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EVIDENCE FOR STEROL CARRIER PROTEIN$_2$-LIKE ACTIVITY IN HEPATIC, ADRENAL AND OVARIAN CYTOSOL

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Purified sterol carrier protein$_2$ (SCP$_2$) from rat liver stimulated utilization of endogenous cholesterol for pregnenolone synthesis by adrenal mitochondria. Cytosolic preparations of rat liver, adrenal and luteinized ovary were also stimulatory in mitochondrial pregnenolone synthesis to different extents. Treatment of all preparations with rabbit anti-rat SCP$_2$ IgG neutralized the stimulatory effects, and immunoprecipitated proteins gave similar patterns on SDS-gradient polyacrylamide gel electrophoresis. Treatment with rabbit pre-immune IgG had no effect on these parameters. Thus, proteins which are immunochemically compatible with hepatic SCP$_2$ appear to be present in steroidogenic tissues and may play a role in control of mitochondrial cholesterol side chain cleavage activity.

The rate limiting step in steroidogenesis is mitochondrial side chain cleavage of cholesterol, and current evidence suggests that availability of the sterol is a primary determinant of this regulation (1,2). Extra mitochondrial cholesterol must be translocated to the cytochrome P 450_{scc} system which is associated with the inner membrane of these organelles (2).

Since cholesterol is relatively insoluble in aqueous solutions and will partition into polar lipid-enriched membranes, a factor which can facilitate cytoplasmic cholesterol transport, or will affect distribution of cholesterol in mitochondrial membranes could be of importance in the regulation of steroidogenesis. There is evidence that cytosol preparations from steroidogenic tissues can stimulate mitochondrial steroid synthesis (3-7), and recent evidence suggests that the active factor may be a protein of relatively low molecular weight (6,7).

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Sterol carrier protein$_2$ is a basic protein with a molecular weight of 13,500, and has been purified to homogeneity from liver cytosol (7). This protein facilitates exchange of cholesterol between liposomes and cell organelles and enhances the activity of enzymes involved in cholesterol and cholesterol ester synthesis (8-10). We have recenty demonstrated the stoichiometric binding of cholesterol to SCP_2, and the net transfer of cholesterol to adrenal mitochondria (11). Furthermore, SCP_2 can directly enhance utilization of endogenous cholesterol by isolated adrenal mitochondria (12). This direct effect appears to involve improved translocation of cholesterol to cytochrome P 450_{scc} without directly affecting the activity of the side chain cleavage system (13).

In the present report, we have demonstrated the presence of a stimulatory factor (s) for mitochondrial pregnenolone synthesis in cytosolic preparations from rat liver, adrenal and luteinized ovary. The stimulatory activity was completely neutralized by antiserum IgG against homogeneous hepatic SCP_2 but not with pre-immune IgG. SDS-polyacrylamide gel electrophoretic patterns of the dissociated IgG-antigen complexes suggest that the factor present in the tissue cytosols has a comparable molecular size as authentic SCP_2.

METHODS

SCP_2 was purified as previously described (7). Antiserum was raised in rabbit using SCP_2 which was electrostatically complexed to phosphorylated bovine serum albumin (13). Pre-immune and immune IgG were prepared by protein-A sepharose affinity chromatography (14), and were further purified by albumin sepharose affinity chromatography. The characteristics and the specificity of the antisera are described elsewhere (12).

To obtain highly luteinized ovaries, female rats were treated with 50 IU pregnant mare serum at 23 days age and 25 IU human chronic gondaotropin 60 h later. The rats were killed 4 days later. The ovaries and liver, as well as adrenal glands from quiescent-killed animals (11) were homogenized (300 mg tissue/ml) in 50 mM phosphate buffer, pH 7.4, containing 5 mM $MgCl_2$ and 154 mM NaCl. Homogenates were centrifuged at 104,000 xg for 50-60 min at 4°C, and following removal of the floating fat layer, the supernatant was collected.

Where indicated the cytosolic preparations were incubated with IgG (160 µg) in a total volume of 200 µl at 25°C for 1 h. This was followed by a 24 h incubation at 5°C. The mixtures were centrifuged at 9000 xg for 20 min, and aliquots of the supernatant were used for studies on biological activities.

Resolution of cytosolic and immunoprecipitated proteins was accomplished by SDS-gradient (10-15%) polyacrylamide slab gel electrophoresis (15) using the buffer system of Laemmli (16). Samples were prepared in a mixture of 0.4% SDS, 1% 2-mercaptoethanol and 10 mM Tris-HCl, pH 6.8, and were heated at 90°C for 5 min. Approximately 100 µg of cytosolic proteins, the entire immunoprecipitates and 10 µg SCP_2 were subjected to electrophoresis. Molecular weights were estimated by the method of Weber et al (17).

For mitochondrial preparations, adrenals from 20-30 rats were homogenized in cold 0.25 M sucrose containing 1mM EDTA, 20 nM nicotinamide and 15 mM Tris-HCl, pH 7.4. Mitochondria were sedimented from an 800 xg supernatant by centrifugation at 5,000 xg for 10 min. Incubations were carried out at 37°C for 25 min and contained in a final volume of 2 ml: 1.7 ml mitochondrial preparation (1-2 mg protein) pH 7.5; 0.35 mg of indicated additions. Pregnenolone was determined by radioimmunoassay (18), and protein was estimated by the method of Lowry et al (19).

RESULTS

Addition of SCP_2 (20 µg) to a suspension of adrenal mitochondria routinely resulted in a 3-4 fold stimulation (mean 3.9 ± 0.3) of pregnenolone synthesis from endogenous cholesterol (Table 1). These results are completely compatible

TABLE 1

Stimulation of mitochondrial pregnenolone synthesis by homogeneous SCP_2 and cytosolic preparations of rat liver, adrenals and luteinized ovaries neutralization by treatment with anti rat SCP_2 IgG.

ADDITIONS/EXPERIMENTS	PREGNENOLONE SYNTHESIS ng/mg mitochondrial protein/25 min 1	2	3
None	95	161	145
SCP_2 (20 µg)	423	593	520
IgG_c (160 µg)		144	
IgG_i (160 µg)		132	
SCP_2 + IgG_c		459	430
SCP_2 + IgG_i		142	160
Adrenal Cytosol (1.5 mg protein)	927	462	
Adrenal Cytosol + IgG_c		431	
Adrenal Cytosol + IgG_i	98	148	
Ovarian Cytosol (10.6 mg protein)			390
Ovarian Cytosol + IgG_c			345
Ovarian Cytosol + IgG_i			140
Hepatic Cytosol (2.8 mg protein)			405
Hepatic Cytosol + IgG_c			390
Hepatic Cytosol + IgG_i			140

Rat adrenal mitochondria were prepared and incubated as described in the text. Incubations were carried out for 25 min at 37°C and included, in a final volume of 2.0 ml: 1.7 ml mitochondrial suspension (1-2 mg protein); 0.3 ml of the indicated additions. Treatment of SCP_2 or cytosol preparations with rabbit serum IgG fractions (160 µg protein) was as described in the text and 0.3 ml of the supernatant prepared following IgG treatment was added to mitochondria. Results are means from duplicate incubations from 3 separate experiments. Pregnenolone formation in each set of duplicate tubes varied less that 10%.

with those reported earlier (12), in which it was shown that the pregnenolone response is proportional to SCP_2 concentrations. Addition of either pre-immune IgG_c or anti SCP_2 IgG_i alone to the mitochondria had no dramatic effect on control levels of pregnenolone synthesis. The stimulatory effect of SCP_2 was completely abolished by pretreatment with anti-SCP_2, but was not affected by treatment with pre-immune IgG_c (compare with appropriate IgG_c control, experiment 2).

Addition of cytosolic preparations of adrenal, luteinized ovaries or liver to adrenal mitochondria all resulted in different levels of stimulation of pregnenolone synthesis. As previously reported (11), the factor (s) in adrenal cytosol responsible for this stimulation is progressively lost by repeated freezing and thawing of the preparation. This is demonstrated by comparing the responses of different mitochondrial preparations to the addition of the same adrenal cytosolic preparation after a single freeze and thaw (experiments 1 and 2). The cytosolic fractions used in experiment 3 were freshly prepared.

Treatment of adrenal, ovarian or hepatic cytosols with pre-immune IgG_c had little effect on the ability of these preparations to stimulate mitochondrial pregnenolone synthesis. In contrast pretreatment of cytosols with anti SCP_2 IgG_i completely neutralized the stimulatory activities of each of the cytosolic preparations.

Since the mitochondrial pregnenolone response is proportional to amounts of SCP_2 added (12), it is possible to estimate by this bioassay, the amounts of immunoreactive SCP_2-like protein (s) in the cytosolic preparations of each tissue. For adrenal cytosol, this was estimated as 9.3 µg SCP_2-equivalents/mg cytosolic protein, or 0.93% of the total protein. In liver, the estimated SCP_2-equivalents was 5.1 µg/mg protein, and in luteinized ovaries, the value averaged 1.2 µg/mg protein.

Since immunoprecipitates in mixtures of anti SCP_2 IgG and SCP_2 or tissue cytosolic preparations were often not visible, the mixtures were spun at 9000 xg for 20 min and the supernatants were used in the mitochondrial incubations shown in Table 1. The precipitates were carefully rinsed with 1 ml Tris-HCl buffer and the resuspended residue was layered over a 3 ml cushion of 1.5 M sucrose. This was centrifuged at 20,000 xg for 60 min and the supernatant was aspirated. The protein residue was solubilized in SDS sample buffer, treated as described under "Methods", and subjected to SDS-polyacrylamide gradient (10-15%) slab gel electrophoresis (16). The gels were initially stained with Coomassie blue (Fig. 1a) and subsequently with silver stain (Upjohn Diagnostics, Kalamazoo, MI) (Fig. 1b).

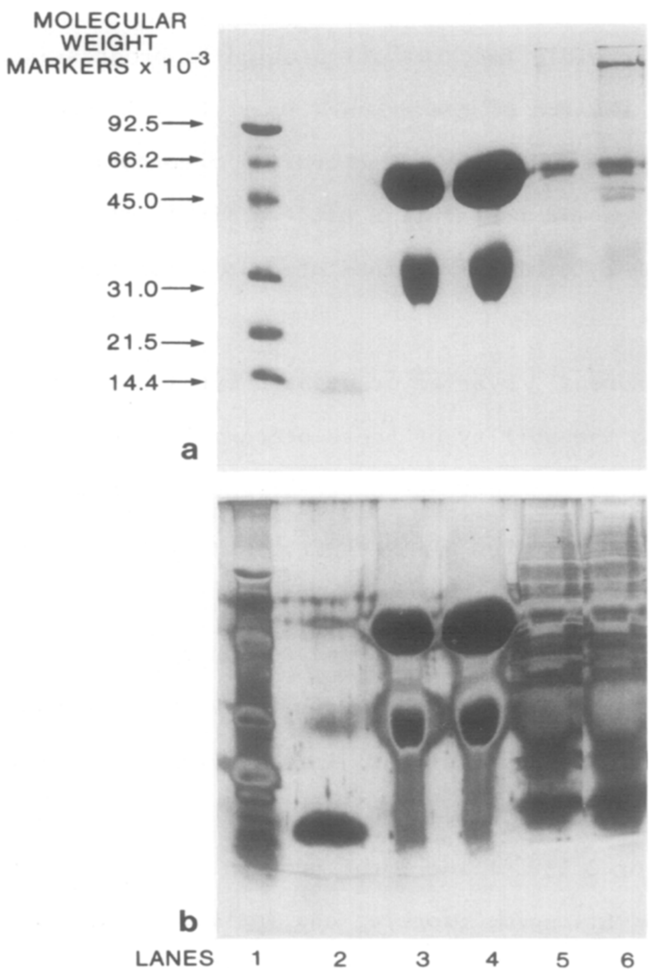

Figure 1 Protein patterns of authentic SCP_2 and adrenal cytosol fractions by SDS-gradient (10-15%) polyacrylamide slab gel electrophoresis. Samples were prepared in 0.4% SDS, 1% 2-mercaptoethanol and 10 mM Tris-HCl, pH 6.8, and were heated at 90°C for 5 min. Lane 1, molecular size markers; lane 2, hepatic SCP_2; lane 3, non immune rabbit IgG; lane 4, rabbit anti-rat SCP_2 IgG; lane 5 immunoprecipitate of adrenal SCP_2 and anti-rat liver SCP_2 IgG; adrenal cytosolic supernatant after immunoprecipitation with anti SCP_2 IgG.

As shown in Fig. 1 a, Coomassie blue staining of samples of SCP_2, adrenal cytosol, IgG fractions and immunoprecipitates was not sufficiently sensitive to detect the low levels of SCP_2 in immunoprecipitates of authentic SCP_2 and adrenal cytosol. By overstaining the Coomassie-stained gels with silver (Fig. 1b), the presence of proteins with electrophoretic characteristics comparable to authentic SCP_2 became apparent.

As shown in Fig. 1b, the preparation of SCP_2, which has been reported to be homogeneous by several criteria, including the unique amino acid composition (7), was dissociated into two discernable bands, under these electrophoretic and staining conditions, with approximate molecular weights of 13,700 and 14,300 (1 and 2). With silver staining, these bands were green suggesting that the difference in molecular sizes might relate to the number of sialic acid residues. Warne et al (5) have recently purified an active fraction from bovine adrenal cytosol which stimulates cholesterol side chain cleavage in a reconstituted system The electrophoretic pattern of the proteins in this fraction appears identical to that obtained for SCP_2, namely two closely associated bands with molecular weights $< 15,000$. Similarly, Trzaskos and Gaylor (10) have described the presence of two electrophoretic protein bands by heat treatment of homogeneous SCP_2. The electrophoretic patterns of the IgG preparations (Fig. 1a and 1b, lanes 3 and 4) demonstrated the presence of 2 major bands corresponding to the heavy and light chains of IgG. Electrophoresis of the dissociated SCP_2 -IgG_i complex (1 and 5) indicated the presence of the two small molecular weight materials observed during the electrophoresis of SCP_2 alone. There appeared, however to be a predominance of the higher molecular weight peptide. The same electrophoretic pattern was observed with the dissociated immunoprecipitate from adrenal cytosol (lane 6). Similar results were obtained with immunoprecipitates from liver and ovarian cytosols.

DISCUSSION A variety of low molecular weight proteins have been implicated in lipid transfers or exchanges between intracellular organelles (e.g. 7-10). Among these are sterol carrier proteins (7,9,10), fatty acid binding protein (z-proteins), which is chemically distinct from SCP_2 (7), and phospholipid exchange proteins (e.g. 20). Current evidence suggests that SCP_2 and at least one of the phospholipid exchange protein (PEP) have identical amino acid compositions and electrophoretic characteristics, and may be the same protein (7). Furthermore, antisera to the purified PEP cross reacts with a pure preparation of a sterol carrier protein (10). The cellular roles of these proteins have yet to be described.

The SCP_2 used in the current studies has been shown to sequester cholesterol in a stoichiometeric relationship and can quantitatively transfer this sterol to adrenal mitochondria, in which conversion of cholesterol to pregnenolone is blocked by aminoglutethimide (11). Furthermore, the protein can stimulate utilization of endogenous mitochondrial cholesterol in the absence of an extra-mitochondrial source of substrate (11,12). This finding has proven useful as a bioassay for SCP_2-like proteins in adrenal cytosolic preparations (12), and has been applied in the present studies.

The stimulatory effect of tissue cytosols in pregnenolone synthesis by adrenal mitochondria is completely neutralized following treatment with specific anti-SCP_2 IgG. Although these finding do not reflect on the number and nature of the immunoreactive proteins in these cytosolic preparations, it is clear that SCP_2 or related proteins are present in these tissues with high rates of cholestero turnover. In steroidogenic tissues, these proteins may effect cytosolic transfer of cholesterol to membrane organelles, and appear to affect distribution of cholesterol within mitochondrial membranes (12).

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