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Regulation of triacylglycerol synthesis in permeabilized rat hepatocytes

Role of fatty acid concentration and diacylglycerol acyltransferase

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Abstract

Isolated hepatocytes from fed and starved rats, permeabilized with *Staphylococcus aureus* α-toxin, were incubated with increasing concentrations of radiolabelled fatty acids, in the presence of a saturating concentration of 3-GP. Incorporation of label into LPA, PA and DAG was lower in cells from starved rats than in cells from fed rats, apparently reflecting the lower activity of GPAT after starvation. This enzyme approached saturation at high fatty acid levels and determined the overall flux through the esterification pathway. TAG synthesis, however, was the same in both nutritional states and could not be saturated with fatty acid under the given conditions. Taken together with the observed accumulation of DAG, these data suggest that the rate of TAG synthesis is controlled by the fatty acid supply and, more particularly, by the affinity of DGAT for acyl-CoA.

Key words: Triacylglycerol synthesis; Regulation, Rat hepatocyte

1. Introduction

The hepatic synthesis of TAG's proceeds mainly along a sequence of four reactions [1] : acylation of 3-GP to LPA ; acylation of LPA to PA ; hydrolysis of the phosphate ester bond of PA, yielding DAG ; finally, acylation of DAG to TAG. These reactions are catalysed by, respectively, GPAT, LPAT, PPH and DGAT.

We have recently shown that rat hepatocytes permeabilized with *Staphylococcus aureus* α-toxin are a very useful model for the study of TAG synthesis and its interaction with other pathways such as phosphatidylcholine synthesis [2]. At fixed fatty acid and increasing 3-GP concentrations, the total flux (i.e. the combined rates of synthesis of TAG, phosphatidylcholine and, in some instances, DAG) was shown to be determined by the activity of GPAT. Phosphatidylcholine and TAG appeared to derive from the same pool of DAG and their relative rates of synthesis were dependent on the level of CDP-choline. However, since the hepatic 3-GP concentration in vivo is saturating for GPAT [3], it was concluded that the fatty acid supply is the rate-limiting factor under physiological conditions.

In the current work we have confirmed and extended these observations by carefully analysing the rate of synthesis of TAG and the levels of the different intermediates of the esterification pathway, as a function of fatty acid concentration, in permeabilized hepatocytes from fed and starved rats.

2. Experimental

2.1. Animals

Male Wistar rats weighing 150–200 g were used in all experiments. The animals had unlimited access to water and were fed a standard pelleted diet ad libitum, or starved for 24 h.

2.2. Preparation and incubation of hepatocytes

Hepatocytes were prepared and the plasma-membrane was permeabilized for hydrophilic low molecular weight compounds as described previously [2]. Briefly, the 'Incubation medium' consisted of 40 mM MOPS pH 7.2, 10 mM $KHCO_3$, 3% (w/v) Dextran F70, 2% (w/v) fatty acid free BSA, 5 mM GSH, 140 mM potassium L-glutamate, 4 mM ATP, 4 mM $MgSO_4$, 0.025% (w/v) *Staphylococcus aureus* α-toxin, 1 mM 3-GP, 0.5 mM L-carnitine, 0.1 mM CoA and increasing concentrations (0.25–1 mM) of [1-^{14}C]long chain fatty acid (palmitate/oleate, 1 : 1, 1 Ci/mol). Incubations were started by adding $2{,}5 \times 10^6$ cells/ml and lasted 6 or 12 min. In some experiments labelled 3-GP (1 Ci/mol) was used in combination with unlabelled long chain fatty acid (palmitate/oleate, 1 : 1).

2.3. Esterification and oxidation

The rates of TAG synthesis and β-oxidation and the incorporation of label into the intermediates of TAG synthesis (LPA, PA and DAG)

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Abbreviations: 3-GP, *sn*-glycerol 3-phosphate; LPA, lysophosphatidate; PA, phosphatidate; DAG, diacylglycerol; TAG, triacylglycerol(s); GPAT, glycerophosphate acyltransferase (EC 2.3.1.15); LPAT, lysophosphatidate acyltransferase (monoacylglycerolphosphate acyltransferase, EC 2.3.1.51); PPH, phosphatidate phosphohydrolase (EC 3.1.3.4); DGAT, diacylglycerol acyltransferase (EC 2.3.1.20); CPT I, carnitine palmitoyltransferase I (EC 2.3.1.21).

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at 6 and 12 min were measured as described [2]. As explained in the text, the data for starved rats in Fig. 1 are taken from the same reference. The rate of β-oxidation (Fig 1a) is shown as nmol fatty acid incorporated into acid soluble oxidation products per min and per 10^8 hepatocytes. TAG synthesis (Fig. 1b) is expressed as (nmol fatty acid incorporated per min and per 10^8 cells)/3 ; DAG accumulation (Fig. 1c) is shown as (nmol fatty acid incorporated per min and per 10^8 cells)/2. The steady-state labelling of LPA (Fig. 1d) and PA (Fig. 1e) is shown as, respectively, nmol fatty acid incorporated per 10^8 cells, (nmol fatty acid incorporated per 10^8 cells)/2. The divisors 2 or 3 refer to the number of fatty acid molecules incorporated per molecule of glycerol ester. For the purpose of clarity, the ordinates of Fig. 1 (panels b–e) are labelled in terms of nmol of the different glycerol esters.

2.4. Other methods

Radiolabelled acyl-CoA was determined as described previously [4].

2.5. Reagents

The source and quality of the materials has been described [2].

3. Results and discussion

Studying the effect of fatty acid concentration on TAG synthesis is rather difficult because three enzymes of this pathway link fatty acid to 3-GP: GPAT, LPAT and DGAT. The poor water solubility of all intermediates of TAG synthesis and their association with intracellular proteins and membranes precludes the measurement of true concentrations, complicating the task. Furthermore, it is not known to what extent hydrolases might limit the accumulation of the pathway intermediates. In spite of all this and as explained in previous work [2], we believe that measuring the incorporation of labelled 3-GP or fatty acid into the lipids is a valid approach. An additional control was performed by comparing the incorporation, into DAG and TAG, of 0.2 mM labelled fatty acid in the presence of 1 mM unlabelled 3-GP on the one hand, and of 1 mM labelled 3-GP in the presence of 0.2 mM unlabelled fatty acid on the other. For cells from starved rats the ratio (mean ± S.E.M.) of nmol fatty acid incorporated over nmol 3-GP incorporated was 1.84 ± 0.22 (n = 6) for DAG and 2.95 ± 0.51 (n = 6) for TAG. For cells from fed rats the corresponding ratios were 2.02 ± 0.41 (n = 4) and 2.90 ± 1.08 (n = 4). These numbers are not significantly different from the theoretical ratios (2 for DAG, 3 for TAG), indicating that there was no significant dilution of labelled fatty acid by endogenous, unlabelled, fatty acid.

Hepatocytes from fed rats were incubated in the presence of increasing concentrations of radiolabelled fatty acid together with optimal concentrations of other cofactors necessary for β-oxidation and TAG synthesis (see section 2). Notably, 3-GP was added at a concentration of 1 mM, assuring saturation of GPAT with this substrate [2]. The same experiments have been done with cells from starved rats and have been published in a previous paper [2]. However, in that report the fatty acid curves (see Fig. 3 in the cited reference) were drawn by simply connecting the means of the individual data points at each fatty acid concentration and obscured the fact that LPA and PA verged on saturation at high fatty acid levels. This led to an underestimation of the importance of the fatty acid concentration and the data have been replotted here in Fig. 1, together with the new data obtained for the fed state, and subjected to non-linear regression.

Fig. 1, panel a, shows that the rate of β-oxidation was higher in hepatocytes from starved rats, as compared to fed rats. This effect of the nutritional state probably cannot be ascribed to differences in cytosolic malonyl-CoA levels since permeabilization of the hepatocytes should entail an approximately hundred-fold dilution of small and hydrophilic intracellular molecules in the incubation medium [2]. The levels of labelled acyl-CoA in both nutritional states were not different (not shown). Rather, an increase in the intrinsic activity of CPT I in starvation [5] may explain the observed increase in the rate of β-oxidation in the starved state. Fig. 1b shows the rate of incorporation of labelled fatty acid into TAG. It can be seen that the rates in the fed and the starved state are the same, as has been shown in intact rat hepatocytes [6]. The relationship between TAG synthesis and fatty acid concentration can be approximated by a straight line; there is no indication for a plateau at high fatty acid levels.

On the other hand, the incorporation of fatty acid into the intermediates of TAG synthesis, LPA (Fig. 1d), PA (Fig. 1e) and DAG (Fig. 1c), is lower in the fasted than in the fed state and clearly levels off at high fatty acid concentrations, in both nutritional states. The labelling of LPA and PA reaches a steady state after approx. 4 min of incubation [2] and Fig. 1d and e show the means of the values obtained at 6 and 12 min. DAG, however, accumulates and Fig. 1c represents the rate of synthesis of this intermediate between 6 and 12 min of incubation. It has been shown that the activity of GPAT, measured in liver homogenates or subcellular fractions, is up to 50% lower after starvation [3,7], and plateaus at a high acyl-CoA supply (high acyl-CoA:albumin ratio) [8]. These observations are in accordance with the present results, showing lower fatty acid incorporation into the esterification intermediates in the starved state and saturation at high fatty acid levels.

The steady-state levels of LPA and PA correlate in a linear fashion (compare Fig. 1d and e), suggesting that the activity of LPAT is determined by the supply of LPA and that this enzyme, under our conditions, is saturated with acyl-CoA, even at the lowest concentration of fatty acid used (0.25 mM). The curves in Fig. 1e would appear sigmoidal if LPAT were not saturated with one of its substrates. Barden and Cleland [9] have shown that the K_m of LPAT for acyl-CoA is less than 100 nM in rat liver microsomes. The alternative possibility, that LPAT would be saturated with LPA and that the PA labelling would be determined by the supply of acyl-CoA, cannot

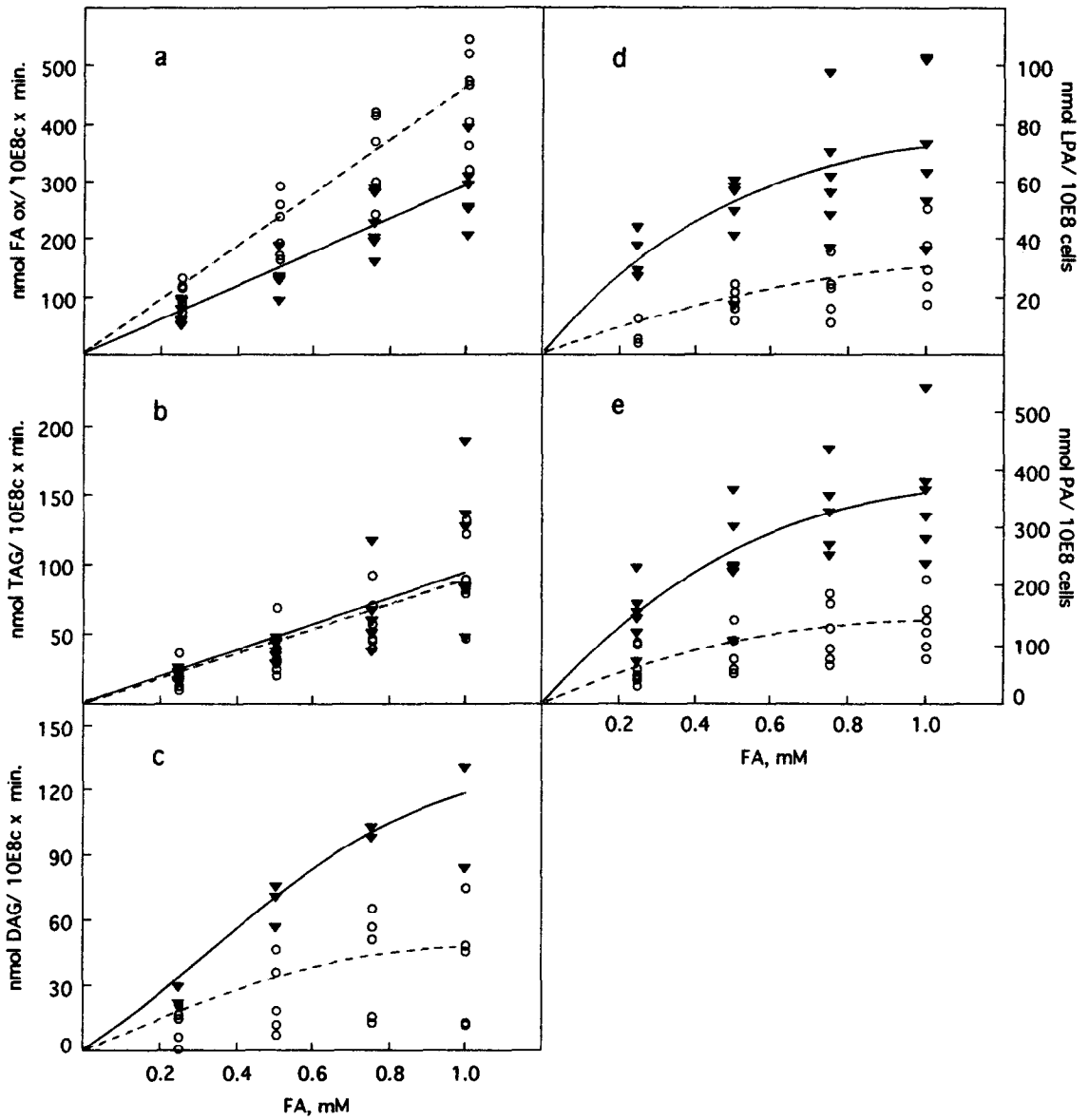

Fig. 1. Permeabilized hepatocytes, prepared from starved (open circles, broken lines) or fed (closed triangles, solid lines) rats, were incubated with increasing concentrations of labelled fatty acid, in the presence of 1 mM 3-GP and other cofactors, as described in section 2. Rates of β-oxidation (panel a) and TAG synthesis (panel b), incorporation of label into LPA (panel d) and PA (panel e), as well as the accumulation of labelled DAG (panel c) are determined and expressed as described in section 2. The results are individual data points and regression curves, calculated as described [2]. Selection of best fitting curve (linear – hyperbolic – sigmoidal) was based on the residual sum of squares. The curves for the fed and starved state are significantly different at the 95% confidence level (F-test) for all panels, except b.

be excluded but seems less likely given the striking similarity of the curves in Fig. 1d and e.

Fig. 1c shows a fatty acid dependent accumulation of DAG, the labelling of which does not reach a steady state (see above). This agrees with previous experiments with permeabilized hepatocytes [2], in which we have shown that DGAT and cholinephosphotransferase draw from the same pool of DAG and that this pool only reaches a steady state in the presence of exogenous CDP-choline. The choline nucleotide is absent in the present experiments and phosphatidylcholine is synthesized at very low rates (not shown).

Based on the present data and the results presented in the previous paper [2] the regulatory role of the different enzymes of the TAG synthesis pathway can be delineated as follows.

GPAT sets the maximal flux for the synthesis of glycerolipids (i.e. all lipids derived from 3-GP) since this enzyme is saturated with 3-GP under physiological conditions [2] and becomes saturated at high fatty acid concentrations (present experiments). The saturation of GPAT with both its substrates does not necessarily limit the rate of TAG synthesis (see below). The influence of the nutritional state on the incorporation of fatty acid

into LPA, PA and DAG appears to be due to the lower GPAT activity in the starved state. Since the cofactors for phospholipid synthesis are not present or diluted out in the incubation medium, the flux through the esterification pathway can be calculated here as the sum of the rates of synthesis of TAG and DAG (Fig. 1b and c) and is lower in the starved state than in the fed state (not shown).

LPAT propagates the flux and plays no regulatory role: its activity is determined by the LPA supply, which is dependent on the activity of GPAT.

PPH does not appear to be rate-limiting under our conditions. This conclusion is based on several data : the linear relationship between PA levels and flux [2]; the strong correlation between LPA and PA levels in both nutritional states (this paper) and the lower PA levels in the starved state in the face of unaltered TAG synthesis rates (this paper).

At 1 mM 3-GP, which is a near-physiological level [2], and in the absence of CDP-choline DGAT is saturated with DAG, since this intermediate accumulates. Under these conditions the rate of TAG synthesis is determined by the acyl-CoA supply and the activity of DGAT, which depends, at least in part, on its affinity for acyl-CoA. Indeed this enzyme appears to have the highest K_m for acyl-CoA, as compared to the other acyltransferases of the pathway, and is not saturated with acyl-CoA, even at a fatty acid concentration of 1 mM. This can be deduced from the linear relationship between the TAG synthesis rate and the fatty acid concentration (Fig. 1b) combined with the saturation or near-saturation of LPAT and GPAT with acyl-CoA (Fig. 1d and e, and discussion above). On the basis of experiments with cultured rat hepatocytes Mayorek and Bar-Tana [10] also concluded that the apparent K_m for palmitate with respect to DAG production is lower than the apparent K_m for overall palmitate incorporation into TAG. Finally, Fig. 1b also indicates that the activity of DGAT is not affected by the nutritional state. There is no firm evidence in the literature to contradict this. Haagsman et al. [11] demonstrated that DGAT can be regulated by a phosphorylation-dephosphorylation mechanism and, in an earlier report, the same group showed that the specific activity of DGAT, measured in microsomes from rat liver with endogenous DAG as substrate, was not changed by the nutritional state of the animal, although the total activity was lower after starvation [12].

Taken together, the data strongly suggest that DGAT is saturated with DAG and that the rate of TAG synthesis is determined by the fatty acid supply and the relatively low affinity of DGAT for acyl-CoA. The higher level of labelled DAG in cells from fed rats is presumably caused by the higher activity of GPAT in the fed state. The fact that the DAG accumulation levels off at high fatty acid concentrations may be due to the saturation of GPAT on the one hand and the continuing increase of DGAT activity with increasing fatty acid concentrations on the other hand. The experiments presented in Fig. 1 were reiterated using radiolabelled 3-GP and unlabelled fatty acid. The results (not shown) were similar to those presented in Fig. 1.

In summary, the data indicate that in permeabilized rat hepatocytes the overall flux through the glycerolipid synthesis pathway is limited by the supply of fatty acid and the activity of GPAT, while the major control point for TAG synthesis is at the level of DGAT. Through separate lines of evidence, other investigators [13,14] also concluded that DGAT is the rate limiting step in hepatic TAG synthesis. In agreement with them, we did not find any indication that PPH plays a regulatory role, although this enzyme has repeatedly been designated as the rate limiting enzyme of hepatic glycerolipid synthesis [15,16].

The results presented here and elsewhere [2] are the first to provide a broad kinetic profile of the TAG synthesis pathway obtained under near-physiological conditions, and offer a comprehensive framework for further studies on the regulation of TAG synthesis.

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References

[1] Bell, R.M. and Coleman, R.A. (1980) Annu. Rev. Biochem. 49, 459–487.
[2] Stals, H.K., Mannaerts, G.P. and Declercq, P.E. (1992) Biochem. J. 283, 719–725.
[3] Declercq, P.E., Debeer, L.J. and Mannaerts, G.P. (1982) Biochem. J. 204, 247–256.
[4] Stals, H.K. and Declercq, P.E. (1992) Anal. Biochem. 202, 117–119.
[5] Saggerson, E.D. and Carpenter, C.A. (1982) Biochem. J. 208, 673–678.
[6] Debeer, L.J., Declercq, P.E. and Mannaerts, G.P. (1981) FEBS Lett. 124, 31–34.
[7] Van Tol, A. (1974) Biochim. Biophys. Acta 357, 14–23.
[8] Rider, M.H. and Saggerson, E.D. (1983) Biochem. J. 214, 235–246.
[9] Barden, R.E. and Cleland, W.W. (1969) J. Biol. Chem. 244, 3677–3684.
[10] Mayorek, N. and Bar-Tana, J. (1985) J. Biol. Chem. 260, 6528–6532.
[11] Haagsman, H.P., de Haas, C.G.M., Geelen, M.J.H. and Van Golde, L.M.G. (1982) J. Biol. Chem. 257, 10593–10598.
[12] Groener, J.E.M., Klein, W. and Van Golde, L.M.G. (1979) Arch. Biochem. Biophys. 198, 287–295.
[13] Mayorek, N., Grinstein, I. and Bar-Tana, J. (1989) Eur. J. Biochem. 182, 395–400.
[14] Rustan, A.C., Christiansen, E.N. and Drevon, C.A. (1992) Biochem. J. 283, 333–339.
[15] Brindley, D.N. (1984) Progr. Lipid Res. 23, 115–133.
[16] Sessions, V.A., Martin, A., Gomez Munoz, A., Brindley, D.N. and Salter, A.M. (1993) Biochim. Biophys. Acta 1166, 238–243.