Decreased Cytochrome c Oxidase IV Expression Reduces Steroidogenesis^[S]

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ABSTRACT

Steroidogenic acute regulatory protein facilitates the translocation of cholesterol to the inner mitochondrial membrane, thereby initiating steroidogenesis. At the inner mitochondrial membrane, cytochrome P450 side-chain cleavage enzyme converts cholesterol to pregnenolone, an oxidative process requiring electrons from NADPH. Pregnenolone then serves as the substrate for the formation of progesterone or dehydroepiandrosterone by downstream enzymes. Studies have shown that cigarette smoke (CS) influences steroid hormone levels. To better understand the underlying mechanisms, we used a mouse model to study the effects of chronic CS exposure on steroidogenesis. Through radioimmunoassay and metabolic

conversion assays, we found that CS reduced progesterone and dehydroepiandrosterone without affecting cytochrome P450 side-chain cleavage enzyme or 3 β -hydroxysteroid dehydrogenase 2 expression. However, CS did reduce expression of cytochrome c oxidase IV (COX IV), a component of the mitochondrial complex that serves as the last enzyme in the electron transport chain. Small interfering RNA-mediated COX IV knockdown indeed decreased progesterone synthesis in steroidogenic cells. In summary, COX IV likely plays a role in steroidogenesis, and passive smoking may negatively affect steroidogenesis by disrupting the electron transport chain.

Introduction

The mitochondrial environment is important for the transport of metabolites across its subcompartments and for proper steroidogenesis in specific steroid-producing cells. Mitochondria have four subcompartments: the outer mitochondrial membrane (OMM), inner mitochondrial membrane (IMM), intermembrane space, and matrix. Steroidogenesis

begins with the transfer of cholesterol from the OMM to the IMM, where cytochrome P450 (P450) side-chain cleavage enzyme (P450scc) converts it to pregnenolone, the substrate for the synthesis of the other steroid hormones in the $\Delta 4$ and $\Delta 5$ pathways (Fig. 1A). Movement of cholesterol through the aqueous phase of the mitochondria to the IMM is facilitated by steroidogenic acute regulatory protein (StAR) (Miller and Auchus, 2011). StAR, a 37-kDa cytoplasmic phosphoprotein, undergoes processing into a 32-kDa intermediate before entering the mitochondria where it is cleaved once again to form a 30-kDa mature protein. Processing requires StAR to interact with OMM-associated voltage-dependent anion-selective channel protein 1 (VDAC1) and phosphate carrier protein (Bose et al., 2008b).

P450scc uses a pair of electrons donated from NADPH to catalyze the conversion of cholesterol to pregnenolone, thereby acting as a terminal oxidase in the mitochondrial electron transport system. Sequentially, electrons from NA-

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ABBREVIATIONS: OMM, outer mitochondrial membrane; 3 β HSD2, 3 β -hydroxysteroid dehydrogenase 2; P450, cytochrome P450; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; COX IV, cytochrome c oxidase IV; CS, cigarette smoke; DHEA, dehydroepiandrosterone; IMM, inner mitochondrial membrane; P450scc, cytochrome P450 side-chain cleavage enzyme; PAGE, polyacrylamide gel electrophoresis; RT, retention time; siRNA, small interfering RNA; StAR, steroidogenic acute regulatory protein; TLC, thin-layer chromatography; VDAC1, voltage-dependent anion-selective channel protein 1.

Fig. 1. Measurement of physiological differences after chronic exposure to CS. A, schematic representation of the steroidogenic pathways. Preg, pregnenolone; Prog, progesterone; 17-OH-P, 17-hydroxypregnenolone; 17-OH-Prog, 17-hydroxyprogesterone. B and C, measurement of endogenous pregnenolone synthesis. Mitochondria from adrenal glands (B) and ovaries (C) were incubated at 37°C for 2 h, and pregnenolone was measured by radioimmunoassay. Exposure to CS reduced pregnenolone synthesis by 38.4% in adrenal tissue (B) and by 33.3% in ovarian tissue (C). Data presented are the mean $\pm$ S.E.M. from three independent experiments performed in triplicate.

DPH first are accepted by ferredoxin reductase associated with the IMM. Next, ferredoxin reductase transfers the electrons to an iron/sulfur protein, ferredoxin, associated with the IMM and facing the matrix. Finally, adrenodoxin then transfers the electrons to P450_{scc}. Within the electron transport process, adrenodoxin reductase and adrenodoxin both serve as universal electron transfer proteins for all of the mitochondrial P450s. To transfer electrons, adrenodoxin forms a 1:1 complex with adrenodoxin reductase and then dissociates to form a 1:1 complex with any mitochondrial P450 (e.g., P450_{scc} or P450_{c11}). Thus, these universal electron transfer proteins function as indiscriminate diffusible electron shuttling proteins.

Multiple studies have shown that cigarette smoke (CS) affects hormone levels. For example, Kirschbaum et al. (1992) demonstrated that active or passive smoking leads to prolonged elevation of steroid levels. In addition, active or passive smoking affects both fetal development and young premenopausal women due to an elevation of follicle-stimulating hormone and gonadotropin; decreased luteinizing hormone, androstenedione, and dehydroepiandrosterone sulfate levels; and a decreased duration of menstruation (Khaw et al., 1988; Velasco et al., 1990; Hautanen et al., 1993; Baron et al., 1995; Cooper et al., 1995; Trummer et al., 2002). In male smokers, levels of adrenocorticotropin-stimulated androstenedione, 17-hydroxyprogesterone, and dehydroepiandrosterone (DHEA) are decreased (Salvini et al., 1992; Hautanen and Adlercreutz, 1993) due to increased secretion of adrenal androgen that inhibits 21- or 11 α -hydroxylase within the adrenal cortex (Kapoor and Jones, 2005).

Secondhand exposure to CS only has minor acute effects, but if continuous, it can lead to long-term complications. To better understand the mechanisms by which passive smoking affects steroidogenesis, we have developed a mouse model in which animals are exposed to CS. Using radioimmunoassay and metabolic conversion assays, we showed that CS reduced synthesis of pregnenolone and DHEA. Rather than affecting expression of the biosynthetic enzymes, CS appeared to alter steroidogenesis by reducing the expression of cytochrome *c* oxidase IV (COX IV), an integral mitochondrial protein that is the last enzyme in the electron transport chain. On the basis of our observations, we have concluded that COX IV likely plays a role in steroidogenesis and that passive smoking may negatively affect steroidogenesis by disrupting electron transport.

Materials and Methods

Reagents and Chemicals. We purchased all of the chemicals from Sigma-Aldrich (St. Louis, MO) or Thermo Fisher Scientific (Waltham, MA) unless noted otherwise. Small interfering RNA (siRNA) oligonucleotides were purchased from Ambion (Austin, TX). VDAC1-specific antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and Calbiochem (San Diego, CA). Pregnenolone assay reagents were obtained from MP Biomedicals (Solon, OH), [³⁵S]methionine was purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA), [¹⁴C]cholesterol and [³H]pregnenolone were purchased from PerkinElmer Life and Analytical Sciences, and the rabbit reticulocyte lysate kit was from Promega (Madison, WI).

Animals. Female C57BI mice, 8 to 9 weeks of age, were purchased from Harlan (Indianapolis, IN) and fed Purina chow (Harlan Teklad, Madison, WI) and water. We randomly divided the mice into two groups: control animals exposed to filtered ambient air and experimental animals exposed to CS for 4 h per day, 5 days per week for 8 consecutive weeks in a whole-body exposure system maintained at a concentration of approximately 40 mg/m³ of total suspended smoke particulates (Gairola et al., 2001). Mitochondria isolated (Bose et al., 2007) from excised adrenal and ovarian tissues were flash-frozen in liquid nitrogen and stored at -86°C. All of the procedures were approved by the Institutional Animal Care and Use Committee of the University of Kentucky (Lexington, KY).

Metabolic Conversion Assays. To measure the conversion of pregnenolone to progesterone, we incubated isolated mitochondria (300 mg) in potassium phosphate buffer with 3×10^6 cpm of [³H]pregnenolone and chased with 30 μ g of radioactive unlabeled progesterone. The reaction was initiated by adding NAD (Simard et al., 2005) and then incubated at 37°C for 2 h in a shaking water bath. To measure the conversion of cholesterol to pregnenolone, mitochondria were incubated with 80,000 cpm of [¹⁴C]cholesterol. In this case, the reaction was initiated by the addition of NADPH (Miller, 1988) and then incubated as above. For complete conversion, we used a 5-fold excess of radioactive carrier to reach the saturation point. For both reactions, the steroids were extracted with 9:1 ether/acetone (v/v), and an equal amount of 50:50 radioactive pregnenolone/progesterone (Sigma-Aldrich) mixture in dichloromethane was added as a carrier. The extracts were concentrated under a stream of nitrogen or air and then separated by thin-layer chromatography (TLC) (Whatman, Clifton, NJ) using a chloroform/ethyl acetate (3:1) mobile phase. The radioactivity was enhanced by a tritium-labeled enhancer (EN3HANCE; GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK), and the amounts of each steroid were determined using a phosphorimager.

Identification of Metabolites by High-Performance Liquid Chromatography. To confirm the identity of each steroid, the radioactive spots on the silica plates were scraped and extracted with a solvent mixture of ether/chloroform (3:1). The solvent was evaporated under nitrogen, and the extract was dissolved in 50 to 100 μ l of

methanol and then separated by high-performance liquid chromatography (Wiebe et al., 2000) using UV and radioisotope detectors in series with a C18 column (Ultrasphere ODS, 5 μ m, 4.6 $\times$ 250 mm; Beckman Coulter, Fullerton, CA). For the purposes of identifying the radiolabeled metabolites, 3:1 methanol/water (v/v) was used at a flow rate of 1 ml/min. An aliquot of each sample was injected along with 10 to 20 μ g of the authentic unlabeled standard, and the retention time (RT) of the radioactive peak was compared with that of the UV absorbance peak at 210 nm of the simultaneously run standard.

Fractionation of StAR-Associated Complexes. Cell-free synthesis of [35 S]Met-labeled StAR was performed with a TNT rabbit reticulocyte system. Ribosomes and associated (incompletely translated) polypeptides were removed (Schwartz and Matouschek, 1999), and then [35 S]StAR was incubated with isolated mitochondria for 10 min at 26°C to allow import of the protein. For the analysis of assembled protein complexes, mitochondria were reisolated and lysed with a digitonin buffer for 15 min on ice (Schägger et al., 1994). The digitonin lysate was combined with native polyacrylamide gel electrophoresis (PAGE) sample buffer [5% Coomassie Brilliant Blue G-250, 100 mM BisTris (pH 7.0), and 500 mM 6-aminocaproic acid] and subjected to 3 to 16% gradient native PAGE at 100 V overnight at 4°C (Bose et al., 2007).

Mass Spectrometry Analysis of StAR-Associated Mitochondrial Complexes. For proteolytic digestion to generate peptides from the complexes isolated by native PAGE, [35 S]Met-labeled bands were excised from the gel, reduced with dithiothreitol (Roche, Basel, Switzerland), alkylated with iodoacetamide, and digested overnight with trypsin (sequencing grade modified; Promega) (Rosenfeld et al., 1992). The resulting peptide fragments were analyzed via liquid chromatography tandem mass spectrometry on a nanoAcquity UPLC (Waters, Milford, MA) coupled with a Q-TOF Premier mass spectrometer (Micromass, Manchester, UK; Waters). The peptides were separated using a linear water/acetonitrile gradient (0.1% formic acid) on a nanoAcquity column (Atlantis column, 3 μ m dC18, 100 Å pore size, 75 μ m i.d. $\times$ 15 cm; Waters) with an inline loading/desalting column (Symmetry column, 5 μ m C18, 180 μ m i.d. $\times$ 20 mm; Waters). Peak lists were generated using the Waters Protein-Lynx Global Server (version 2.2.5). Background subtraction for mass spectrometry and tandem mass spectrometry data used the adaptive method with two iterations of Savitzky-Golay smoothing over three channels. Tandem mass spectrometry data were centroided and deisotoped with a setting of 80% top, signal-to-noise threshold of 3%, minimum peak width of four channels, and resolution of 10,000 full width at half-maximum. Proteins were identified from tandem mass spectrometry spectra by unrestricted searching of the National Center for Biotechnology Information nonredundant database (NCBI nr 2010.06.22; 11,238,375 sequences), allowing for one missed cleavage and using trypsin as the cleavage enzyme. After an initial search, the data were filtered to remove hits for keratin and then resubmitted using a *Mus musculus* (146,469 sequences) species filter. Searches were performed using the Mascot MS/MS Ion Search program (version 2.2; Matrix Science, London, UK), taking carbamidomethylated cysteine and oxidation of methionine into consideration with a mass tolerance of ± 0.1 Da for precursor ions and ± 0.2 Da for fragment ions. Only ion scores with $p < 0.05$ were considered relevant when listing the number of unique peptides. For data matching multiple accession numbers, preference was given to an accession number from mitochondria or where a more descriptive protein name was given in the database.

COX IV Knockdown. COS-1 and MA-10 cells were transfected with either 30 or 60 pmol of three siRNAs targeting COX IV, siRNA1 (5'-CCAAGCGAAUGCUGGACAUTT-3'), siRNA2 (5'-GGGAUGA-GAAAGUUCAGUUTT-3'), and siRNA3 (5'-CCAGUUUACGUG-GAAAAAATT-3'), using Oligofectamine (Invitrogen, Carlsbad, CA). Nontargeting siRNA (Ambion) served as a control in all of the experiments. A combination of two nontargeting scrambled siRNAs (Ambion) also was included as a control in all of the experiments. We

used Western blot analysis to demonstrate siRNA-mediated knockdown of COX IV expression.

Results

Decreased Pregnenolone and DHEA Synthesis upon CS Exposure. Studies have shown that the expression of several steroid hormones decreases upon CS exposure (Kirschbaum et al., 1992; Kapoor and Jones, 2005). This likely is the result of an inhibitory effect of CS on the steroidogenesis pathway (Fig. 1A). To evaluate this possibility, we first examined components of the pathway in a mouse model that we developed to mimic mild, chronic exposure to CS. Control mice were exposed to filtered ambient air, whereas experimental mice were exposed to 40 mg/m³ of total suspended smoke particles for 4 h per day, 5 days per week. After 8 weeks, we found no significant change in body weight (data not shown). Because most steroid synthesis takes place in the gonad tissues and adrenal glands, we excised these tissues from both mouse groups. Although it is known that addition of biosynthetic StAR to isolated mitochondria will increase pregnenolone production 7- to 9-fold over buffer alone or those deficient in StAR (Bose et al., 2000, 2008b), this method does not reflect the endogenous steroidogenic activity of the tissues. Therefore, we determined the endogenous steroidogenic capacity by measuring the pregnenolone synthesis of isolated mitochondria from these tissues using radioimmunoassays (Bose et al., 1996). Pregnenolone synthesis of the adrenal glands from CS-exposed animals (200 ng/ml) was 38.4% lower than that of control animals (325 ng/ml) (Fig. 1B). Likewise, pregnenolone synthesis of the ovarian tissues from CS-exposed animals (380 ng/ml) was 33.3% lower than that of control animals (570 ng/ml) (Fig. 1C). Thus, chronic exposure to CS reduced mitochondrial function of steroidogenic tissues as demonstrated by the decrease in endogenous pregnenolone synthesis capacity.

We next expanded analysis of steroidogenesis by using metabolic conversion assays to evaluate the synthesis of the steroids downstream of pregnenolone. For these studies, mitochondria were isolated from steroidogenic mouse Leydig (MA-10) and human adrenal (NCI-H295) cells lines as well as from mouse ovarian and adrenal tissues. We measured the conversion of pregnenolone to progesterone, 17-OH-pregnenolone, and DHEA by incubating the mitochondria with [3 H]pregnenolone and separating the resulting metabolites by TLC. Metabolites were identified initially according to their polarity on the TLC plates followed by confirmation via high-performance liquid chromatography. The results show that pregnenolone is actively converted into progesterone by mitochondria from MA-10 and NCI-H295 cells, thus indicating active 3 β -hydroxysteroid dehydrogenase 2 (3 β HSD2) (Fig. 2A). Furthermore, addition of the 3 β HSD2 inhibitor trilostane (Bose et al., 2000) significantly reduced the conversion of pregnenolone to progesterone. Mitochondria isolated from ovarian tissue of both control and CS-exposed mice synthesized progesterone, 17-OH-pregnenolone, and DHEA (Fig. 2B), although CS exposure resulted in a 40% reduction in DHEA production (Fig. 2, B and C). Mitochondria isolated from adrenal tissues of both animal groups also synthesized progesterone and 17-OH-pregnenolone but not DHEA because the enzyme needed to catalyze this reaction, P450C17, is not available (Staels et al., 1993). Each of the

Fig. 2. Metabolic synthesis of steroids. A, metabolic conversion of pregnenolone to downstream steroid hormones in the mitochondria isolated from mouse Leydig MA-10 and human adrenal NCI-H295 cells. Reactions were initiated with NAD and [3 H]pregnenolone. Resulting steroid products were separated through silica-coated plates. Mitochondria from both cell types converted pregnenolone (Prog) to progesterone (Prog). Trilostane inhibited 3β HSD2 activity. The relative conversion to 17-OH-pregnenolone is higher in MA-10 cells than that in NCI-H295 cells. B, similar metabolic conversion from the mitochondria isolated from mouse adrenal and ovary tissues, as described for control experiments in A. Similar levels of progesterone are seen in both control and CS-exposed tissues. C, phosphorimager quantification of the DHEA level in B. Data presented are the mean $\pm$ S.E.M. from three independent experiments performed in triplicate.

spots from the TLC plates was then extracted and analyzed by high-performance liquid chromatography to compare their RTs with those of authentic steroids to validate their identities. The RT for the pregnenolone marker recorded by UV absorbance was 13.1 min, and the TLC-extracted tritium-labeled band eluted at 13.2 min, indicating that this band was pregnenolone (Supplemental Fig. 1A). Likewise, the RTs of authentic progesterone, DHEA, and 17-OH-pregnenolone coincided with the RTs of the radioactive compounds extracted from the TLC plate, thus confirming their identities (Supplemental Fig. 1, B–D). On the basis of these experiments, tissues from animals exposed to CS synthesize pro-

gesterone, but synthesis of steroids in the $\Delta 5$ pathway also is reduced.

We next checked the expression levels of VDAC1, P450_{scc}, and 3β HSD2 by Western blot analysis to determine whether changes in these enzymes could account for the decreased endogenous pregnenolone production seen with CS exposure. Glyceraldehyde-3-phosphate dehydrogenase served as an internal control. Expression of VDAC1, P450_{scc}, and 3β HSD2 did not differ between control and CS-exposed mouse ovarian or adrenal tissues (Fig. 3, A–C). Thus, the reduced conversion of cholesterol to pregnenolone is likely due to a different disruption in the mitochondrial electron transport system.

Fig. 3. Western blot analysis of steroidogenic protein expression levels. In each lane, 12.5 μ g of mitochondrial proteins was separated by 10% (P450_{scc}) or 15% (VDAC1 and 3β HSD2) SDS-PAGE and blotted onto polyvinylidene difluoride membrane. "C" represents the control and "CS" represents the CS-exposed animals. Bottom panels showed detection of glyceraldehyde-3-phosphate dehydrogenase on the same membrane, as described above. D, characterization of mitochondrial high-molecular-mass complexes isolated from control and CS-exposed tissues. Native gradient gel electrophoresis of mitochondrial complexes isolated from ovarian and adrenal tissues and detected with [35 S]StAR. The result shows formation of complexes in both control and CS-exposed tissues with an approximate molecular mass of 650 kDa. E and F, Western blot analysis of ovary and adrenal tissue lysates, respectively, for COX IV expression. The right panel shows quantitative expression of COX IV. The expression of COX IV was reduced in the CS-exposed tissues.

Native Mitochondrial Complexes from CS-Exposed Animals Lack COX IV. Matrix-targeting StAR acts at the OMM (Bose et al., 2002), is imported into the intermembrane space (Tsujishita and Hurley, 2000), and finally resides in the mitochondrial matrix (Clark et al., 1994), thus indicating that StAR can form complexes with several different proteins present within the various mitochondrial compartments (Bose et al., 2008a). Identification of unknown protein complexes with a specific cell-free synthesized protein has been used recently to identify low abundant proteins present in a high-molecular-mass complex (Otera et al., 2007; Bose et al., 2008a,b). Thus, we isolated stable StAR-containing complexes from ovarian and adrenal tissues to determine whether exposure to CS altered the composition of these complexes. After the import of cell-free synthesized [35 S]StAR into mitochondria, complexes were isolated by digitonin lysis of the cells, separated by native PAGE (Bose et al., 2008a,b), and detected by autoradiography. One high-molecular-mass complex of approximately 650 kDa was isolated from ovarian tissue from both control and CS-exposed animals (Fig. 3D). In addition, two high-molecular-mass complexes, close in size, were isolated from the adrenal tissue of the CS-exposed animals (Fig. 3D). To determine whether differences exist between the complexes isolated from the two animal groups, we subjected complexes to in-gel tryptic digestion and then analyzed the resulting peptides by liquid chromatography tandem mass spectrometry. Proteins

were deemed part of the complex only if at least two significant peptide matches were found for each protein in each sample, and these are listed in Supplemental Tables 1 to 4. In the adrenal tissues, we identified three unique COX IV peptides in the control and CS-exposed samples, providing 18 and 20% sequence coverage of the protein, respectively. As the last enzyme in the electron transport chain, COX IV potentially could play a role in supporting P450 activity. The mass spectrometry technique used did not provide quantitative results, so we performed a Western blot analysis with COX IV antibody and found that CS-exposed ovarian and adrenal tissues expressed 8- to 10-fold less COX IV than control tissues (Fig. 3, E and F).

COX IV Knockdown. We next used siRNA to knock down COX IV expression in MA-10 cells to determine whether changes in its expression would lead to altered steroidogenesis. We generated three siRNA oligonucleotides, siRNA1, siRNA2, and siRNA3, but only siRNA3 reduced COX IV expression to below detectable levels as shown by Western blot analysis (Fig. 4A). In MA-10 cells treated with siRNA3, isolated mitochondria synthesized 57% less progesterone than controls as determined by metabolic conversion assays (Fig. 4, B and C). Furthermore, addition of the 3β HSD2 inhibitor trilostane reduced the progesterone level an additional 15%, thus indicating that 3β HSD2 was active in the siRNA3-treated cells and not responsible for the decrease in

Fig. 4. Knockdown of COX IV expression by siRNA. A, Western blot analysis of siRNA-treated MA-10 cells for COX IV expression. siRNA3 was the most effective in reducing COX IV expression. B, metabolic conversion in MA-10 cells after COX IV knockdown. Mitochondria from control and COX IV knockdown cells were incubated with [3 H]pregnenolone in the presence of NAD, and steroid production was determined by TLC. COX IV knockdown resulted in progesterone reduction. C, quantification of progesterone conversion shown in B. D, metabolic conversion of [14 C]cholesterol to pregnenolone in control and COX IV knockdown MA-10 cells. The pregnenolone level was reduced 30% in COX IV knockdown cells. E, quantification of steroid production shown in D. In C and E, data are the mean $\pm$ S.E.M. from three independent experiments performed in triplicate. F, Western blot analysis of StAR expression in COX IV knockdown COS-1 cells. The result shows the absence of precursor and intermediate StAR and an accumulation of the mature form of StAR.

progesterone caused by COX IV knockdown (Fig. 4C). Taken together, these results strongly suggest that the COX IV protein plays a substantial role in the $\Delta 4$ pathway of steroid hormone biosynthesis.

We next sought to determine the mechanism for decreased pregnenolone synthesis in CS-exposed animals. Given that exposure to CS did not alter expression of VDAC1, P450_{scc}, or β HSD2 (Fig. 3, A–C), the possibility exists that the CS-mediated reduction in COX IV slows the rate of cholesterol to pregnenolone conversion. In fact, we observed that the conversion of [¹⁴C]cholesterol to pregnenolone in MA-10 cells with COX IV knockdown was reduced by approximately 30% compared with that of control cells (Fig. 4, D and E). Trilostane had no effect. Taken together, the knockdown through CS eventually reproduced results similar to those with the siRNA knockdown cells performed In Vitro. Because StAR is responsible for transporting cholesterol to the IMM, the site of pregnenolone synthesis, we next examined StAR expression and processing in cells lacking COX IV. StAR is expressed as a 37-kDa precursor that undergoes processing to become a 32-kDa intermediate before it reaches the mitochondria as a mature 30-kDa protein. Using an antibody that detects all three forms, we found that COX IV knockdown in StAR/F2-transfected COS-1 cells resulted in a severely reduced level of the 37- and 32-kDa StAR products but higher expression of the mature 30-kDa StAR (Fig. 4F). In general, the 37-kDa StAR is very short-lived (Pon and Orme-Johnson, 1986; Epstein and Orme-Johnson, 1991; Stocco and Sode-man, 1991; Stocco and Clark, 1996), and in the absence of COX IV, it is possible that the 37-kDa form of StAR was imported into the mitochondria faster, thus resulting in quicker formation of the mature 30-kDa form without increased transport of cholesterol.

Discussion

The transport of cholesterol into the mitochondria initiates steroidogenic activity. Although the exact mechanism of cholesterol transport remains unknown, studies have shown clearly that a substantial amount of cholesterol is transported by the protein StAR (Artemenko et al., 2001) and that the active confirmation of StAR requires its association with VDAC1 (Bose et al., 2008b). Once transported in the mitochondria, cholesterol then serves as the initial substrate for the synthesis of steroid hormones in a reaction that requires the transfer of two electrons. Because CS reduces steroidogenic activity, we sought to determine whether an alteration in mitochondrial complex composition or a disruption in the electrochemical potential of the electron transport chain could account for the decrease in the hormones. Mitochondria from control and CS-exposed animals expressed similar levels of VDAC1, P450_{scc}, and β HSD2 (Fig. 3). We also found that the adrenal tissues isolated from control and CS-exposed animals had native mitochondrial complexes of similar sizes (Fig. 3D). Although the complexes isolated from ovarian tissues were smaller in size, it is likely that this is due to a breakdown from a higher molecular mass complex because mass spectrometry did not show a difference in which proteins were present in the complexes. Thus, it is likely that the conformation of preexisting mitochondrial OMM-associated translocases remains unchanged, but the organization of the complex(s) with the mitochondrial membrane may be altered.

The possibility exists that the composition of each mitochondrial complex dynamically regulates electron transport. The conversion of cholesterol to pregnenolone requires electrons to be transferred from NADPH, through the flavoprotein ferredoxin and ferredoxin reductase, to P450_{scc}. Although this electron system is part of complex I, it influences the function of all of the mitochondrial complexes. In addition, two differentially expressed isoforms of ferredoxin are expressed in humans (Sheftel et al., 2010). Other laboratories have shown that protein kinase A, which becomes activated with oxidative stress, phosphorylates cytochrome *c* oxidase, thereby modulating its activity (Prabu et al., 2006). Moreover, Galati et al. (2009) demonstrated that the Vb subunit of cytochrome *c* oxidase, reduced under hypoxia, plays a key role in complex stability and function such that loss of this subunit can lead to decreased membrane potential and mitochondrial function.

Tobacco smoke is a major source of exogenous pro-oxidants: reactive oxygen species and free radical generators are present in both its gas and particulate phases. Smoking results in an elevation of reactive oxygen species and the depletion of its scavengers in circulating blood. Reactive oxygen species also have been shown to inhibit steroidogenesis in MA-10 cells at the level of transporting cholesterol into the mitochondria (Stocco et al., 1993) without affecting the expression of P450_{scc} or ATP (Diemer et al., 2003). The reduction in transport may be due to the activation of the p38 mitogen-activated protein kinase pathway, which occurs with oxidative stress and is associated with decreased steroidogenesis (Abidi et al., 2008).

Our results suggest that disruption of electron transport led to the observed decrease in steroid hormone synthesis. We observed that CS resulted in an increase in the mature 30-kDa form of StAR, so its import is not necessary for its cholesterol transport activity (Arakane et al., 1996), and thus an increase in the 30-kDa form should not reduce pregnenolone synthesis. Thus, it appears likely that CS reduced the capacity of the mitochondria to metabolize cholesterol into pregnenolone by decreasing COX IV expression and disrupting the electron transport chain. Further investigation is necessary to determine how COX IV energetically participates in the steroidogenic pathway.

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Authorship Contributions

Participated in research design: Pawlak, Prasad, and Bose.

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Wrote or contributed to the writing of the manuscript: Bose and Whittall.

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