Regulation of Triacylglycerol Synthesis in the Liver

MODULATION OF DIACYLGLYCEROL ACYLTRANSFERASE ACTIVITY IN VITRO

(Received for publication, March 3, 1982)

Hendrik P. Haagsman, Catharina G. M. de Haas, Math J. H. Geelen, and Lambert M. G. van Golde

From the Laboratory of Veterinary Biochemistry, State University of Utrecht, Biltstraat 172, 3572 BP Utrecht, The Netherlands

Acyl-CoA:1,2-diacylglycerol O-acyltransferase (EC 2.3.1.20) of rat liver microsomes could be inactivated in vitro by incubation with ATP, Mg++, and a 105,000 x g rat liver supernatant. Of the nucleotides tested, ATP was the most effective in inactivating diacylglycerol acyltransferase.

The rate of this inactivation was not influenced by the addition of cAMP. Treatment of rat liver microsomes with high concentrations of the catalytic subunit of cAMP-dependent protein kinase did not result in the inactivation of diacylglycerol acyltransferase.

The activity of diacylglycerol acyltransferase was lower in microsomes isolated in the presence of fluoride (50 mM) than in control microsomes isolated from homogenates containing chloride (50 mM) instead of fluoride. Interestingly, the activity of diacylglycerol acyltransferase was inhibited in the presence of fluoride and that in control microsomes were both reduced to the same level upon treatment of the microsomes with Mg++, ATP, and 105,000 x g supernatant.

In order to investigate whether the inactivated diacylglycerol acyltransferase could be reactivated the microsomes were reisolated, washed, and subsequently incubated with 105,000 x g supernatant of rat liver. Microsomal diacylglycerol acyltransferase could indeed be reactivated by a factor present in the 105,000 x g supernatant. The activating factor appeared to be heat-labile, nondialyzable, and trypsin-sensitive. The reactivation of microsomal diacylglycerol acyltransferase was inhibited in the presence of fluoride (50 mM).

Both inactivation and reactivation of diacylglycerol acyltransferase appeared to be reversible processes; reactivated microsomal diacylglycerol acyltransferase could be inactivated again by incubation with Mg++, ATP, and 105,000 x g supernatant and subsequently reactivated again in the presence of 105,000 x g supernatant.

These findings are consistent with a model in which microsomal diacylglycerol acyltransferase is interconvertible between catalytically inactive and active sites, possibly via a phosphorylation-dephosphorylation mechanism.

The final step in the de novo synthesis of triacylglycerols in the liver takes place in the endoplasmic reticulum (1, 2) and

* This investigation was supported in part by the Netherlands Foundation for Chemical Research (SON), with financial aid from the Netherlands Organization for the Advancement of Pure Research (ZWO). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom all correspondence should be addressed.

† The abbreviation used is: Bq, becquerel (1 Bq = 1 disintegration/s).
ice-cold 0.9% NaCl solution, minced, and homogenized (20%, w/v) with the aid of a Potter-Elvehjem homogenizer. Microsomes were thoroughly washed (see under "Experimental Procedures"), no inactivation of diacylglycerol acyltransferase inactivation was much less pronounced in the presence of some components of the subcellular fraction were estimated by the method of Bradford (7). Freshly isolated microsomes were used in all experiments.

**Inactivation of Microsomal Diacylglycerol Acyltransferase**

- Diacylglycerol acyltransferase was routinely inactivated by incubating rat liver microsomes (3-4 mg of protein/ml) with 10 mM Mg²⁺, 5 mM ATP, and 105,000 × g supernatant from rat liver (8-12 mg of protein/ml) during 15 min at 25 °C in the presence of 50 mM NaF (added as last component). Inactivation of diacylglycerol acyltransferase does not occur in washed microsomes if Mg²⁺ and ATP are added and 105,000 × g supernatant is omitted, which means that a cytosolic factor is needed for inactivation. After the inactivation procedure the microsomes were collected by centrifugation for 60 min at 105,000 × g and subsequently resuspended with the aid of a Potter-Elvehjem homogenizer in medium I.

**Reactivation of Microsomal Diacylglycerol Acyltransferase**

- After inactivation as described in the preceding paragraph, the activity of microsomal diacylglycerol acyltransferase could be restored by incubation of the microsomes with 105,000 × g supernatant obtained from livers of fed rats at 25 °C. In some experiments, microsomes were chromatographed on Sephadex G-50 (medium) prior to the reactivation procedure. The 105,000 × g supernatant was desalted in some cases by Sephadex G-50 chromatography.

**Measurements of Enzyme Activities**

- Diacylglycerol acyltransferase was assayed in the microsomal fraction, using endogenous diacylglycerols as substrates. Microsomes (0.3-0.4 mg of protein) were incubated at 37 °C in a medium which contained the following components: 50 mM potassium phosphate, pH 6.5, 1.0 mM dithiothreitol, 50 mM NaF, bovine serum albumin (5 mg/ml, dialyzed (8) and charcoal-treated (9)) and 50 gm [1-¹⁴C]palmitoyl-CoA (240 Bq/mol) in a total volume of 0.5 ml. The incorporation of palmitoyl-CoA into triacylglycerols was a linear function of time up to 1.5 min. The incubations were terminated after 1 min by the addition of 2 ml of methanol:chloroform (2:1, v/v). After extraction of the lipids (10), the triacylglycerols were isolated by thin layer chromatography on Silica G using petroleum ether (b.p. 40-60 °C), diethyl ether (85:15, v/v) as developing solvent in an atmosphere saturated with NH₃. The silica, containing the triacylglycerol fragments, was scraped from the plate and assayed for radioactivity. Cholinephosphotransferase was assayed in the microsomal fraction as described previously, using endogenous diacylglycerols as substrates (11). Assays were terminated after 2 min of incubation. The incorporation of CPD choline was a linear function of time up to 6 min. Phosphatidic acid phosphohydrolase was measured as described earlier (11) using microsomal bound [¹⁴C]phosphatidic acid as substrate.

**Analysis of Lipids**

- Diacylglycerols were isolated by thin layer chromatography on Silica G using petroleum ether (b.p. 40-60 °C), diethyl ether (85:15, v/v) as developing solvent. The amounts of diacylglycerols were determined as described earlier (12).

## RESULTS

**Inactivation of Diacylglycerol Acyltransferase**

- In agreement with an earlier report from our laboratory (3), diacylglycerol acyltransferase activity could be reduced rapidly by incubating microsomes in the presence of 5 mM Mg²⁺, 1 mM ATP, and a 105,000 × g supernatant fraction from rat liver. During this inactivation, the levels of diacylglycerols in the microsomes remained unchanged. In preliminary experiments (3) we found some inactivation of diacylglycerol acyltransferase by incubation of microsomes with Mg²⁺ and ATP in the absence of a 105,000 × g supernatant. However, if the microsomes were thoroughly washed (see under "Experimental Procedures"), no inactivation of diacylglycerol acyltransferase was observed in the presence of Mg²⁺ and ATP alone.

ATP can be replaced by ADP, GTP, and ITTP, although inactivation was much less pronounced in the presence of these nucleotides (Table I). On the other hand, dATP, β,γ-methylene ATP, AMP, and adenosine were not able to replace ATP. Interestingly, the activity of diacylglycerol acyltransferase was enhanced 2-fold in the presence of CTP. It is important to emphasize that none of the nucleotides affected the assay of diacylglycerol acyltransferase activity (not shown).

Inactivation is dependent on the presence of 105,000 × g supernatant (Fig. 1). No inactivation of diacylglycerol acyltransferase occurred when microsomes were incubated with high concentrations of cAMP-dependent protein kinase (5-15 units/ml) in the presence of 10 mM Mg²⁺ and 5 mM ATP. Inclusion of cAMP (10⁻⁴ or 10⁻³ M) in the inactivation medium had no effect on the rate of inactivation. Also, preincubation of the cytosolic fraction with cAMP for 10 min at 25 °C, before using this fraction to inactivate microsomal diacylglycerol acyltransferase, had no effect on the inactivation (Fig. 2).

Earlier we reported that homogenization of livers in a medium containing 50 mM NaF yielded diacylglycerol acyltransferase activities that were lower than those of control microsomes isolated in a medium without fluoride present. A possible explanation for this observation could be that fluoride

### TABLE I

<table>
<thead>
<tr>
<th>Nucleotide Specificity for Inactivation of Diacylglycerol Atyltransferase</th>
<th>Activity (expressed as percentage of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>ATP</td>
<td>53 ± 7</td>
</tr>
<tr>
<td>ADP</td>
<td>86 ± 1</td>
</tr>
<tr>
<td>AMP</td>
<td>136 ± 12</td>
</tr>
<tr>
<td>Adenosine</td>
<td>121 ± 10</td>
</tr>
<tr>
<td>β,γ-Methylene ATP</td>
<td>129 ± 9</td>
</tr>
<tr>
<td>dATP</td>
<td>162 ± 19</td>
</tr>
<tr>
<td>GTP</td>
<td>70 ± 11</td>
</tr>
<tr>
<td>ITP</td>
<td>76 ± 6</td>
</tr>
<tr>
<td>UTP</td>
<td>83 ± 5</td>
</tr>
<tr>
<td>CTP</td>
<td>208 ± 24</td>
</tr>
</tbody>
</table>

*Average of 4 incubations ± S.D.*
would prevent the action of phosphoprotein phosphatases which would bring the enzyme in a more active state. If indeed a phosphorylation-dephosphorylation mechanism exists that regulates diacylglycerol acyltransferase activity, the fluoride-homogenized livers would contain diacylglycerol acyltransferase that is more phosphorylated. In that case, inactivation of diacylglycerol acyltransferase in control microsomes most eventually lead to the same activities as those found after inactivation of microsomes isolated in the presence of fluoride.

**Fig. 2.** Lack of cAMP to affect inactivation of microsomal diacylglycerol acyltransferase (DGAT). 105,000 x g supernatant was incubated for 10 min at 25 °C in the presence of CAMP (10^-4 M). Subsequently the supernatant was used to inactivate microsomal diacylglycerol acyltransferase. Microsomes (1.67 mg of protein) were incubated in the presence of 10 mM Mg^2+ and 5 mM ATP with control (O) or cAMP-treated (●) 105,000 x g supernatant (10.2 mg of protein/ml). After the indicated periods of incubation time at 25 °C in a total volume of 0.5 ml, the diacylglycerol acyltransferase activity was determined immediately.

**Fig. 3.** Effects of fluoride in the homogenization medium on the inactivation of diacylglycerol acyltransferase (DGAT) in microsomes derived from ad libitum fed and 48-h starved rats. Livers from each group of rats (3 rats per group) were divided into two parts. One part was homogenized in medium I + 50 mM NaF, the other part in medium I + 50 mM NaF. After isolation of the microsomes, diacylglycerol acyltransferase was inactivated (in the presence of fluoride (50 mM) in both preparations), immediately followed by the diacylglycerol acyltransferase assay. For all inactivations 105,000 x g supernatants from livers of fed rats were used. Zero time values of diacylglycerol acyltransferase activities were 82, 86, and 66 pmol-min^{-1} per mg of protein in A (fed rats, livers homogenized in the presence of NaCl), 53, 50, and 50 pmol-min^{-1} per mg of protein in B (fed rats, livers homogenized in the presence of F^-), 40, 40, and 90 pmol-min^{-1} per mg of protein in C (starved rats, livers homogenized in the presence of Cl^-), and 32, 25, and 43 pmol-min^{-1} per mg of protein in D (starved rats, livers homogenized in the presence of F^-). S.D. of the other time points were calculated via the S.D. of the percentage decrease compared to the zero time value of each rat.

**Fig. 4.** Reactivation of diacylglycerol acyltransferase (DGAT). A, diacylglycerol acyltransferase was first inactivated as described under "Experimental Procedures." Subsequently, after re-isolation of the microsomes (by centrifugation for 1 h at 105,000 x g), diacylglycerol acyltransferase was activated by incubation with the cytosolic fraction (10.0 mg of protein/ml) in the presence (●) or absence (○) of 50 mM NaF at 25 °C, immediately followed by the diacylglycerol acyltransferase assay. C, basal activation (i.e., activation in the presence of medium I). Microsomal protein concentration in the activation was 4.3 mg/ml. B, microsomes, incubated for 15 min with only 10 mM Mg^2+ and 105,000 x g supernatant, were reisolated and treated like the inactivated microsomes as described above. Microsomal protein concentration was 5.7 mg/ml. Data are from one experiment out of 3 experiments with similar results.

**Fig. 5.** Activation of diacylglycerol acyltransferase (DGAT) as a function of the amount of added 105,000 x g supernatant. After inactivation of microsomal diacylglycerol acyltransferase (see under "Experimental Procedures") the microsomes were reisolated. Subsequently the microsomes (3.4 mg/ml) were incubated for 15 min at 25 °C with various amounts of 105,000 x g supernatant. Immediately hereafter, diacylglycerol acyltransferase activity was determined.
TABLE II
Effects of various treatments of the cytosolic fraction on the activation of diacylglycerol acyltransferase

Microsomal diacylglycerol acyltransferase was inactivated as described under "Experimental Procedures." After reisolation, microsomes (3.5 mg of protein/ml) were incubated for 15 min at 25 °C with various additions. Activity of diacylglycerol acyltransferase at t = 0 was 28 pmol-min⁻¹ per mg of microsomal protein.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Diacylglycerol acyltransferase activity pmol/min/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>67</td>
</tr>
<tr>
<td>S₁₀₅ (9.4 mg of protein/ml)</td>
<td>244</td>
</tr>
<tr>
<td>S₁₀₅, treated for 5 min at 100 °C (9.4 mg of protein/ml)</td>
<td>74</td>
</tr>
<tr>
<td>S₁₀₅, treated for 60 min at 37 °C (9.4 mg of protein/ml)</td>
<td>365</td>
</tr>
<tr>
<td>S₁₀₅, stored for 9 days at 4 °C (9.2 mg of protein/ml)</td>
<td>672</td>
</tr>
<tr>
<td>S₁₀₅, trypsin-treated (9.4 mg of protein/ml)</td>
<td>66</td>
</tr>
</tbody>
</table>

*Values are from a representative experiment with duplicate assays. The different experiments were repeated 2-3 times with similar results.

S₁₀₅ = 105,000 × g supernatant, gel filtered on Sephadex G-50.

Trypsin-treated; S₁₀₅ was incubated for 60 min at 37 °C with trypsin (1:25, w/w), whereafter trypsin inhibitor was added before incubation with microsomes.

Indeed, incubation of control microsomes with Mg²⁺, ATP, and 105,000 × g supernatant for 10 min reduces the activity of diacylglycerol acyltransferase to the same value as that measured in microsomes isolated in the presence of fluoride (Fig. 3). This does not only hold for rats fed ad libitum but also for rats that were starved for 48 h. These findings suggest a connection between the Mg²⁺-ATP-dependent inactivation of diacylglycerol acyltransferase and the prevention of diacylglycerol acyltransferase activation during homogenization in the presence of fluoride.

Reactivation of Diacylglycerol Acyltransferase—Fig. 4A shows that inactivated diacylglycerol acyltransferase could be reactivated by incubating the microsomes in the presence of a 105,000 × g supernatant. Fluoride (50 mM) inhibited this reactivation. Interestingly, microsomes which had not been inactivated before could not be activated (Fig. 4B).

Reactivation was dependent on the amount of 105,000 × g supernatant protein added (Fig. 5). Sephadex G-50 chromatography of the 105,000 × g supernatant did not affect the activating potential. In addition, during conventional bag dialysis the activating factor was retained in the bag. The activating factor disappeared when the supernatant was boiled for 5 min (Table II). Also trypsin treatment resulted in a disappearance of the activating factor. On the other hand, incubation of the 105,000 × g supernatant

Fig. 6. Reversibility of diacylglycerol acyltransferase (DGAT) activation. Microsomal diacylglycerol acyltransferase was inactivated as described under "Experimental Procedures." After reisolation of the microsomes, diacylglycerol acyltransferase activity could be restored in an activation reaction, requiring 105,000 × g supernatant. After resedimentation the microsomes (1.7 mg of protein/ml) were subjected to a second inactivation reaction. This resulted again in a time-dependent inactivation of diacylglycerol acyltransferase. After centrifugation and resuspension, the microsomes were finally incubated in an activation reaction, which again resulted in activation of the enzyme. During inactivation and activation steps the concentration of 105,000 × g supernatant was 10.2 mg of protein/ml. Aliquots were taken from the inactivation and activation reactions to assay activity of the enzyme immediately. O, activation of diacylglycerol acyltransferase without 105,000 × g supernatant.
Modulation of Diacylglycerol Acyltransferase Activity

Table III

<table>
<thead>
<tr>
<th>Additions</th>
<th>Diacylglycerol acyltransferase activity ( \text{pmol/min/mg protein} )</th>
<th>Phosphatidylic acid phosphohydrolase activity ( \text{nmol/min/mg protein} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (basal activation)</td>
<td>109</td>
<td>0.26</td>
</tr>
<tr>
<td>( S_{100} )</td>
<td>207</td>
<td>5.05</td>
</tr>
<tr>
<td>( S_{nd} ) (10.2 mg of protein/ml, 60 min, 37 °C)</td>
<td>224</td>
<td>1.55</td>
</tr>
<tr>
<td>( S_{nd} ) (11.2 mg of protein/ml, 13 days, 4 °C)</td>
<td>721</td>
<td>0.22</td>
</tr>
</tbody>
</table>

a Activation factor expressed as ability to reactivate diacylglycerol acyltransferase in activation reaction (15 min at 25 °C).

for 1 h at 37 °C led to an increase in reactivating potential (Table II). Prolonged storage of the supernatant at 4 °C resulted in an even more pronounced increase in reactivating potential. At the same time, the activity of soluble phosphatidic acid phosphohydrolase (EC 3.1.1.34) decreased (Table III).

The reaction of diacylglycerol acyltransferase should be a reversible process in order to have physiological relevance. The following experiment was designed to investigate this question. Microsomal diacylglycerol acyltransferase was inactivated by incubation with \( \text{Mg}^{2+}, \text{ATP}, \) and \( 105,000 \times g \) supernatant and subsequently reactivated by treatment with \( 105,000 \times g \) supernatant as described in Fig. 4. After the reactivation, the microsomes were resuspended and incubated again with \( \text{Mg}^{2+}, \text{ATP}, \) and supernatant. Fig. 6 shows that this treatment decreased the activity of diacylglycerol acyltransferase again to the same value as that observed after the first inactivation reaction. After resedimentation and resuspension in medium I, diacylglycerol acyltransferase could be reactivated again (Fig. 6).

The results shown in Fig. 4 suggested that activation of microsomal diacylglycerol acyltransferase is much more pronounced after preincubation of the microsomes with \( \text{Mg}^{2+}, \text{ATP}, \) and \( 105,000 \times g \) supernatant (to inactivate the enzyme) than for control microsomes. Diacylglycerol acyltransferase could be activated directly, i.e., without prior inactivation, in microsomes from livers homogenized in the presence of fluoride and in microsomes from livers of fasted rats (data not shown). This is probably due to the lower diacylglycerol acyltransferase activity in these microsomes (Fig. 3).

Discussion

The results presented here demonstrate that the activity of diacylglycerol acyltransferase in rat liver microsomes can be modulated in a reversible way. Until so far these observations are in line with an already suggested mechanism (3), in which diacylglycerol acyltransferase activity is regulated between catalytically active and inactive states, possibly via a phosphorylation-dephosphorylation mechanism. Because diacylglycerol acyltransferase is an integral membrane protein which uses membrane-bound diacylglycerols as substrates and has not been purified until now, it is very difficult to determine whether diacylglycerol acyltransferase is a protein that indeed can be functionally phosphorylated. Nevertheless, the results suggest that diacylglycerol acyltransferase can be inactivated in a mode consistent with a phosphorylation mechanism.

This inactivation requires ATP, although some inactivation occurs in the presence of ADP, GTP and ITP. AMP, adenosine, dATP, and \( \beta,\gamma\)-methylene ATP were ineffective in replacing ATP in the inactivation reaction. The fact that \( \beta,\gamma\)-methylene ATP cannot replace ATP suggests that cleavage of a phosphate group may play a role in the inactivation reaction. No satisfactory explanation can as yet be offered for the stimulation of diacylglycerol acyltransferase activity by ATP in the inactivation reaction. Like the other nucleotides tested, CTP did not affect the assay of diacylglycerol acyltransferase activity.

Previous studies (3) indicated that diacylglycerol acyltransferase activity can be decreased upon incubation of hepatocytes in the presence of glucagon or cAMP. Yet, in freshly isolated microsomes cAMP had no effect on the inactivation of the enzyme. It is of course unknown at which level cAMP affects diacylglycerol acyltransferase activity.

Some enzymes that are inactivated by a phosphorylation mechanism require cAMP-independent protein kinases in the inactivation reaction (13). An example in lipid metabolism is the microsomal enzyme hydroxymethylglutaryl-CoA reductase (EC 1.1.1.34), which is thought to be an important regulatory enzyme in cholesterol biosynthesis. This enzyme is regulated by reversible phosphorylation (14). Dibutylryl cAMP and glucagon diminish hydroxymethylglutaryl-CoA reductase activity in isolated hepatocytes (15, 16). Yet, the enzyme is not a substrate for cAMP-dependent protein kinase but is inactivated by a specific reductase kinase (17).

Another finding that points in the direction of a phosphorylation-dephosphorylation mechanism is that the presence of fluoride, an anion known to inhibit phosphoprotein phosphatases, in the homogenization medium results in a lower diacylglycerol acyltransferase activity. A similar finding was observed with hydroxymethylglutaryl-CoA reductase (18, 19). Activation would not take place during homogenization under these conditions. Interestingly, diacylglycerol acyltransferase in microsomes isolated from liver of fed and 48-h fasted rats in the presence of fluoride could be inactivated to the same end values as diacylglycerol acyltransferase from control microsomes. This suggests that fluoride protects against dephosphorylation during homogenization.

Inactivated diacylglycerol acyltransferase could be activated upon incubation with \( 105,000 \times g \) supernatant from rat liver, although always some basal activation was observed during incubation at 25 °C in the absence of supernatant (see Figs. 4 and 6 and Table II). As pointed out earlier (3), activation was dependent on the presence of dithiothreitol in the medium. Fluoride inhibited this activation, again suggesting that a phosphatase is the activating factor. The activating factor is non-dialyzable, heat-labile, and trypsin-sensitive, indicating that it is a protein. Interestingly, the factor can be activated upon storage at 4 °C. It is not likely that soluble phosphatidic acid phosphohydrolase is the activating factor in the cytosolic fraction, because this enzyme loses its activity upon storage at 4 °C (Table III). In addition, phosphatidic acid levels in microsomes are low, and consequently microsomal diacylglycerol levels will not be affected by incubation with the cytosolic fraction. Indeed no change in microsomal diacylglycerol levels could be observed. The conversion of diacylglycerols into triacylglycerols was reported to be stimulated by fatty acid binding protein (20). However, the activation of diacylglycerol acyltransferase could not be inhibited...
by flavaspidic acid, an inhibitor of the fatty acid binding protein (21). Like inactivation, activation of diacylglycerol acyltransferase is a reversible process, indicating that this enzyme can be regulated rapidly by interconversion between metabolically active and inactive states. Diacylglycerol acyltransferase activity has not reached its maximum after 15 min of incubation in the presence of 105,000 × g supernatant. It appears that diacylglycerol acyltransferase has a large reserve capacity which can be used when for example the fatty acid concentration is high which results in a higher diacylglycerol acyltransferase activity and a higher rate of triacylglycerol synthesis (6).

Work is in progress to purify the activator protein and inactivation factor. Furthermore, we will investigate the physiological significance of the regulation of diacylglycerol acyltransferase activity as described in this report.

REFERENCES
Regulation of triacylglycerol synthesis in the liver. Modulation of diacylglycerol acyltransferase activity in vitro.

H P Haagsman, C G de Haas, M J Geelen and L M van Golde