 37 kDa protein into 30 kDa protein more slowly than do adrenal cells [39]. The half-life of StAR pre-protein in COS-1 cells estimated from our results is almost the same as that reported by Arakane et al. [34]. The 37 kDa pre-StAR protein disappeared more slowly in COS-1 cells that had been transfected with STS expression plasmids than in COS-1 cells that had not been transfected with pSTS expression plasmids. StAR protein binds cholesterol, and this binding may change the conformation of the protein and increase the protein life-span. Nuclear receptors change to an activation conformation by combining with a ligand. Some nuclear receptors combine

with ligands to make a stable structure that modulates biological activation due to the conformation change [40–42]. StAR protein may have a longer life-span due to change of its conformation and stimulate steroid hormone production.

In StAR-gene-knockout mice, it has been reported that HDL-derived cholesterol accumulates in steroidogenic cells [43]. In the early stage of congenital lipoid adrenal hyperplasia, cholesterol is transported into mitochondria by a StAR-independent mechanism [44]. The fact that CS increased the amount of pregnenolone produced by STS-transfected COS-1 cells in the absence of StAR protein suggests that STS not only desulphates CS, but also has a StAR-independent effect on steroidogenesis. The existence of a large amount of free cholesterol may result in an increase in cholesterol movement into the inner mitochondrial membrane not only via StAR protein, but also via a StAR-independent mechanism, such as transport by peripheral benzodiazepine receptors [45] or by sterol-carrier protein 2 [46,47], in steroid-hormone-producing cells.

It has been reported that MLN64 is directly involved in mobilization of lysosomal cholesterol to mitochondria [48]. Much is now known about intracellular cholesterol trafficking, but there is little information on the trafficking of CS. The mechanism by which StAR gene expression is controlled has been analysed, and the promoter region of the StAR gene has become clear [3,32,49–52]. The human StAR promoter is responsive to SREBPs (sterol-element-binding proteins) [32,53], which regulate genes associated with cholesterol homoeostasis, including 3-hydroxy-3-methylglutaryl-CoA reductase and LDL receptor [54,55]. Since COS-1 cells have endogenous cholesterol hydroxylase activity, free cholesterol produced by STS may affect the expression levels of SREBP in steroidogenic cells. Human StAR gene expression is thought to be affected via transcription directly or indirectly by STS. It is thought that STS expression is important for regulation of StAR gene expression. However, the mechanism by which the expression of STS in steroid-hormone-producing cells is controlled is not known. To clarify the actions of StAR protein in steroid-hormone-producing cells, we will investigate the trafficking of CS and the molecular mechanism of the control of STS gene expression in future studies.

In conclusion, the increase in StAR protein action is a result of both an increase in translation and a longer half-life of the 37 kDa pre-StAR protein. STS increases StAR protein levels and stimulates steroid production in cells.

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