Folding, activity and import of steroidogenic acute regulatory protein into mitochondria changed by nicotine exposure

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Abstract

Nicotine, a pharmacologically active constituent of tobacco smoke, decreases sex steroid production and impairs reproductive function. The rate-limiting step in steroid hormone biosynthesis is the transport of substrate cholesterol from the outer to inner mitochondrial membrane by the steroidogenic acute regulatory protein (StAR). StAR is a 37 kDa cytoplasmic phosphoprotein processed as a 32 kDa intermediate to a mature 30 kDa inactive mitochondrial protein. StAR's cholesterol transport capacity is proportional to its residency time at the outer mitochondrial membrane (OMM). Nonsteroidogenic COS-1 cells transfected with StAR/F2, steroidogenic MA-10 cells induced with cAMP or transfected with StAR or the isolated steroidogenic mitochondria preincubated with nicotine reduced StAR expression, import and activity. Mitochondria isolated from steroidogenic tissues or cells, pretreated with nicotine, also reduced the association of StAR with the OMM, but had no effect on the import of signal sequence substituted SCC/N-62StAR. The fluorescence emission maximum of StAR was unchanged with nicotine, but StAR's free energy of unfolding and the surface area (m) increased in the presence of nicotine. Nicotine also blocked StAR from proteolysis with trypsin, suggesting that nicotine partially stabilised protein conformation by insertion into the molten globule conformation of StAR.

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Introduction

Smoking causes ~4 million deaths in the United States every year. Nicotine, one of the main components of cigarette smoke, is delivered through smoking and is not only a potent cause of lung cancer but also causes low birth rate, preterm delivery and abortion in women addicted to smoking. Nicotine causes menstrual irregularities and pregnancy complications, decreases fertility in women (Weisberg 1985), inhibits spermatogenesis and causes decreased steroidogenesis (Aydos *et al.* 2001, Mlynarcikova *et al.* 2005). From the study of laboratory animals, nicotine affects normal functioning of accessory reproductive organs in male albino rats (Patil *et al.* 1999). Although nicotine's mechanism of action is not clearly understood, it inhibits the release of pituitary follicle-stimulating hormone and luteinizing hormone through its action on the hypothalamo-hypophyseal axis (Blake *et al.* 1972, Blake 1974, 1978). Cigarette smoke contains an average of 0.5–1.6 mg nicotine per cigarette (Calafat *et al.* 2004, Shihadeh & Saleh 2005), which was found to be 0.01 µg/ml (0.022 µM) in the blood stream and 1 µg/ml or 2.2 µM in the tissues of smokers (Hakki *et al.* 2001). Nicotine dependence results from binding of nicotine to acetylcholine receptors in the central nervous system (Tapper *et al.* 2004), thus it influences the central nervous system and also increases the oxidative stress of

mitochondria, which in turn affects normal respiratory function of the mitochondrion, a key organelle in the first step of steroid hormone biosynthesis.

In an acute response, steroid hormones are synthesised and released within minutes of tropic hormonal stimulation. Steroidogenic tissues do not store pre-synthesised steroids but respond rapidly to increase the conversion of cholesterol to steroid hormones under acute conditions (Miller 1988). The substrate cholesterol is insoluble in water and is transported into the mitochondria by an unknown mechanism. The rate-limiting step is the transport of cholesterol from the outer to inner mitochondrial membrane and is fostered by a cytoplasmic phosphoprotein called the steroidogenic acute regulatory protein or StAR (Stocco & Clark 1996). StAR is a 37 kDa phosphorylated 'active pre-protein', and is transported into mitochondria as a 30 kDa mature protein (Stocco & Clark 1996). Mutant StAR cannot transport cholesterol, resulting in an inborn error in metabolism, called congenital lipoid adrenal hyperplasia (lipoid CAH), in which fetuses die shortly after birth due to a salt-losing crisis (Lin *et al.* 1995, Bose *et al.* 1996, 2000, Nakae *et al.* 1997). StAR is a 285 amino acid protein, but deletion of the first 62 amino acids retains complete activity when compared with full-length StAR in the transfected cells or when biosynthetic N-62 StAR or cell-free synthesised (CFS) StAR is incubated with mitochondria isolated from

MA-10 cells. On the contrary, carboxy-terminal deletion of only 10 amino acids reduce StAR activity by 50% and deletion of 28 amino acids completely abolishes the same (Lin *et al.* 1995, Sugawara *et al.* 1995, Arakane *et al.* 1996). Biochemical and structural studies have shown that the StAR amino acid substitution mutations that cause lipoid CAH have a gross error in folding (Bose *et al.* 1998). Therefore, the movement of cholesterol into mitochondria is dependent on two factors: first, the expression of properly folded StAR, and second, the interaction of StAR with mitochondrial membranes. Thus, any hindrance of StAR association with the OMM will reduce cholesterol transport into the mitochondria and will reduce pregnenolone synthesis. Environmental factors, such as exposure to tobacco smoke which contains nicotine, may reduce the interaction of StAR with the OMM, resulting in reduced transport of the substrate cholesterol and finally reduced pregnenolone. In this article, we report that the presence of nicotine inhibits StAR processing and cholesterol transport into mitochondria, thereby decreasing pregnenolone synthesis. We also provide evidence that StAR import occurs in a unique fashion through the OMM and that nicotine changes StAR-specific mitochondrial import processing at the OMM.

Materials and methods

Cell culture and transfection

COS-1 cells (monkey kidney) were grown in 10 cm plates in Dulbecco's modified Eagle medium supplemented with 10% foetal bovine serum and $1 \times$ penicillin-streptomycin at 37 °C in a humidified incubator with 5% CO₂. Cells were plated at a density of 1×10^6 in six-well plates 24 h prior to transfection, and co-transfected with 1.0 µg StAR and F2 (P450_{scc}-Adrenodoxin reductase-adrenodoxin; Harikrishna *et al.* 1993) using lipofectamine (Invitrogen). The cells were washed with serum-free media 12 h after transfections, and supplemented with media containing appropriate antibiotics, 10% serum and 1 pmol trilostane. The media was collected after 48 h and the accumulated pregnenolone was measured by RIA (RIA kit, MP Biomedicals, Irvine, CA, USA). To expose cells with nicotine, we incubated either COS-1 or MA-10 (mouse leydig) cells in serum-free media containing 0.1, 0.5, 1.0, 2.5, 5.0 and 10 mM nicotine for 6 h, then removed the nicotine and allowed the cells to recover for 2 h in complete growth media. After 2 h, both nicotine-treated and -untreated cells were co-transfected with plasmids containing StAR and F2 vectors, and accumulated pregnenolone was measured as described above. For PCR amplification of the nicotine-exposed cells, RNA was isolated using TRIZOL reagent (Invitrogen), and reverse transcribed with Superscript II with 'oligodT' primer at

42 °C. Two microlitres of cDNA were amplified by 5'-AGCTATGGAAGAGACTCTCTA-3' sense and 5'-AGC-TAAGCTTCAACACCTGGCT-3' anti-sense primer for 25 cycles at 94 °C for 1 min, 72 °C for 2 min and 55 °C for 1 min. An aliquot of 10 µl amplified DNA was analysed in a 1.6% agarose gel stained with ethidium bromide.

Western blot

The protein concentration was determined using Bio-Rad's dye reagent (Bio-Rad). Protein samples (12.5 µg) from transfected cells were electrophoresed in 15% SDS-PAGE, electroblotted to a polyvinylidene fluoride (PVDF) membrane and processed for western blotting with N-62 StAR (Bose *et al.* 1999), cytochrome P450 side chain cleavage enzyme (Hsu *et al.* 2006) and adrenodoxin reductase, antibodies independently (Brentano *et al.* 1992). As a control for StAR, we used *Escherichia coli* expressed N-62 StAR and CFS P450_{scc} as a control for F2. The band intensity was quantitated by digitising with the 'Unscan-it' program (Silk Scientific Corporation, Orem, UT, USA).

Isolation and purification of mitochondria

Protein import and competition experiments require large quantities of mitochondria; since milligram quantities of mitochondria can be isolated from a pair of adrenals, we preferred to use mitochondria from sheep adrenal tissues for all import and direct bioactivity measurements. The sheep adrenals, from control groups, were available from the laboratory of Dr Charles Wood, University of Florida, immediately after killing. To isolate the mitochondria from adrenal glands, the tissues were transferred to mitochondria isolation buffer (250 mM sucrose, 10 mM HEPES (pH 7.4), 1 mM EGTA) immediately after killing and chopped into small pieces on ice. The cells or the tissue fractions were homogenised in a hand-held all-glass Dounce homogeniser with ten gentle up and down strokes, and the cell debris was removed by spinning at 3500 g for 10 min. The supernatant containing the mitochondrial fraction was centrifuged at 10 000 g for 10 min, and the pellet was washed with the mitochondrial isolation buffer, resuspended in an energy regeneration buffer (125 mM sucrose, 80 mM KCl, 5 mM MgCl₂, 10 mM NaH₂PO₄, 10 mM isocitrate, 1.0 mM ATP, 1.0 mM NADP, 0.1 mM ADP and 25 mM HEPES (pH 7.4)) and stored at either –86 °C or in liquid nitrogen.

In vitro steroidogenic activity with biosynthetic N-62 StAR

To isolate the biologically active form of N-62 StAR from *E. coli* cytosol, the bacterial pellet was sonicated in

a glycerol buffer (10 mM HEPES (pH 7.4), 10% glycerol and 1 mM phenylmethylsulphonyl fluoride (PMSF)) on ice for 10 min. The collected supernatant was centrifuged at 18 000 *g* for 10 min, passed through a Ni-NTA superpose column, washed with glycerol buffer and eluted with the same buffer containing 100 mM imidazole. The final purification was done by passing through a gel filtration column equipped with a fast protein liquid chromatography (FPLC) system (Pharmacia, AKTA). To determine the StAR activity, we incubated 1 µg biosynthetic StAR with 20 µg sheep adrenal mitochondria in an energy regeneration buffer (Bose *et al.* 2002) for 1 h at 37 °C and measured the pregnenolone synthesis by RIA.

Protein import

[³⁵S]-Met labelled StAR and SCC/StAR were synthesised in a cell-free system (CFS) using a transcription/translation (TNT) coupled reticulocyte lysate systems (Promega). Ribosomes and associated incompletely translated polypeptide chains were removed by centrifugation at 150 000 *g* for 15 min at 4 °C (Schwartz & Matouschek 1999). For all protein import experiments, 100 µg isolated mitochondria was incubated in a 26 °C water bath with ³⁵S-StAR or SCC/StAR to a final volume of 100 µl, and terminated by the addition of 1 mM mCCCP and an equal volume of boiling 2× SDS-sample buffer. The import reactions were analysed by electrophoresing through SDS-PAGE, fixed in methanol/acetic acid (40:10), dried and exposed to a phosphorimager screen.

For the isolation of the StAR-OMM complex, [³⁵S]StAR and P450_{sc}c were imported into isolated mitochondria, extracted with digitonin buffer (1% (w/v) digitonin, 20 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 50 mM NaCl, 10% (w/v) glycerol, 1 mM PMSF) for 15 min on ice and subjected to gradient blue native (BN)-PAGE (Schagger & von Jagow 1993, Schagger *et al.* 1994) at 4 °C and 100 V overnight. An aliquot of 100 µl digitonin lysate was applied on a 30–10% sucrose density gradient, centrifuged for 4 h at 4 °C at 286 000 *g* in a Beckman ultracentrifuge, aliquoted into equal fractions and analysed by SDS-PAGE.

Cigarette smoke condensate (CSC)

The CSC was prepared at the University of Kentucky using Reference Cigarettes, 1R4F (9 mg tar and 0.8 mg nicotine/cig; Pillsbury & Bright 1972). Briefly, the smoke particulates were collected on a Cambridge filter pad from cigarettes smoked under the standard Federal Trade Commission protocol (35 ml puff volume of a 2 s duration; Pillsbury & Bright 1972) and dissolved in dimethylsulfoxide (DMSO) at 40 mg/ml. Control cells

were treated with a medium containing an equivalent amount of DMSO.

Fluorescence spectroscopy

For fluorescence spectroscopy, Tryptophan (Trp) was excited at 295 nm and the spectrum was recorded at 0.2 nm intervals from 310 to 450 nm at 25 °C. The buffer (20 mM NaH₂PO₄, 50 mM NaCl, pH 7.4) and protein spectra were recorded under identical conditions. Emission maxima and intensity were plotted as a function of increasing amounts of nicotine. Because of the high photon counts from the N-62 StAR, we employed only 10 ng protein throughout the experiment. For unfolding studies, N-62 StAR was incubated with 0.01, 0.1, 1.0, 5 and 10 mM nicotine for 2 h and then melted by incubating with 8.0 M urea overnight at room temperature. Wavelength maxima of each fluorescence spectrum were determined after subtraction of buffer blank, plotted as a function of urea concentration and the free energy (ΔG) of unfolding was calculated by following the linear extrapolation (Pace 1986).

Results

Nicotine reduces pregnenolone synthesis by inhibiting StAR expression and processing

To study the effect of nicotine on pregnenolone synthesis, we incubated COS-1 cells with different concentrations of nicotine prior to transfection with StAR and F2 vector. Nonsteroidogenic COS-1 cells do not express the P450_{sc}c system, so we co-transfected with F2 vector which expresses the complete P450_{sc}c system as a single fusion protein (P450_{sc}c-adrenodoxin reductase-adrenodoxin; Harikrishna *et al.* 1993). As a positive control, we transfected the COS-1 cells with F2 and water soluble 22R-hydroxycholesterol, which bypasses the action of StAR and shows the maximum capacity of a cell to synthesise steroid hormones. Nicotine showed no effect on pregnenolone synthesis up to 1.0 mM concentration, but increasing the nicotine concentration to 2.5 mM decreased pregnenolone from 480 to 320 ng/ml, a loss of 33% in comparison to the untreated cells (Fig. 1A). At 5 mM nicotine, pregnenolone was reduced to 220 ng/ml and at 10 mM the activity was decreased to the level of buffer control, suggesting a strong inhibition of pregnenolone synthesis at higher concentrations of nicotine. StAR expression was unchanged with <1 mM nicotine (Fig. 1B), but with higher concentrations and especially at 2.5 mM concentrations of nicotine, the 37 and 32 kDa StAR expressions (Fig. 1C) were completely inhibited. Expression of F2, which is a 120 kDa protein, monitored by western blotting with anti-P450_{sc}c (Fig. 1D) and

Figure 1 Effect of nicotine on pregnenolone synthesis. (A) COS-1 cells were preincubated with 0.1, 0.5, 1.0, 2.5, 5.0 and 10 mM of nicotine and co-transfected with StAR/F2. (B) Western blot with StAR antibody of the cells in (A). (C) Intensity of expression of StAR proteins, where (■) is 30 kDa, (▨) is 32 kDa and (□) is 37 kDa StAR. (D and E) Western blot of the cells (A) with cytochrome P450scc and adrenodoxin reductase antibodies. Last lane, in (D), is the cell-free synthesised P450scc acting as positive control. (F) COS-1 cells preincubated with 0.1, 1.0, 2.5, 5.0 and 10 mM nicotine, were transfected with F2 and incubated with 22R-hydroxycholesterol. (G) Direct measurement of bioactivity from the mitochondria isolated from MA-10 cells preincubated with 0.1, 1.0,

anti-adrenodoxin reductase (Fig. 1E) antibodies independently. The lower 45 kDa band (Fig. 1E) with adrenodoxin reductase antibody may be due to a leaky expression of adrenodoxin reductase. This band is shorter than the full-length adrenodoxin reductase-expressed band. In the F2 vector, adrenodoxin reductase is fused with the carboxy terminus of P450_{scc} sequence without its N-terminal amino acids, suggesting a possible reason for shorter expression than the full-length adrenodoxin reductase (Harikrishna *et al.* 1993). The pregnenolone synthesising capacity was determined by co-transfecting COS-1 cells with StAR and F2 vector, where the expression of F2 and StAR are under the control of the cytomegalovirus (CMV) promoter. To determine if nicotine affected the mitochondrial electron transport system, we measured pregnenolone synthesis with the F2-transfected COS-1 cells, incubated with 22R-hydroxycholesterol and different concentrations of nicotine. Only 10 mM nicotine reduced the pregnenolone synthesis from 820 to 390 ng/ml with no effects observed at any lower concentrations (Fig. 1F). Similar results were observed when we transfected MA-10 cells with StAR in the presence of nicotine (Fig. 1G). The effect of nicotine depends on the mode of delivery (Benowitz 1996), and the *in vitro* experiments shows reduced pregnenolone synthesis only with higher concentrations of nicotine (Sarasin *et al.* 2003). Because of the faster hydrolysis of nicotine in the tissue, an accurate determination of nicotine concentration is not possible (Sarasin *et al.* 2003). The induction of MA-10 cells with cAMP in presence of nicotine, decreased StAR expression in a similar fashion as observed for COS-1 transfected with StAR and F2 (Fig. 1H). However, StAR mRNA expression remained unchanged, as determined by RT-PCR of MA-10 cells RNA (Fig. 1G, right panel), indicating that nicotine directly inhibited StAR expression and processing into mitochondria without affecting the gene expression.

Nicotine directly blocks cholesterol transport into mitochondria

StAR's activity is directly proportional to its residency time at the OMM. We directly measured pregnenolone synthesis by incubating biosynthetic N-62 StAR with the mitochondria isolated from MA-10 cells and sheep adrenal tissues. The activity, as measured by the level of pregnenolone synthesis, remained at the similar level between the two sources of mitochondria (Fig. 2A) and showed no activity when incubated with heat-inactivated StAR, validating our mitochondrial preparation.

To understand nicotine-induced inhibition of cholesterol transport, we incubated sheep adrenal mitochondria with the biosynthetic N-62 StAR in the presence of 0.1–10 mM nicotine, and measured pregnenolone synthesis. Mitochondria incubated with the biosynthetic N-62 StAR synthesised 600 ng/ml pregnenolone (Fig. 2B), which was ninefold more than the mitochondria or buffer control. The pregnenolone level was reduced to 285 ng/ml with 2.5 mM nicotine indicating that direct incubation of nicotine inhibited 47% of pregnenolone synthesis, which was significantly greater than the transfected cells. Increasing the nicotine concentration to 5.0 mM reduced the pregnenolone to 75 ng/ml, and with 10 mM nicotine the pregnenolone synthesis was reduced to the level of buffer, suggesting that nicotine completely inhibited pregnenolone synthesis at higher concentrations. Incubation of biosynthetic N-62 StAR with the mitochondria isolated from MA-10 cells preexposed to nicotine also showed reduced pregnenolone (Fig. 2C). However, the pregnenolone synthesis was unaffected in presence of 10 mM cotinine, a primary metabolite of nicotine demonstrating that indeed nicotine entered into the cell (Fig. 2C).

A direct incubation of nicotine may affect the mitochondrial electron transport system as nicotine depolarises the mitochondrial architecture (Banzet *et al.* 1999). Therefore, we determined the concentration-dependent effect of nicotine by incubating isolated adrenal mitochondria with 22R-hydroxycholesterol. The activity in the absence of nicotine was 780 ng/ml, and remained unchanged up to 5 mM (Fig. 2D) but decreased to 550 ng/ml (Fig. 2D) at 10 mM concentration. On western blotting, the expression of P450_{scc} (61 kDa; Fig. 2E) and adrenodoxin reductase (54 kDa; Fig. 2F) remain unchanged. One shorter band of 55 kDa with the P450_{scc} antibody (Fig. 2E) is the signal sequence cleaved P450_{scc}, and two smaller bands with the adrenodoxin antibody (Fig. 2F) are due to two-step cleavage of adrenodoxin reductase (Brentano *et al.* 1992), confirming that at lower concentrations nicotine have no effect on the mitochondrial electron transport system. The partial disappearance of the shorter band with the increase in nicotine concentration may be due to partial inhibition of the cleavage of the N-terminal signal sequence of adrenodoxin reductase.

Nicotine inhibits StAR import

To determine the effect of nicotine on StAR import through the OMM, we first characterised StAR import into isolated mitochondria by measuring the cleavage of

2.5, 5.0 and 10 mM nicotine and transfected with StAR (left-hand panel). RT-PCR of the RNA from MA-10 cells incubated with 0.1, 1, 2.5, 5 and 10 mM nicotine (right-hand panel). (H) Western blot with StAR antibody of the cAMP induced MA-10 cell incubated with indicated concentrations of nicotine. Data in (A, C, F and G) (left panel) are the means ($\pm$ S.E.M.) of three separate experiments, each performed in triplicate.

Figure 2 (A) Direct measurement of pregnenolone synthesis with the mitochondria isolated from MA-10 cells and adrenal tissues incubated with biosynthetic N-62 StAR before and after heat inactivation. (B) Effect of nicotine on the isolated mitochondria. Mitochondria were incubated with the biosynthetic N-62 StAR and 0.1, 0.5, 1.0, 2.5, 5.0 and 10 mM of nicotine. (C) Direct measurement of bioactivity with the mitochondria isolated from MA-10 cells. An aliquot of 4.0 μ g mitochondria isolated from MA-10 cells incubated 1.0, 2.5, 5.0 and 10 mM nicotine. One aliquot was also incubated with 10 mM cotinine. (D) Incubation of 22R-hydroxylcholesterol with the mitochondria isolated from adrenal tissues in the presence of 0.1, 0.5, 1.0, 2.5, 5 and 10 mM nicotine. (E and F) Western blot with cytochrome P450scc and adrenodoxin reductase antibodies independent of the mitochondria exposed to the indicated concentrations of nicotine. Data in (A–D) are the mean ($\pm$ S.E.M.) of three separate independent experiments, each performed in triplicate.

37 to 30 kDa StAR. We also determined the membrane association of StAR by Na_2CO_3 extraction, as Na_2CO_3 breaks the protein–protein interaction (Li & Shore 1992), but not the lipid–protein interaction. Further, we compared StAR import efficiency with SCC/N-62 StAR by substituting its leader sequence with a mitochondrial matrix-targeting protein, P450scc. Import of ^{35}S -StAR and SCC/N-62 StAR onto the isolated mitochondria for 2 h (Fig. 3A) followed by extraction with sodium carbonate showed that StAR and SCC/N-62 StAR remained with the pellet fraction, suggesting that after import StAR and SCC/N-62 StAR were integrated into the membrane. However, the import efficiency of SCC/StAR is higher than StAR as determined by the intensity of the lower 26 kDa band (Fig. 3B). Phosphorimager quantitation of the cleaved bands showed that the SCC/StAR was imported fivefold faster than StAR (Fig. 3C), demonstrating that protein import was kinetically controlled.

To identify the effect of nicotine on StAR import, we incubated the isolated mitochondria with 0.1–10 mM nicotine, and imported ^{35}S -StAR and ^{35}S -SCC/StAR. Nicotine concentrations up to 1 mM had no effect on StAR import, but at higher concentrations the intensity of the 30 kDa band was reduced (Fig. 3D). However, at any concentration of nicotine the import of SCC/StAR was unaffected. Incubation of additional fresh 100 μg mitochondria to the mitochondria pre-exposed with nicotine (5 or 10 mM) restored StAR import (Fig. 3E), suggesting that nicotine indeed specifically inhibits StAR import. This also demonstrates that nicotine possibly blocked the StAR-specific mitochondrial import process, and that increasing the amount of mitochondria increased the free sites required for StAR import. To confirm this observation, we preincubated the isolated mitochondria with nicotine for 30 min, reisolated, washed and then incubated with ^{35}S -StAR at 26 °C for 2 h (Fig. 3F). Measurement of import efficiency (3G) showed that the 5 mM or higher concentrations of nicotine inhibited StAR import, suggesting that temporary incubation of nicotine also inhibited the StAR-specific mitochondrial import process, but had no effect on the import of SCC/StAR (3F, bottom panel).

Effect of CSC on StAR activity and import

Nicotine is delivered to the lungs and blood stream as a complex of several components during cigarette smoking. To determine the effect of the nicotine present in cigarette smoke, we incubated COS-1 cells with CSC as an index to cigarette smoke exposure and transfected them with StAR and F2 vector. Incubation of StAR/F2-transfected COS-1 cells with 0.1 $\mu\text{g}/\text{ml}$ of CSC had no effect, but 1 $\mu\text{g}/\text{ml}$ CSC, which is equivalent to 70 ng nicotine (RJ Reynolds Tobacco Co. 1988), reduced pregnenolone synthesis from 520 to 350 ng/ml ($\sim 33\%$ less), and 5 $\mu\text{g}/\text{ml}$ CSC reduced

pregnenolone synthesis to the level of nontransfected cells (Fig. 4A). The tenfold increase in effect of CSC when compared with nicotine alone is due to the difference in delivery method of nicotine to the cells (Benowitz 1996). Western blotting of the transfected cells with StAR antiserum showed that 1 $\mu\text{g}/\text{ml}$ CSC reduced the expression of 37 kDa StAR by 75% and also reduced the expression of 30 kDa StAR by 40%. At higher concentrations of CSC the expression of 37 kDa StAR was completely ablated, suggesting that the decrease in pregnenolone synthesis was due to the absence of 37 kDa StAR (Fig. 4B and C). Nicotine binds directly with the mitochondrial membrane (Xie *et al.* 2005), and thus StAR may not have a chance to fold properly to bind with cholesterol and foster it to mitochondria. As a result, StAR was directly imported into mitochondria as a mature 30 kDa protein without any increase in pregnenolone synthesis.

Interaction of StAR with OMM is inhibited by nicotine

To identify whether the presence of nicotine changes StAR's stability and its interaction with the OMM, we briefly imported full-length ^{35}S -StAR and P450scc into the mitochondria isolated from sheep adrenals, extracted with digitonin and electrophoresed on a 3–20% BN-PAGE (BN Gel electrophoresis; Schagger & von Jagow 1993, Schagger *et al.* 1994). We found only one complex with full-length StAR (Fig. 5A). To determine the inhibitory effect of nicotine we incubated the mitochondria in the presence and absence of nicotine, imported ^{35}S -StAR, extracted with digitonin (Wiedemann *et al.* 2003) and fractionated the complex(es) by sucrose density gradient ultracentrifugation. Analysis of each fraction on SDS-PAGE (Fig. 5B and C) showed two different distribution patterns of StAR complex: one with imported 30 kDa StAR (bottom band) and one with unimported 37 kDa StAR (top band). The nicotine-treated and -untreated mitochondria (Fig. 5B and C) had a similar pattern of density distribution up to reaction number 7 (Fig. 5B), and the pattern changed from fraction 8 onwards with a reduction in intensity of the unimported 37 kDa StAR complex, indicating that nicotine blocked StAR's interaction with the OMM.

Binding of StAR with nicotine

Protein folding is a dynamic process and folding changes upon binding or interaction with another protein. To understand whether a reduction in StAR expression is associated with its binding to nicotine, we used fluorescence spectroscopy probing with the Trp residues. Depending on the polarity of the Trp microenvironment, the fluorescence emission

Figure 3 Mitochondrial StAR import. (A) Cell-free synthesised (CFS) ³⁵S-StAR and SCC/N-62 StAR were imported into isolated mitochondria. (B) Import kinetics. StAR and SCC/N-62 StAR were imported kinetically from 5, 15, 30 and 60 min. (C) Phosphorimager quantitation of the import efficiency of SCC/StAR and StAR. (D) Import of ³⁵S-StAR (top panel) and SCC/N-62 StAR (bottom panel) in the presence of 0, 1, 2.5 and 10 mM nicotine. (E) Additional unexposed 100 µg mitochondria was added to the incubation mixture of ³⁵S-StAR and mitochondria preincubated with the indicated concentrations of nicotine. (F) StAR and SCC/StAR import onto mitochondria preincubated with 0, 1, 2.5, 5 and 10 mM nicotine. (G) Quantitative estimation of the imported 30 kDa StAR (from (E), top section) and shows the means (± S.E.M.) of three separate experiments.

Figure 4 Pregnenolone synthesis in the presence of cigarette smoke condensate (CSC) transfected with StAR and F2. (A) COS-1 cells preincubated with 0.1, 1.0 and 5 µg/ml of CSC. Data are the means ($\pm$ S.E.M.) of three separate experiments, each performed in triplicate. (B) Western blot of the CSC-induced StAR transfected COS-1 cells. The *E. coli* expressed N-62 StAR is a control present in the last lane. (C) Quantitation of the intensity of the 37, 32 and 30 kDa bands from western blot and show the means ($\pm$ S.E.M.) of three separate experiments. The Y-axis is the intensity of the expressed protein and X-axis is the concentrations of CSC.

maximum of the spectrum appears between 335 and 354 nm and reflects the compactness of a protein. In the absence of nicotine, biosynthetic N-62 StAR showed an emission maximum at 337.2 ± 0.2 nm, and in the presence of increasing concentrations of nicotine from 0.01 to 10 mM, the emission maximum remained unchanged (Fig. 6A), but the fluorescence intensity decreased, suggesting that nicotine possibly

interacted with StAR. Therefore, we determined the binding of StAR with nicotine by measuring the change in free energy (ΔG) of StAR (Pace 1986, 1992). The unfolding of N-62 StAR (Fig. 6B) started at 1.75 M urea with a transitional midpoint at 2.5 M urea and completed melting at 4.0 M urea. These curves fitted a two-state model where the free energy difference between the native (N) and unfolded (U)

Figure 5 Effect of nicotine on the StAR-mitochondrial import complex. (A) Digitonin extraction of the ³⁵S-StAR and P450_{sc} on the BN-PAGE. (B and C) Analysis of the StAR density gradient centrifugation by SDS-PAGE, where B and C represent mitochondria untreated and treated with nicotine respectively.

Figure 6 Effect of nicotine on N-62 StAR conformation. (A) Fluorescence spectra of N-62 StAR in the presence of 0.1, 0.01, 1, 5 and 10 mM nicotine. The inset is the plot of emission maxima with different concentrations of nicotine. (B) Effect of nicotine on the stability of N-62 StAR in the absence (solid line) and presence of nicotine (broken line). (C) Determination of free energy of unfolding (ΔG) of N-62 StAR in the absence (■) and presence (○) of nicotine. (D) A plot of the surface area m value plotted against nicotine concentration. (E) Partial proteolysis of StAR in the presence of indicated concentrations of nicotine.

states depends on the urea concentration. Thus, $\Delta G = \Delta G_{H_2O} - m$ (Urea), where ΔG is the free-energy difference at any urea concentration. ΔG_{H_2O} is the free energy difference in the absence of urea and m is a solvent denaturant value that measures the rate of change of ΔG as a function of urea concentration

(Wrabl & Shortle 1999); therefore the m value is proportional to the new surface area of protein that becomes accessible upon unfolding (Wrabl & Shortle 1999). The ΔG of unfolding of N-62 StAR in the presence and absence of nicotine was 3.143 and 2.467 kcal/mol (Fig. 6C), and the m value changed

from -0.87 to -1.241 kcal/mol per m with increasing nicotine concentrations (Fig. 6D), suggesting a partial stabilisation of StAR.

To determine the partial stabilisation of StAR in the presence of nicotine, we proteolysed StAR and analysed the protection of StAR domains in SDS-PAGE (Fig. 6E). Trypsin is a highly specific enzyme that digests after lysine and arginine residues at or above pH 7.0. Therefore, we incubated biosynthetic N-62 StAR in the presence of different concentrations of nicotine but with a fixed concentration of trypsin for a limited time at room temperature. To visualise the proteolysed band by Coomassie blue dye, we incubated microgram quantities of biosynthetic N-62 StAR and with higher concentrations of nicotine during this proteolysis reactions. In the absence of nicotine or at lower concentrations of nicotine, the proteolytic pattern of N-62 StAR was similar to our earlier findings (Bose *et al.* 1999), and at higher nicotine concentrations N-62 StAR became resistant to proteolysis (Fig. 6E, last lane), indicating that nicotine stabilised StAR's conformation.

Discussion

Steroidogenic activity and cholesterol transport are related to the stability and expression of StAR. This activity depends on the availability of the active protein at the OMM and only newly synthesised StAR can foster cholesterol (Artemenko *et al.* 2001). Western blotting of the nicotine-treated and -untreated cells with different antibodies confirmed that nicotine did not affect the outer mitochondrial and matrix proteins. Therefore, the mitochondrial general protein import pore (GIP; Neupert 1997) for protein import and sorting assembly remains unaffected. The import of StAR is reduced but not the signal sequence substituted StAR, so there is a StAR-specific transport mechanism through the OMM. Thus, the OMM-associated protein that StAR interacts with during its import and the transport of cholesterol, is the key factor affected by nicotine.

The view that StAR may act as an intracellular hormone fostered the idea that mitochondria might have specific receptor(s) that are required for StAR's action, other than those involved in protein import. When N-62 StAR is expressed in transfected cells, it transfers cholesterol to the peroxisomal and other membranes in addition to the OMM (Kallen *et al.* 1998), suggesting that the principal function of the mitochondrial leader is to target StAR exclusively to the OMM for its import. N-62 StAR equally transports cholesterol similar to full-length StAR to the mitochondrial matrix, and works in a partially open conformation. Therefore, the organisation of OMM proteins is crucial for mitochondrial function. This is especially true of mitochondrial targeted nuclear-encoded proteins which may use preexisting

OMM-associated proteins as receptors or may associate with the OMM, as is the case with OMM-associated β -barrel proteins (Koehler 2004). Therefore, the import receptors at the OMM may be affected by nicotine (Hinsch *et al.* 2001). As StAR's conformation remained unchanged with increasing concentrations of nicotine, it is likely that nicotine affected the conformation of the cytoplasmic proteins associated with the OMM, thereby inhibiting StAR's interaction with the OMM.

The model structure suggested that StAR should work at the inner mitochondrial space to shuttle cholesterol into the matrix. StAR's co-crystal structure (Roderick *et al.* 2002) and the three-dimensional crystal structure of StARD4 (Romanowski *et al.* 2002) suggested that StAR must work at the cytoplasmic side of the OMM by partially opening its conformation. The absence of any significant difference in the CD spectrum between nicotine-treated and -untreated StAR indicated no interaction or minor interaction of StAR with nicotine. The small change in free energy (-0.67 kcal/mol) suggested that nicotine is associated with StAR in a molten globule state where StAR is in a partially open conformation due to the presence of more water molecules inside the protein core (Privalov 1996). The unimported StAR at the OMM was most likely proteolysed by the proteosomal proteases (Granot *et al.* 2003) or through ubiquitin-mediated proteases, which is a mandatory mechanism of the degradation of proteins for proper functioning of the cellular process (Lee *et al.* 2001). Thus, a lesser availability of StAR to transport adequate cholesterol into the mitochondria resulted in reduced pregnenolone.

Incubation of mitochondria with nicotine reduced StAR import, but not the import of SCC/N-62 StAR. The difference between StAR and SCC/StAR is the leader sequence, and both of them are imported into the mitochondrial matrix. Thus, there is a StAR-sequence leader-specific mechanism associated with StAR import into the mitochondria. All proteins must be imported through the mitochondrial import channel Tom40, where the outer mitochondrial Translocases (Tom) act as receptors for the incoming mitochondrial-targeted proteins for their further association with the OMM or their translocation and sorting through the IMM using the smaller Tim proteins. As β -barrel proteins like Tom40 or porin (also known as VDAC) do not always use the preexisting Tom40 and VDAC channel for association with the OMM, it is possible that the StAR import process was hindered either with voltage dependent anion channel (VDAC) or with Tom40 after nicotine exposure. The minor import inhibition of SCC/N-62 StAR rules out the possibility of an alteration of the Tom40 channel. Thus, we hypothesise that the most abundant OMM-associated protein, VDAC, is affected by nicotine.

In summary, in this manuscript we have provided the following evidence: 1) StAR expression is reduced due to the presence of toxins like nicotine, 2) reduction of pregnenolone synthesis is due to the damage or blockage of the OMM, 3) StAR is stabilised by nicotine resulting in decreased interaction with the OMM, 4) temporary exposure to nicotine blocks both StAR and cholesterol transport, 5) the effect of cigarette smoke is stronger than that of nicotine alone and decreases the expression of protein at the OMM and 6) the import mechanism for StAR is different from other mitochondrial proteins imported into the same mitochondrial compartment.

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