Triacylglycerol synthesis in goat mammary gland

The effect of ATP, Mg^{2+} and glycerol 3-phosphate on the esterification of fatty acids synthesized *de novo*

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Goat mammary-gland microsomal fraction by itself induces synthesis of medium-chain-length fatty acids by goat mammary fatty acid synthetase and incorporates short- and medium-chain fatty acids into triacylglycerol. Addition of ATP in the absence or presence of Mg^{2+} totally inhibits triacylglycerol synthesis from short- and medium-chain fatty acids, and severely inhibits synthesis *de novo* of medium-chain fatty acids. The inhibition by ATP of fatty acid synthesis and triacylglycerol synthesis *de novo* can be relieved by glycerol 3-phosphate. The effect of ATP could not be mimicked by the non-hydrolysable ATP analogue, adenosine 5'-[β,γ -methylene]triphosphate and could not be shown to be caused by inhibition of the diacylglycerol acyltransferase by a phosphorylation reaction. Possible explanations for the mechanism of the inhibition by ATP are discussed, and a hypothetical model for its action is outlined.

Goat milk fat contains about 25 mol% of short-chain (C_4 – C_6) and medium-chain (C_8 – C_{12}) fatty acids (Marai *et al.*, 1969). These fatty acids are all synthesized in the mammary gland and esterified into triacylglycerol (Moore & Christie, 1977). Goat mammary-gland fatty acid synthetase can synthesize fatty acids of short, medium and long chain length (Grunnet & Knudsen, 1979). The termination mechanism for short- and medium-chain fatty acids is a transacylation reaction, resulting in release of the synthesized fatty acids as acyl-CoA (Hansen & Knudsen, 1980; Knudsen & Grunnet, 1982). The synthesis of medium-chain fatty acids is dependent on a simultaneous removal of the acyl-CoA produced (Grunnet & Knudsen, 1981). Long-chain fatty acids are released as free fatty acids by a thioesterase (Grunnet & Knudsen, 1983). The microsomal fraction of goat mammary gland is able to remove medium-chain acyl-CoA from the fatty acid synthetase complex. Grunnet & Knudsen (1981) originally showed that goat mammary microsomal fraction could only induce medium-chain-length fatty acid synthesis and incorporate the fatty acids into triacylglycerol in the presence of ATP, Mg^{2+} and glycerol 3-phosphate. However, other experiments showed that microsomal fractions preloaded with diacylglycerol could induce medium-chain fatty acid synthesis by goat mammary fatty acid synthetase and incorporate these

fatty acids into triacylglycerol (Grunnet & Knudsen, 1981). In the above experiments it was observed that ATP alone or in combination with Mg^{2+} completely inhibited induction of synthesis of medium-chain fatty acids by the microsomal fraction (I. Grunnet & J. Knudsen, unpublished work). These observations led us to investigate further the effects of ATP, Mg^{2+} and glycerol 3-phosphate on the ability of the microsomal fraction to induce medium-chain fatty acid synthesis.

Materials and methods

Materials

NADPH, L-glycerol 3-phosphate, adenosine 5'-[β,γ -methylene]triphosphate and dithiothreitol were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Acetyl-CoA synthetase, myokinase, pyruvate kinase, ATP and CoA were obtained from Boehringer, Mannheim, West Germany, and sodium [1- ^{14}C]acetate was from The Radiochemical Centre, Amersham, Bucks., U.K. Silica-gel HR and all other reagents were obtained from E. Merck, Darmstadt, West Germany.

Methods

Malonyl-CoA was synthesized by the method of Eggerer & Lynen (1962). [1- ^{14}C]Acetyl-CoA was synthesized enzymically. Acetyl-CoA synthetase

(0.25 mg; 3 units/mg), myokinase (50 μ g; 360 units/mg) and pyruvate kinase (50 μ g; 200 units/mg) were incubated at room temperature in 1.0 ml of a mixture containing 80 mM-Tris/HCl, pH 8.0, 1 mM-EDTA, 150 mM-KCl, 4 mM-MgCl₂, 10 mM-phosphoenolpyruvate, 3 mM-ATP, 4.7 mg of CoA and 4.6 mM-sodium [1-¹⁴C]acetate (54 Ci/mol). The incubation continued for 70 min, and the end product was then purified by high-performance liquid chromatography, on a stainless-steel column (25 cm $\times$ 0.41 cm) packed with Lichrosorb RP-18 (10 μ m). The eluent consisted of a mixture of 50 mM-potassium phosphate, pH 5.0, and ethanol, and the flow rate was 2.8 ml/min. The elution was performed with a gradient (increasing by 0.6%/min) from 2% to 9% ethanol. The fraction containing [1-¹⁴C]acetyl-CoA was collected and freeze-dried overnight. Unlabelled acetyl-CoA was added before desalting on a Sephadex G-10 column (2.5 cm $\times$ 55 cm), eluted with water (20 ml/h), and the sample was finally adjusted to pH 4.5 with HCl and stored at -80°C. Recovery of [1-¹⁴C]acetate from the synthesis was typically 70%.

Lactating goats 2-3 weeks *post partum* were used. Subcellular fractionation of mammary gland and purification of fatty acid synthetase were performed as described by Knudsen (1972). Preincubation of the microsomal fraction was performed as described by Haagsman *et al.* (1982).

Fatty acid synthetase was incubated in 1 ml of 0.1 M-potassium phosphate buffer, pH 7.0, containing 30 μ M-[1-¹⁴C]acetyl-CoA (4.2-17.1 Ci/mol), 1 mM-EDTA and 240 μ M-NADPH. Malonyl-CoA was infused continuously (2 nmol/min) as described by Grunnet & Knudsen (1979). The incubations were divided into two portions, of 250 μ l and 750 μ l. An equal volume of 5 M-NaOH was added to the 250 μ l portion of each incubation to stop the reaction, and the pattern of fatty acids synthesized was determined by radio-g.l.c. (Knudsen *et al.*, 1981). Chloroform/methanol (1:2, v/v; 3 ml) was added to the remaining 750 μ l of the incubation mixture to stop the reaction, and lipids were extracted by the method of Bligh & Dyer (1959), except that 0.1 M-HCl was used instead of water. Individual neutral lipids were separated by t.l.c. on silica-gel HR plates (20 cm $\times$ 20 cm; 0.5 mm-thick coating) developed with hexane/diethyl ether/methanol/acetic acid (90:20:3:2, by vol.). Phospholipids were separated on similar plates developed in chloroform/methanol/acetic acid/water (50:25:7:3, by vol.). Lipid spots were detected by iodine vapour. The fatty acids in the lipid classes were analysed by radio-g.l.c. as described above. Radioactivity was counted as described by Marshall & Knudsen (1977). Protein was precipitated with 15% (w/v) trichloroacetic acid and measured

by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Results

Addition of increasing amounts of microsomal fraction, without added ATP, Mg²⁺ and glycerol 3-phosphate, to incubations containing goat mammary-gland fatty acid synthetase stimulated synthesis of all individual fatty acids, especially medium-chain fatty acids (Fig. 1a). However, at high concentration of the microsomal fraction (4.3 mg/ml) a strong inhibition of fatty acid synthesis was observed. The synthesis of short-chain (C_{4:0}-C_{6:0}) and long-chain (C_{14:0}-C_{16:0}) fatty acids was independent of their incorporation into triacylglycerol. In contrast, the synthesis of C_{10:0} fatty acid was closely related to its incorporation into triacylglycerol (Fig. 1b).

Long-chain fatty acids (C_{14:0}-C_{16:0}) were not incorporated into triacylglycerols, probably owing to the lack of ATP and Mg²⁺, necessary for their activation, in the incubation medium. Lauric acid was incorporated at low concentration of microsomal fraction, indicating that the goat mammary fatty acid synthetase can terminate at this chain length by the transacylation mechanism. Addition of ATP in the presence of Mg²⁺, but in the absence of glycerol 3-phosphate, totally inhibited incorporation of short- and medium-chain fatty acids synthesized *de novo* into triacylglycerols and synthesis of medium-chain fatty acids *de novo* (Table 1). The inhibition of medium-chain fatty acid synthesis is most probably secondary to the inhibition of incorporation of these acids into triacylglycerols. Only long-chain fatty acids synthesized *de novo* were incorporated into triacylglycerols, showing that the ATP inhibition of triacylglycerol synthesis is specific for medium- and short-chain fatty acids.

The inhibitory effect of ATP on synthesis of medium-chain fatty acids and incorporation of short- and medium-chain fatty acids could be observed at low ATP concentration in the absence of added Mg²⁺, and in the presence of 1 mM-EDTA (Fig. 2a). The inhibition of medium-chain fatty acid synthesis and incorporation into triacylglycerol was almost maximal at 0.5 mM-ATP (Fig. 2b). The synthesis of butyric acid was unaffected by the ATP addition, but the synthesis of long-chain fatty acids, especially C_{14:0}, was increased, probably owing to the decreased termination at medium-chain length. Addition of glycerol 3-phosphate in the presence and absence of Mg²⁺ could partly or completely relieve the ATP effect on synthesis of medium-chain fatty acids (Table 1 and Fig. 3a) and on incorporation of short- and

Fig. 1. Effect of microsomal fraction on fatty acids synthesized by lactating goat mammary-gland fatty acid synthetase: (a) total fatty acids synthesized; (b) fatty acids incorporated into triacylglycerols

The incubation system is described in the Materials and methods section. The incubations (1.0 ml) contained 105 μ g of fatty acid synthetase (specific activity 960 nmol of NADPH oxidized/min per mg of protein), microsomal fraction as indicated and 30 μ M-[1- 14 C]acetyl-CoA (sp. radioactivity 4.1 Ci/mol). Malonyl-CoA was infused at a rate of 2.0 nmol/min for 15 min. The values for acetate incorporations are means $\pm$ half the difference between duplicates. Synthesis of individual fatty acids is marked as follows: $\circ$, $C_{4:0}$; $\square$, $C_{6:0}$; $\triangle$, $C_{8:0}$; ∇ , $C_{10:0}$; $\bullet$, $C_{12:0}$; $\blacksquare$, $C_{14:0}$; $\blacktriangle$, $C_{16:0}$. The values shown are typical results from at least three individual experiments with microsomal preparations and fatty acid synthetase from at least two separate goats.

medium-chain fatty acids into triacylglycerol (Table 1 and Fig. 3b).

When glycerol 3-phosphate was added in the presence of ATP, but in the absence of Mg^{2+} , incorporation of fatty acids synthesized *de novo* was observed into both phosphatidic acid and diacylglycerol as well as into triacylglycerol (Fig. 4). Under these conditions decanoic acid is the main fatty acid incorporated into all the lipid classes. About 80% of the synthesized decanoic acid was recovered in the analysed lipid classes, and decanoic acid could not be detected in the non-esterified fatty acids fraction. The large incorporation into phosphatidic acid seen in this experiment is due to the lack of added Mg^{2+} , which is necessary for the conversion of phosphatidic acid into diacylglycerol (Marshall & Knudsen, 1977). When Mg^{2+} was added to the medium, only insignificant amounts of labelled fatty acids were found in phosphatidic acid, whereas the content of fatty acids synthesized *de novo* in diacylglycerol was mainly unchanged (results not shown). Fig. 4 also shows a different incorporation pattern of fatty acids in the various lipid classes. Short-chain fatty acids are almost exclusively incorporated into triacylglycerols, whereas medium- and longer-chain fatty acids are incorporated into both triacylglycerols and diacylglycerols and into phosphatidic acid.

To gain more insight in the nature of the ATP interaction with the incorporation of short- and medium-chain fatty acids into triacylglycerols, incubations with the non-hydrolysable ATP analogue adenosine 5'-[β , γ -methylene]triphosphate were performed. Surprisingly this compound had no effect at all, either on fatty acid synthesis or on the incorporation of fatty acids synthesized *de novo* into triacylglycerols (Table 2).

The above result indicates that the effect of ATP involves hydrolysis in a Mg^{2+} -independent reaction or that sufficient Mg^{2+} is sequestered in the membrane to support this process. Haagsman *et al.* (1982) have reported evidence for inhibition of rat liver diacylglycerol acyltransferase by a phosphorylation/dephosphorylation mechanism. One possible explanation for the observed ATP inhibition of short-chain fatty acid esterification into triacylglycerols would therefore be an ATP inhibition of the diacylglycerol acyltransferase by a phosphorylation mechanism. Short-chain fatty acids are found exclusively in the *sn*-3 position of goat milk triacylglycerols (Marai *et al.*, 1969). We therefore investigated the effect of preincubation of microsomal preparations with ATP- Mg^{2+} in the presence of 50 mM-NaF, but were unable to show any effect of the treatment on the ability of the microsomal fraction to synthesize triacylglycerol and induce medium-chain fatty acid synthesis (Table 3).

Fig. 2. Effect of ATP on (a) fatty acid synthesis, and (b) triacylglycerol synthesis in a system where goat mammary-gland fatty acid synthetase is incubated with goat mammary-gland microsomal fraction

The incubation conditions are described in the Materials and methods section. The incubation mixtures (1.0 ml) contained 174 μ g of fatty acid synthetase (specific activity 832 nmol of NADPH oxidized/min per mg of protein), microsomal fraction (2 mg), 30 μ M-[1-¹⁴C]acetyl-CoA (sp. radioactivity 17.1 Ci/mol), and ATP was added as indicated. Malonyl-CoA was infused at a rate of 2 nmol/min for 15 min. The values for acetate incorporation are means $\pm$ half the difference between duplicates. Synthesis of individual fatty acids is indicated as follows: ○, C_{4:0}; □, C_{6:0}; △, C_{8:0}; ▽, C_{10:0}; ●, C_{12:0}; ■, C_{14:0}; ▲, C_{16:0}. The values shown are

Fig. 3. Effect of glycerol 3-phosphate, in the presence of ATP, on (a) fatty acid synthesis and (b) triacylglycerol synthesis when goat mammary-gland fatty acid synthetase is incubated with the microsomal fraction

The incubation conditions and symbols were as described in Fig. 2 legend, except that 5 mM-ATP was present and glycerol 3-phosphate was added as indicated. The values for acetate incorporation are means $\pm$ half the difference between duplicates. The values shown are typical results from at least three individual experiments with microsomal preparations and fatty acid synthetase from at least two separate goats.

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Table 1. *Effect of ATP and glycerol 3-phosphate on fatty acid synthesis and triacylglycerol synthesis when goat mammary-gland fatty acid synthetase is incubated in the presence of goat mammary-gland microsomal fraction*

The incubation system was as described in the Materials and methods section. Incubations (1.0 ml) contained 105 µg of fatty acid synthetase (specific activity 960 nmol of NADPH oxidized/min per mg of protein), 2 mg of microsomal fraction, 4 mM-MgCl₂ and 30 µM-[1-¹⁴C]acetyl-CoA (sp. radioactivity 4.1 Ci/mol); malonyl-CoA was infused at a rate of 2.0 nmol/min for 15 min; ATP (5 mM) and glycerol 3-phosphate (5 mM) were added as indicated. The values for the radioactivity distribution in fatty acids are means of two determinations. The values for the acetate incorporation are means ± half the difference between duplicates. The values shown are typical results from at least three individual experiments with microsomal preparations and fatty acid synthetase from at least two separate goats. Abbreviations: TG, triacylglycerol synthesis; TFA, total fatty acid synthesis.

Additions				Distribution of radioactivity in fatty acids (mol%)							Acetate incorporated (nmol) from [1- ¹⁴ C]acetyl-CoA into	
ATP	Glycerol 3-phosphate	Microsomal fraction		C _{4:0}	C _{6:0}	C _{8:0}	C _{10:0}	C _{12:0}	C _{14:0}	C _{16:0}	Total fatty acids	Triacylglycerol
—	—	—	TG									
—	—	—	TFA	47	9	1	3	5	22	13	3.74 ± 0.04	—
—	—	+	TG	19	14	18	47	1	1	1		
—	—	+	TFA	40	10	10	23	4	8	6	6.16 ± 0.15	1.23 ± 0.10
+	—	+	TG	—	—	—	—	—	28	72		
+	—	+	TFA	69	9	3	3	5	6	6	4.58 ± 0.01	0.20 ± 0.03
+	+	+	TG	33	20	16	23	5	3	1		
+	+	+	TFA	65	12	8	9	3	2	1	5.51 ± 0.20	2.43 ± 0.33

Fig. 4. *Fatty acids incorporated into various lipid classes by goat mammary microsomal fraction*
The incubation conditions were as described in Fig. 2, except that 5 mM-ATP and glycerol 3-phosphate were present. Values for acetate incorporation are means ± half the difference between duplicates. The values shown are typical results from at least three individual experiments with microsomal preparations and fatty acid synthetase from at least two separate goats. Symbols: □, phosphatidic acid; ▨, diacylglycerol; ■, triacylglycerol.

Table 2. *Effect of ATP and adenosine [β,γ -methylene]triphosphate on fatty acid synthesis and triacylglycerol synthesis when goat mammary-gland fatty acid synthetase is incubated in the presence of goat mammary-gland microsomal fraction* The incubation conditions were as indicated in Fig. 2, except that ATP (1 mM), adenosine 5'-[β,γ -methylene]triphosphate (1 mM) and 8 mM-MgCl₂ were added as shown. The values for the radioactivity distribution in fatty acids are means of two determinations. The values for the incorporation of acetate are means $\pm$ half the difference between duplicates. The values shown are typical results from at least three individual experiments with microsomal preparations and fatty acid synthetase from at least two separate goats. Abbreviations: TG, triacylglycerol synthesis; TFA, total fatty acid synthesis.

Additions			Distribution of radioactivity in fatty acids (mol%)								Acetate incorporated (nmol) from [1- ¹⁴ C]acetyl-CoA into	
ATP (1.0mM)	Adenosine [β,γ-methylene]-triphosphate (1.0mM)	MgCl ₂ (8mM)		C _{4:0}	C _{6:0}	C _{8:0}	C _{10:0}	C _{12:0}	C _{14:0}	C _{16:0}	Total fatty acids	Triacylglycerol
—	—	—	TG	15	6	9	37	28	6			
			TFA	33	11	4	12	12	19	10	4.76±0.40	0.56±0.02
+	—	—	TG			5	16	44	33	3		
			TFA	39	8	4	5	10	24	11	4.49±0.10	0.10±0.01
—	+	—	TG	14	7	9	36	28	7			
			TFA	35	9	5	13	11	18	10	4.52±0.40	0.63±0.03
+	—	+	TG				14	58	28			
			TFA	37	8	4	5	10	25	12	4.23±0.07	0.16±0.01
—	+	+	TG	9	7	10	39	30	6			
			TFA	25	8	5	16	13	22	12	5.52±0.16	0.67±0.01

Table 3. *Effect of preincubation of the microsomal fraction with ATP and NaF on fatty acid synthesis and triacylglycerol synthesis when goat mammary-gland fatty acid synthetase is incubated in the presence of goat mammary-gland microsomal fraction*

The incubation conditions were as described in Fig. 2. A 2 mg sample of pretreated microsomal fraction was added (treatment was described in the Materials and methods section) and the kind of pretreatment was as indicated in the Table. The values for the radioactivity distribution in fatty acids are means of two determinations. The values for the incorporation of acetate are means $\pm$ half the difference between duplicates. The values shown are typical results from at least three individual experiments with microsomal preparations and fatty acid synthetase from at least two separate goats. Abbreviations: TG, triacylglycerol synthesis; TFA, total fatty acid synthesis.

Treatment of the microsomal fraction		Distribution of radioactivity in fatty acids (mol%)							Acetate incorporated (nmol) from [1- ¹⁴ C]acetyl-CoA into	
		C _{4:0}	C _{6:0}	C _{8:0}	C _{10:0}	C _{12:0}	C _{14:0}	C _{16:0}	Total fatty acids	Triacylglycerol
Washed, untreated	TG	25	13	14	38	7	2			
	TFA	42	11	7	18	5	11	6	5.56 $\pm$ 0.22	1.22 $\pm$ 0.11
Preincubated with 5 mM-ATP	TG	22	15	15	36	11	2			
	TFA	36	9	5	16	14	16	5	5.40 $\pm$ 0.13	1.18 $\pm$ 0.02
Preincubated with 5 mM-ATP and 50 mM-NaF	TG	21	13	14	39	13	1			
	TFA	43	8	6	14	11	13	6	5.44 $\pm$ 0.12	1.13 $\pm$ 0.01
Preincubated with 5 mM-ATP, 50 mM-NaF and 5 mM-glycerol 3-phosphate	TG	18	12	14	40	13	2			
	TFA	38	10	7	21	7	12	5	5.65 $\pm$ 0.03	1.22 $\pm$ 0.10

Discussion

The present experiments show that the induction of medium-chain fatty acid synthesis by the microsomal fraction itself is closely linked to the

incorporation of these acids into triacylglycerol. The present experiments, together with previously published results, which showed that either ATP, glycerol 3-phosphate and Mg²⁺ or microsomes pre-

loaded with diacylglycerols (Grunnet & Knudsen, 1981) are necessary for induction, strongly indicate that an active triacylglycerol-synthesizing system is required for induction.

Experiments with albumin or 2,6-di-*O*-methyl- α -cyclodextrin have shown that the induction involves a removal or dissociation of medium-chain acyl-CoA derivatives, synthesized by the goat mammary fatty acid synthetase, from the enzyme complex (Knudsen & Grunnet, 1982). The factors involved *in vivo* in this removal process are not known, but the present experiment shows that the removed acyl-CoA species are not only available for the diacylglycerol acyltransferase, because a large incorporation is observed into both diacylglycerol and phosphatidic acid when ATP and glycerol 3-phosphate are added in the absence of Mg²⁺ to the incubation system (Fig. 4).

The results also show that, although the inhibitory effect of ATP on medium-chain fatty acid synthesis and incorporation of short- and medium-chain fatty acids into triacylglycerol is independent of added Mg²⁺, it still requires the hydrolysis of ATP. The non-hydrolysable ATP analogue adenosine 5'-[β , γ -methylene]triphosphate was unable to mimic the ATP effect.

The experiments did not confirm the possibility of inhibition of the diacylglycerol acyltransferase by a phosphorylation reaction, similar to what has been suggested for rat liver diacylglycerol acyltransferase (Haagsman *et al.*, 1982).

One possible explanation for the ATP inhibition could be an activation of endogenous free fatty acids in the membranes of the microsomal fraction. The activated fatty acids could be incorporated into triacylglycerols in preference to medium- and short-chain acyl-CoA synthesized *de novo*. Such a mechanism could as well explain inhibition of medium-chain fatty acid synthesis as the inhibition of short-chain fatty acid incorporation into triacylglycerol. It has previously been shown that palmitoyl-CoA effectively competes with butyryl-CoA in the diacylglycerol acyltransferase reaction in cow mammary-gland microsomal fraction

(Marshall & Knudsen, 1980). The mechanism outlined above could also explain the glycerol 3-phosphate effect. The added glycerol 3-phosphate can act as an acceptor for the activated fatty acids and thereby prevent acylation of endogenous diacylglycerol with long-chain fatty acids, and supply 1-acylglycerol phosphate, which can be acylated with decanoyl-CoA synthesized *de novo* to give phosphatidic acid containing C_{10:0}, as seen in Fig. 4. The above hypothetical model is tested in the following paper (Hansen *et al.*, 1984).

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