A C T A B I O C H I M I C A P O L O N I C A
Vol. 30 1983 No. 1

BARBARA TIAŁOWSKA, JERZY KLIMEK and LEON ŻELEWSKI

PROGESTERONE BIOSYNTHESIS SUPPORTED BY FATTY ACID OXIDATION IN THE MITOCHONDRIAL FRACTION OF HUMAN TERM PLACENTA*

Department of Biochemistry, Medical School,
80-211 Gdańsk, ul. Dębinki 1, Poland

Oleic acid or oleoyl-CoA in the presence of bovine serum albumin, CoA, ATP, carnitine and malate were found to support progesterone biosynthesis from cholesterol in human term placental mitochondria. This fatty acid-dependent progesterone biosynthesis increased in the presence of adenine nucleotides and decreased after the addition of fluorocitrate. In the presence of rotenone only the stimulatory effect of ATP was maintained, whereas the inhibitory effect of fluorocitrate on the conversion of cholesterol to progesterone was abolished.

Similar results were obtained when pyruvate was used as substrate instead of fatty acids.

In the presence of antimycin, neither ATP nor fluorocitrate affected significantly the fatty acid-supported progesterone biosynthesis.

These results indicate that oxidation of fatty acids stimulates progesterone biosynthesis by increasing the regeneration of the reducing equivalents necessary for the cholesterol side-chain cleavage reaction.

The rate-limiting step of progesterone biosynthesis in human placental mitochondria is the NADPH-dependent side-chain cleavage of cholesterol to yield pregnenolone, the precursor of progesterone (Meigs & Ryan, 1968; Simpson & Miller, 1978). Therefore the regeneration of NADPH indispensable for the cholesterol side-chain mixed-function oxidase system plays an important role in the regulation of progesterone biosynthesis in human placental mitochondria. Our findings have shown that in placental mitochondria NADPH for cholesterol side-chain cleavage may be generated by NADP-

* This work was supported by the Ministry of Higher Education, Science and Technology within the project R.1.9.03.04.

-dependent malic enzyme (Bogusławski *et al.*, 1972; 1974), NADP-dependent isocitrate dehydrogenase (Bogusławski *et al.*, 1976; Klimek *et al.*, 1976) and transhydrogenase activities (Klimek *et al.*, 1979).

Robinson & Stevenson (1971) reported on fatty acid oxidation as a source of electrons for cholesterol hydroxylations in corpora lutea. In our previous publication (Tiałowska *et al.*, 1980) the inhibitory effect of fatty acids on the conversion of cholesterol to progesterone in the presence of isocitrate as a source of reducing equivalents was described. We suggested that the effect of non-metabolized fatty acids is a result of their action on the cholesterol side-chain mixed-function oxidase system but not on the NADPH-generating system. Conditions favouring binding or oxidation of fatty acids restore progesterone biosynthesis in the mitochondrial fraction (Tiałowska *et al.*, 1980). Therefore, we suppose that the oxidation of fatty acids would yield NADH and isocitrate which could subsequently generate NADPH *via* both energy-linked transhydrogenase and isocitrate dehydrogenase.

The present work was undertaken to study the regulatory effect of fatty acids in progesterone biosynthesis in the human placental mitochondria. The data obtained indicate that fatty acid oxidation can support the biosynthesis of progesterone from cholesterol in these mitochondria.

MATERIALS AND METHODS

Oleic acid, oleoyl-CoA, citric acid, pyruvic acid, $NADP^+$, CoA, DL-carnitine·HCl, rotenone and bovine serum albumin essentially fatty acid free (BSA) were purchased from Sigma Chemical Co (St. Louis, Mo., USA); antimycin from Calbiochem (U.S.A.); progesterone from Koch-Light Lab. (Colnbrook, Bucks., England); AMP, ADP, and ATP from the Medical School, Łódź (Poland); dimethylformamide, sucrose "AnalaR" and Tris from BDH Chemicals Ltd (England); [4-^{14}C]cholesterol (spec. act. 58 mCi/mmol), [^{3}H]progesterone (12 Ci/mmol) from the Radiochemical Centre (Amersham, England). All other chemicals used were analytical grade products purchased from POCh (Gliwice, Poland). Four times distilled water from all-quartz apparatus was used throughout.

For isolation of mitochondria, human term placentas were cooled to 0 - 4° C within 30 min after delivery and the following procedure was started immediately. The blood was drained off, membranes removed and the tissue obtained (mainly chorionic villi) was rinsed three times with 0.9% NaCl and then three times with the solution containing 0.25 M-sucrose, 5 mM-EDTA and 10 mM-Tris/HCl buffer (pH 7.4). Approximately 100 g of the tissue was passed through a meat grinder and suspended in 200 ml of the isolation medium containing 0.25 M-sucrose, 5 mM-EDTA and 10 mM-Tris/HCl (pH 7.4). The resulting mince was homogenized manually in a glass Potter-Elvehjem homogenizer with a Teflon pestle. The homogenate was filtered

through three layers of surgical gauze; and the residue was rehomogenized in the same medium. The combined homogenates were centrifuged at 600 *g* for 10 min and the supernatant was centrifuged again at 9000 *g* for 15 min. The pellet obtained was washed twice with about 20 ml of 0.25 M-sucrose *plus* 10 mM-Tris/HCl (pH 7.4) and after each washing centrifuged at 9000 *g* for 15 min.

Incubation was carried out at 37°C in air with constant shaking for 1 h. The incubation mixture contained in a total volume of 2.5 ml: 15 mM-KCl, 50 mM-Tris/HCl (pH 7.4), 10 mM-potassium phosphate buffer (pH 7.4), 5 mM-$MgSO_4$, 1 mM-EDTA, 0.5 mM-NADP, 1.0% bovine serum albumin and 5 mg mitochondrial protein. Incubation was initiated by addition of 0.20 μCi of [4-^{14}C]cholesterol in 20 μl of acetone and terminated by freezing the sample instantaneously at −25°C. Oleic acid was dissolved in 25 μl of dimethylformamide.

Each value presented in the Tables represents the mean of three to five determinations of duplicate samples. The differences between separate experiments were about 10%.

Isolation of steroids and radioactivity measurements were performed as described by Klimek *et al.* (1979).

Protein was determined by the biuret method (Layne, 1957).

RESULTS AND DISCUSSION

The effect of oleic acid on progesterone biosynthesis in placental mitochondria was studied in the presence of BSA. The results presented in Table 1 showed that oleic acid was completely ineffective in supporting cholesterol side-chain cleavage reaction in placental mitochondria. The effect of oleic acid *plus* the fatty acid activating and transporting agents: CoA, ATP and carnitine, on progesterone biosynthesis was very low. However, the addition of malate stimulated progesterone biosynthesis strongly. The compe-

Table 1

The effect of fatty acid-activating and transporting agents on the oleic acid-supported progesterone biosynthesis in placental mitochondria

The incubation mixture was as described in Methods. Final concentrations were: 100 μM-oleic acid, 5 mM-DL-carnitine, 3 mM-ATP, 50 μM-CoA, 0.1 mM-fluorocitrate.

Substrate and cofactors added	Progesterone biosynthesis	
	dpm ^{14}C per flask	% of conversion
Oleic acid	0	0
+ carnitine + ATP + CoA	1 330	0.33
+ carnitine + ATP + CoA + malate	15 980	3.6
+ carnitine + ATP + CoA + malate + fluorocitrate	980	0.22

titive inhibitor of aconitase, fluorocitrate, completely abolished oleic acid-supported progesterone biosynthesis. A possible explanation of the stimulatory effect of malate is an increased availability of oxaloacetate for citrate formation after condensation with acetyl-CoA, originating from β-oxidation of oleoyl-CoA. This explanation is supported by the studies indicating that human term placental mitochondria are able to oxidize fatty acids and that the rate of oxidation is significantly higher in the presence of L-malate (Świerczyński *et al.*, 1976b). These results indicate that the conditions which favour the oxidation of fatty acids increase progesterone biosynthesis. Similar results were obtained when oleoyl-CoA was used instead of oleic acid (Table 2):

Table 2

Oleoyl-CoA-supported progesterone biosynthesis by human term placental mitochondria

The incubation mixture was as described in Methods. Final concentrations were: 100 μM-oleoyl-CoA, 5 mM-DL-carnitine, 3 mM-ATP, 0.1 mM-fluorocitrate.

Substrate and cofactors added	Progesterone biosynthesis	
	dpm ^{14}C per flask	% of conversion
Oleoyl-CoA	440	0.10
Oleoyl-CoA + ATP	880	0.20
Oleoyl-CoA + carnitine	2 260	0.51
Oleoyl-CoA + carnitine + ATP	4 000	0.90
Malate	620	0.14
Malate + carnitine	580	0.13
Malate + ATP	1 600	0.36
Malate + ATP + carnitine	1 640	0.37
Oleoyl-CoA + malate	1 330	0.30
Oleoyl-CoA + malate + carnitine	6 220	1.4
Oleoyl-CoA + malate + carnitine + fluorocitrate	3 110	0.7
Oleoyl-CoA + malate + ATP	2 310	0.52
Oleoyl-CoA + malate + ATP + carnitine	33 740	7.6
Oleoyl-CoA + malate + ATP + carnitine + fluorocitrate	1 780	0.4

oleoyl-CoA alone did not stimulate progesterone biosynthesis and only a slight effect of ATP and carnitine or ATP *plus* carnitine was observed in the presence of oleoyl-CoA. A further addition of malate resulted in stimulation of cholesterol side-chain cleavage activity. Addition of ATP produced a strong stimulation of oleoyl-CoA-supported steroidogenesis in the presence of malate *plus* carnitine. Since in this case oleate was present in the activated form, the stimulatory effect of ATP could be interpreted as pointing to the energy dependence of cholesterol hydroxylation by electrons derived from the oxidation of fatty acids. A similar proposal has been made by Robinson & Stevenson (1971) for pig corpus luteum mitochondria. It is thus possible that

the effect of ATP on the oleic acid-supported progesterone biosynthesis in the presence of cofactors (Table 1) was due to both the activation of oleic acid and the energy-dependent transfer of electrons from the oleate-oxidizing system to the cholesterol side-chain cleavage system.

Data presented in Table 2 indicate that progesterone biosynthesis supported by oleoyl-CoA in the presence of malate, ATP and carnitine was also almost completely inhibited by fluorocitrate. One may assume that in the course of fatty acid oxidation the regeneration of reducing equivalents and citrate synthesis can occur. We have previously shown that energy-dependent transhydrogenase and NADP-dependent isocitric dehydrogenase activities are involved in the generation of NADPH for the cholesterol side-chain cleavage system (Bogusławski *et al.*, 1976; Klimek *et al.*, 1976; 1979).

For further study pyruvate was selected (Tables 3 and 4) as a substrate which, like fatty acids, is metabolized to acetyl-CoA. Acetyl-CoA, derived from fatty acids or pyruvate, condenses with oxaloacetate to produce citrate which can be metabolized in the Krebs cycle. Data presented in Table 3 indicate that pyruvate-supported progesterone biosynthesis was negligible even at 10 mM pyruvate concentration. However, the conversion of cholesterol into progesterone increased substantially when malate was added. This is in agreement with previous studies in which it was shown that in human

Table 3

The effect of pyruvate concentration in the presence of malate or malate plus ATP on the progesterone biosynthesis in placental mitochondria

The incubation mixture was as described in Methods. Final concentrations were: 1 mM-malate, 3 mM-ATP, 0.1 mM-fluorocitrate, and pyruvate as indicated.

Additions	Progesterone biosynthesis	
	dpm ^{14}C per flask	% of conversion
Malate	490	0.11
Pyruvate 1 mM	980	0.22
Pyruvate 5 mM	2 980	0.67
Pyruvate 10 mM	3 370	0.76
Pyruvate 1 mM + malate	15 500	3.5
Pyruvate 5 mM + malate	15 100	3.4
Pyruvate 10 mM + malate	16 400	3.7
Pyruvate 1 mM + malate + fluorocitrate	7 990	1.8
Pyruvate 5 mM + malate + fluorocitrate	7 550	1.7
Pyruvate 10 mM + malate + fluorocitrate	8 880	2.0
Pyruvate 1 mM + malate + ATP	50 600	11.4
Pyruvate 5 mM + malate + ATP	57 700	13.0
Pyruvate 10 mM + malate + ATP	60 400	13.6
Pyruvate 1 mM + malate + ATP + fluorocitrate	14 650	3.3
Pyruvate 5 mM + malate + ATP + fluorocitrate	15 540	3.5
Pyruvate 10 mM + malate + ATP + fluorocitrate	15 980	3.6

Table 4

The effect of AMP, ADP, and ATP and respiratory chain inhibitors on the pyruvate-supported progesterone biosynthesis in placental mitochondria

The incubation mixture was as described in Methods. Final concentrations were: 1 mM-pyruvate, 1 mM-malate, 3 mM-AMP, 3 mM-ADP, 3 mM-ATP, 0.1 mM-fluorocitrate, 8 μM-rotenone.

Additions	Progesterone biosynthesis	
	dpm ^{14}C per flask	% of conversion
Pyruvate	1 470	0.33
+ AMP	1 690	0.38
+ ADP	3 690	0.83
+ ATP	5 330	1.2
+ ATP + fluorocitrate	1 860	0.42
+ rotenone	3 770	0.85
+ AMP + rotenone	1 780	0.40
+ ADP + rotenone	4 260	0.96
+ ATP + rotenone	23 980	5.4
+ ATP + rotenone + fluorocitrate	21 760	4.9
+ malate	18 650	4.2
+ malate + AMP	51 950	11.7
+ malate + ADP	52 400	11.8
+ malate + ATP	53 300	12.0
+ malate + ATP + fluorocitrate	15 500	3.5
+ malate + rotenone	14 210	3.2
+ malate + AMP + rotenone	10 200	2.3
+ malate + ADP + rotenone	27 970	6.3
+ malate + ATP + rotenone	45 300	10.2
+ malate + ATP + rotenone + fluorocitrate	47 100	10.6

placental mitochondria pyruvate alone was metabolized poorly whereas the addition of malate at a low concentration increased the oxygen uptake with pyruvate as substrate (Świerczyński *et al.*, 1976a).

The biosynthesis of progesterone from cholesterol, supported by pyruvate *plus* malate, strongly increased in the presence of ATP and decreased after addition of fluorocitrate. These effects on progesterone biosynthesis with pyruvate as an oxidizable substrate are essentially similar to those obtained with oleic acid or oleoyl-CoA (Tables 1 and 2).

As may be seen from Table 3, maximal stimulation of pyruvate-supported progesterone biosynthesis was achieved in the presence of ATP. It was therefore interesting to investigate the effect of ATP and other adenylate nucleotides on this biosynthesis. The results of experiments reported in Table 4 indicate that the stimulatory effect of ATP exceeded the effect of ADP especially when rotenone was present in the incubation medium. AMP did not stimulate progesterone biosynthesis in the presence of pyruvate. In contrast to the different effects of adenylate nucleotides on progesterone

biosynthesis in the presence of pyruvate, all the adenine nucleotides tested showed a stimulatory effect when pyruvate *plus* malate was present in the incubation medium. This is probably due to higher oxidation of pyruvate in the presence of malate and a corresponding conversion of AMP and ADP to ATP. The addition of rotenone abolished the stimulatory effect of AMP and ADP but not that of ATP on the pyruvate *plus* malate-supported progesterone biosynthesis. These results suggest that the effect of AMP and ADP is mainly a consequence of ATP formation from these nucleotides. Since adenylate kinase activity is present in human term placental mitochondria (Schreiner & Villee, 1965), AMP can be transformed to ADP and then phosphorylated to ATP during pyruvate oxidation.

It is known that the enzyme for cholesterol side-chain cleavage, solubilized from mitochondria of human term placenta, has an absolute requirement for NADPH, which can not be replaced by NADH (Mason & Boyd, 1971). This implies that the stimulatory effect of ATP on pyruvate *plus* malate-supported progesterone biosynthesis is due to the increased ATP-dependent flux of electrons from NADH, generated by pyruvate dehydrogenase, to the NADPH pool. This probably occurs *via* the pyridine nucleotide transhydrogenase, previously reported to be present in placental mitochondria (Villee *et al.*, 1960).

The effect of pyruvate presented here is similar to that of α-ketoglutarate on progesterone biosynthesis in placental mitochondria, described by Klimek *et al.* (1979). The α-ketoglutarate-supported progesterone biosynthesis was also stimulated in the presence of ADP and ATP. Comparison of the effect of α-ketoglutarate *plus* ATP (Klimek *et al.*, 1979) and that of pyruvate *plus* malate and ATP (Table 4) indicates that the conversion of cholesterol into progesterone was higher when pyruvate *plus* malate and ATP were present in the incubation medium. Addition of rotenone caused a slight increase of the α-ketoglutarate *plus* ATP-supported steroidogenesis but no stimulatory effect of rotenone was observed in the presence of pyruvate *plus* malate and ATP. It is possible that NADH produced in the presence of pyruvate *plus* malate significantly exceeded the NADH pool regenerated by α-ketoglutarate.

The addition of fluorocitrate abolished the stimulatory effect of ATP in the presence of both pyruvate and pyruvate *plus* malate (Table 4). The effect of fluorocitrate, which is a known inhibitor of aconitase, might suggest that a further metabolism of citrate generated from acetyl-CoA is responsible for the stimulation of progesterone biosynthesis by pyruvate. It has been found that fluorocitrate in human placental mitochondria decreases citrate-supported progesterone biosynthesis (Klimek *et al.*, 1976). However, a lack of the inhibitory effect of fluorocitrate in the presence of rotenone on progesterone biosynthesis supported by pyruvate *plus* malate and ATP indicates that the inhibition is connected not only with the inhibition of aconitase. Świerczyński *et al.* (1976c) observed in the placental mitochondria

an incomplete inhibition by fluorocitrate of pyruvate *plus* malate oxidation at fluorocitrate concentration which inhibited completely citrate oxidation. Thus the decreased NADH pool regenerated by pyruvate might be an indirect cause of the slight progesterone biosynthesis in the presence of fluorocitrate. This lower NADH pool could be oxidized mainly *via* the respiratory chain to slow down the supply of electrons for the pyridine nucleotide transhydrogenase involved in the cholesterol side-chain cleavage system. When rotenone was added all NADH was available for transhydrogenation. These results clearly indicate that NADH supplied by pyruvate dehydrogenase can be used by placental mitochondria to support progesterone biosynthesis and that energy is required to divert the electrons from NADH to NADPH pool.

Similar results were obtained when oleoyl-CoA *plus* malate and carnitine was used to generate intramitochondrial NADPH for side-chain cleavage. As may be seen in Table 5, AMP, ADP and ATP supported progesterone biosynthesis in the presence of oleoyl-CoA. Similarly as in the presence

Table 5

The effect of AMP, ADP, ATP and respiratory chain inhibitors on the oleoyl-CoA- or citrate-supported progesterone biosynthesis in placental mitochondria

The incubation mixture was as described in Methods. Final concentrations were: 100 μM-oleoyl-CoA, 1 mM-malate, 5 mM-DL-carnitine, 3 mM-AMP, 3 mM-ADP, 3 mM-ATP, 0.1 mM-fluorocitrate, 8 μM-rotenone, 5 μM-antimycin, 1 mM-citrate.

Additions	Progesterone biosynthesis	
	dpm ^{14}C per flask	% of conversion
Oleoyl-CoA *plus* malate and carnitine	6 220	1.4
+ AMP	28 420	6.4
+ ADP	27 970	6.3
+ ATP	28 860	6.5
+ ATP + fluorocitrate	1 330	0.3
+ rotenone	11 540	2.6
+ AMP + rotenone	8 880	2.0
+ ADP + rotenone	11 990	2.7
+ ATP + rotenone	21 760	4.9
+ ATP + rotenone + fluorocitrate	19 540	4.4
+ antimycin	4 880	1.1
+ AMP + antimycin	3 640	0.82
+ ADP + antimycin	3 600	0.81
+ ATP + antimycin	5 330	1.2
+ ATP + antimycin + fluorocitrate	3 640	0.82
Citrate	20 870	4.7
+ AMP	21 760	4.9
+ ADP	22 640	5.1
+ ATP	27 080	6.1
+ ATP + fluorocitrate	580	0.13
+ ATP + fluorocitrate + rotenone	1 780	0.4

of pyruvate *plus* malate, the addition of rotenone abolished the stimulatory effect of both AMP and ADP, however it had no significant effect on oleoyl-CoA-supported progesterone biosynthesis in the presence of ATP. The progesterone biosynthesis supported by oleoyl-CoA (*plus* malate and carnitine) in the presence of ATP was also inhibited by fluorocitrate. This inhibition was not observed when rotenone was added. These results indicate that progesterone biosynthesis supported by oleoyl-CoA is energy dependent. As β-oxidation of fatty acids is associated with the reduction of NAD and flavoprotein, it appeared that both reduced flavoprotein and NADH can supply electrons for cholesterol side-chain cleavage system *via* energy-dependent transhydrogenation. However, in contrast to rotenone, antimycin which prevented flavoprotein oxidation caused an inhibition of oleoyl-CoA-supported progesterone biosynthesis even in the presence of ATP.

The effect of oleoyl-CoA presented here is in general similar to that described by Robinson & Stevenson (1971) in ovarian mitochondria. They also observed a stimulation of fatty acid-supported progesterone biosynthesis by ATP. However, different effects are noted if we compare the inhibitory action of antimycin on the oleoyl-CoA (with malate and carnitine) *plus* ATP-supported progesterone biosynthesis, with the strong stimulation of steroidogenesis by ATP in the presence of antimycin observed by Robinson & Stevenson (1971). These authors suggested that electrons from fatty acids reach cytochrome P_{450}, at least in part, *via* the flavoprotein-dependent acyl-CoA dehydrogenase. Our results suggest that in placental mitochondria under these conditions the electrons for side-chain cleavage are supplied mainly *via* energy-dependent transhydrogenation from NADH generated by oleoyl-CoA oxidation. β-Oxidation of oleoyl-CoA leads to acetyl-CoA production. Acetyl-CoA condenses with oxaloacetate to produce citrate which can be further metabolized in the Krebs cycle. Therefore, it has been examined whether AMP, ADP and ATP stimulate citrate-supported progesterone biosynthesis (Table 5). In contrast to oleoyl-CoA (*plus* malate and carnitine)--supported progesterone biosynthesis there was no stimulation of the conversion of cholesterol to progesterone by either AMP, ADP or ATP. The limiting effect of adenine nucleotides on the conversion of cholesterol to progesterone in the presence of citrate indicates that the generation of NADPH from citrate is energy-independent. Fluorocitrate reduced citrate--supported progesterone formation but this inhibition could not be reversed by the addition of rotenone. This proves that the stimulation by ATP of oleoyl-CoA (*plus* malate and carnitine)-supported progesterone biosynthesis was dependent upon NADH production from the β-oxidation of oleoyl-CoA.

The results presented in this paper provide evidence for an important role of fatty acids in the regulation of progesterone biosynthesis. The conditions leading to the oxidation of fatty acids increase progesterone biosynthesis by generating reducing equivalents.

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BIOSYNTEZA PROGESTERONU ZALEŻNA OD UTLENIAJĄCYCH SIĘ KWASÓW TŁUSZCZOWYCH W MITOCHONDRIALNEJ FRAKCJI Z ŁOŻYSKA LUDZKIEGO

Streszczenie

Stwierdzono, że kwas oleinowy i oleilo-CoA w obecności albuminy surowicy wołu, CoA, ATP, karnityny i jabłczanu stymulują biosyntezę progesteronu z cholesterolu w mitochondrialnej frakcji z łożyska ludzkiego.

Zależna od kwasów tłuszczowych biosynteza progesteronu wzrasta w obecności nukleotydów adenilowych, natomiast ulega obniżeniu po dodaniu fluorocytrynianu.

W obecności rotenonu stwierdza się stymulujący efekt ATP na konwersję cholesterolu do progesteronu, natomiast zniesione zostaje hamujące działanie fluorocytrynianu.

Podobne wyniki otrzymano z pirogronianem użytym jako substrat oddechowy zamiast kwasów tłuszczowych.

W obecności antymycyny zarówno ATP jak i fluorocytrynian są bez wpływu na stymulowaną przez kwasy tłuszczowe biosyntezę progesteronu.

Uzyskane wyniki wskazują, że utleniające się kwasy tłuszczowe stymulują biosyntezę progesteronu przez zwiększoną regenerację ekwiwalentów redukcyjnych niezbędnych w procesie odszczepienia łańcucha bocznego cholesterolu.

Received 28 June, 1982.