CHOLESTEROL ESTERS AND STEROIDOGENESIS IN PIG CORPORA LUTEA

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1. Introduction

The corpus luteum of a number of species, including pig, contains quantities of cholesterol esters [1, 2, 3] which vary in concentration during the oestrus cycle [4]. Since an inverse relationship obtains between the amount of cholesterol esters present, and the progestational secretory activity [1] it was proposed that the sterol esters were stored precursors for ovarian steroidogenesis [4, 5, 6]. That luteinizing hormone, which stimulates steroidogenesis, induces depletion of ovarian cholesterol esters [7, 8] is well established. The present study demonstrates that (i) cholesterol from cholesterol-fatty acids (FA) ester can be converted to pregnenolone and progesterone in luteal mitochondria and (ii) that the FA moiety of the sterol ester provides a potential source of electrons for the steroid hydroxylation reactions necessary for this conversion. The cholesterol side-chain cleavage (SCC) reaction is energy dependent when FA is the sole source of electrons.

2. Materials and methods

4-^{14}C-cholesterol (58 μCi/mg) and 4-^{14}C-cholesteryl-oleate (46 μCi/mg) were obtained from the Radiochemical Centre, Amersham: both were purified before use as described previously [9]. Palmitic, stearic, oleic, linoleic and linolenic acids, carnitine co-enzyme A (CoA), ATP, bovine serum albumin (BSA) (fatty acid free), D,L-isocitrate and succinate were obtained from Sigma (London). Palmityl-carnitine was prepared by the method of Fritz and Yue [10].

Experimental details of the preparation of mitochondria from porcine ovarian corpora lutea, and the SCC assay were described previously [9]. Whole mitochondria (approximately 5 mg protein) were incubated in 1 ml of medium consisting of 200 mM sucrose, 25 mM Tris-HCl (pH 7.4), 10 mM potassium phosphate (pH 7.4) 20 mM KCl, 5 mM $MgCl_2$ and 0.2 mM tetra-sodium EDTA with substrates and inhibitors as required. The reaction, which was started by adding 1 μg 4-^{14}C-cholesterol (100,000 cpm), proceeded at 37° for 1 hr in an atmosphere of air, and was stopped with 1 ml methanol containing 4 μg cholesterol, 2 μg pregnenolone and 2 μg progesterone. An identical assay procedure was used when 4-^{14}C-cholesteryl-oleate was substrate, 100,000 cpm 4-^{14}C-cholesteryl-oleate replacing the 4-^{14}C-cholesterol, and cholesteryl-oleate (4 μg) as well as the other steroids being added as carriers at termination of the reaction. After extraction of the remaining substrates and products of reaction with hot acetone:methanol:ethyl acetate (2:1:1 by vol) followed by partitioning from water into chloroform, they were separated by TLC on silica gel G as before [9]. The TLC system [di-isopropyl ether:petroleum spirit (60°–80°):acetic acid (70:30:1 by vol)] proved adequate for separating 4-^{14}C-cholesteryl-oleate, 4-^{14}C-cholesterol, 4-^{14}C-pregnenolone and 4-^{14}C-progesterone: the ester ran well ahead of free cholesterol and close to the solvent front.

3. Results and discussion

3.1. *Cholesteryl-oleate as source of cholesterol for steroidogenesis*

Since Armstrong et al. [11] showed cholesteryl-oleate to be the most abundant ester in luteal tissue, this substance was chosen as a representative ester for

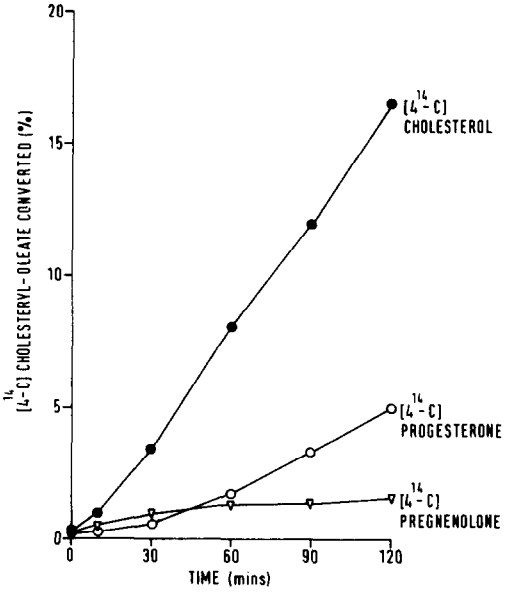

Fig. 1. Time course of 4-^{14}C-cholesteryl-oleate utilization by intact mitochondria from porcine corpora lutea. 4-^{14}-cholesteryl-oleate (100,000 cpm) was incubated with 3 mg of mitochondrial protein in an isotonic buffered sucrose containing 1% BSA, as described in the methods. The electron donor added was D,L-isocitrate (10 mM). The identified products are indicated on the figure.

our *in vitro* experiments. Fig. 1 illustrates the time course of utilization of 4-^{14}C-cholesteryl-oleate by a preparation of porcine luteal mitochondria in terms of the production of 4-^{14}C-metabolites. It can be seen that 4-^{14}C-cholesterol was the first major product of the incubation, while there was a gradual accumulation of the more polar steroids 4-^{14}C-pregnenolone and 4-^{14}C-progesterone. It is clear that cholesteryl-oleate can be hydrolysed by the intact mitochondrial fraction, and the free cholesterol used as substrate for SCC. No compound corresponding to pregnenolone-oleate was isolated: this finding together with the data shown in fig. 1 suggests that hydrolysis of the ester linkage precedes the SCC reaction. Coutts and Stanfield [12, 13] demonstrated cholesteryl esterase (sterol ester hydrolase, EC 3.1.1.13) activity in rat and bovine corpora lutea: this enzyme activity was found predominantly, in the 5000 *g* pellet.

3.2. *Utilization of free fatty acid (FFA) as electron donor for SCC*

Evidence that FFA was the preferred oxidizable substrate in the luteal ovary was obtained by Cooper and Stevenson [14] who showed that the RQ for endogenous substrate of rat luteinized ovary was 0.71 and no other added carbon source could alter this figure. Flint and Denton [15] also showed that oxygen uptake by slices of luteinized rat ovary was unaffected by the addition of glucose to the medium, and later [16] suggested that the main endogenous substrate for respiration in this tissue was FFA liberated from sterol esters.

Our experiments showed that with no added cofactors FFA was completely ineffective in supporting SCC in intact porcine luteal mitochondria. In fact the unsaturated acids, oleic, linoleic and linolenic, which are known to be potent uncouplers of oxidative phosphorylation [17] actually inhibited SCC supported by 500 μM succinate: the sypply of electrons from succinate for steroid hydroxylation is energy dependent [9]. This uncoupling effect of the unsaturated FFA's was overcome by the addition of 1% BSA, in which case there was a slight stimulation of SCC activity by all the previously mentioned FFA as well as palmitic and steraric acid.

The oxidation of FFA by heart mitochondria is enhanced when incubation conditions allow the synthesis of acyl-carnitine intermediates [10]. Our results indicate that the utilization of FFA as electron donor for ovarian luteal steroidogenesis is markedly stimulated under similar conditions. The effect on SCC activity of adding 50 μM oleate together with the FFA activating and transporting agents CoA (50 μM), ATP (3 mM) and carnitine (5 mM) is shown in table 1. The features to note in this table are (i) that ATP was essential for the utilization of oleate as an electron source, (ii) oleate supports SCC more effectively if exogenous CoA as well as ATP is added, and (iii) SCC is most active when all three cofactors are present. The results in the lower section of the table suggest that there is an endogenous supply of FA in the mitochondrial fraction.

Acyl-carnitines can be oxidized directly by mitochondria without prior need for activation [18]. Using

Table 1

The effect of fatty acid activating and transporting agents on cholesterol side-chain cleavage activity in the presence of oleic acid. The method of assay has been described in the methods, activity being expressed as percentage conversion of 4-^{14}C-cholesterol to 4-^{14}C-steroid metabolites. The fatty acid substrate, and cofactors when present were at the following final concentrations: oleic acid, 75 μM; ATP, 3 mM; carnitine, 5 mM; CoA, 50 mM. 3 mg of mitochondrial protein were present in each incubation.

Substrate and cofactors added	Conversion of 4-^{14}C-cholesterol to the following products.		
	4-^{14}C-pregnenolone (%)	4-^{14}C-progesterone (%)	Total conversion (%)
No additions	0.4	0.3	0.7
Oleic acid	0.6	0.8	1.4
Oleic acid + carnitine	0.7	0.6	1.3
Oleic acid + CoA	0.7	0.7	1.4
Oleic acid + ATP	8.4	5.6	14.0
Oleic acid + carnitine + CoA	0.8	0.6	1.4
Oleic acid + carnitine + ATP	11.0	5.9	16.9
Oleic acid + CoA + ATP	17.4	6.4	23.8
Oleic acid + CoA + carnitine + ATP	18.8	9.4	28.2
Carnitine	0.7	0.5	1.2
Carnitine + CoA	1.1	0.2	1.3
Carnitine + ATP	9.0	6.0	15.0
Carnitine + ATP + CoA	13.6	8.0	21.6
CoA	1.0	0.5	1.5
CoA + ATP	10.0	7.2	17.2
ATP	7.5	5.1	12.6

preparations of intact porcine luteal mitochondria, we investigated the relationship between palmityl-carnitine utilization and SCC in the presence of some respiratory inhibitors (fig. 2). Palmityl-carnitine itself supported 6.3% conversion of 4-^{14}C-cholesterol to radioactive products in these incubations. Rotenone (10 μM) markedly stimulated this support, but both amytal (1.8 mM) and antimycin (330 ng/mg mitochondrial protein) inhibited the conversion by approximately 50%. ATP (3 mM) greatly stimulated the SCC reaction in the presence of palmityl-carnitine, and abolished the inhibitory effects of both amytal and antimycin. It appears, therefore, that SCC, when supported by FFA is energy dependent. The β-oxidation of FFA is associated with the reduction of NAD^+, and flavoprotein. Rotenone is known to prevent the oxidation of NADH, while allowing ATP formation from reduced flavoprotein (FPH_2) [19, 20]. Rotenone then, will stimulate FFA supported SCC by allowing ATP formation from FPH_2 while maintaining the electron pressure from NADH to the cytochrome P450 chain via transhydrogenation. Since the transhydrogenase involved in transfer of H^+ from NADH to SCC is not energy dependent in pig luteal mitochondria but support of SCC by succinate is, [9] it is suggested that, in the absence of inhibitors, electrons from FA reach cytochrome P450, at least in part, via the flavoprotein dependent acyl-CoA dehydrogenase. Antimycin and amytal probably inhibit by interfering with ATP formation; the former inhibiting the oxidation of both NADH and FPH_2 while the latter interfere with energy transfer reactions [21, 22].

4. Conclusion

It has been shown that both the cholesterol and FA moieties of cholesteryl esters can play an important role in SCC. The utilization of electrons from FFA for cholesterol hydroxylation reactions appears to be energy dependent.

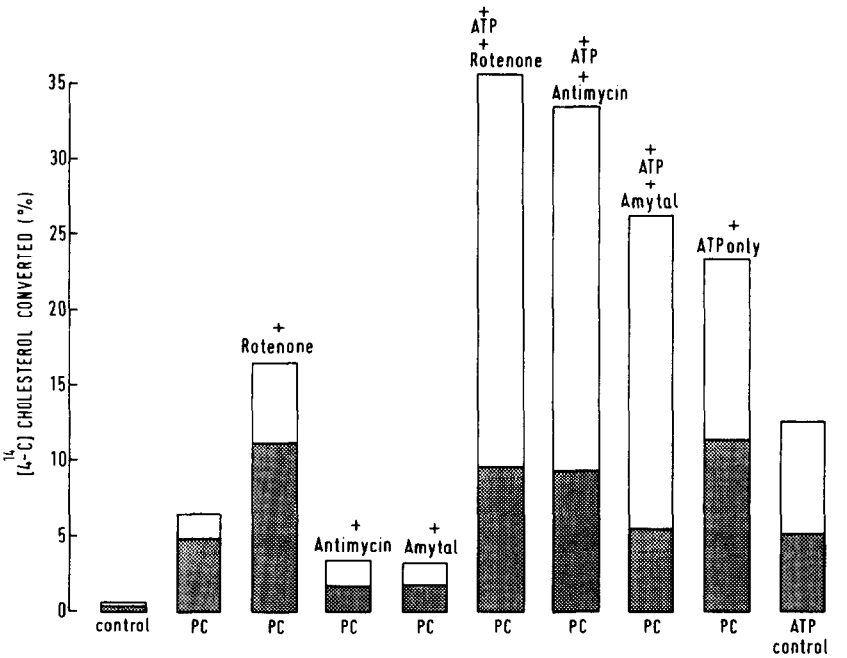

Fig. 2. The effect of respiratory inhibitors on cholesterol side-chain cleavage supported by 50 μM palmityl-carnitine (PC). The assay was described in the text. Rotenone, amytal, antimycin and ATP, present where indicated on the figure, were at final concentrations of 10 μM, 1.8 mM, 330 ng/mg of mitochondrial protein, and 3 mM respectively. The total area in each column represents total 4-^{14}C-steroid metabolites; the hatched areas are 4-^{14}C-pregnenolone, the clear areas 4-^{14}C-progesterone. No PC is present in the controls; the first contains mitochondria and 4-^{14}C-cholesterol, the second has 10 μM ATP added.

Acknowledgements

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