Hydrolysis of Bovine Milk Fat Globules by Lipoprotein Lipase: Inhibition by Proteins Extracted from Milk Fat Globule Membrane

GUNHILD SUNDHEIM
Department of Animal Nutrition
Agricultural University of Norway
P.O. Box 26
N-1432 As-N LH
Norway

GUNILLA BENGTSSON-OLIVECRONA
Department of Physiological Chemistry
University of Umeå
S-901 85 Umeå
Sweden

ABSTRACT

Extraction of membrane proteins from milk fat globules by GuHC1 or by MgCl2 made the lipids more accessible to lipolysis by added lipoprotein lipase. The increase in lipolysis paralleled the loss of membrane proteins and was continuous up to 2.5 M GuHCl, which was the highest concentration used. About twice as much protein was extracted with 2.5 M GuHCl as with buffer only. The amount of protein lost was about 50% of total milk fat globule protein. Lipolysis of milk fat globules was inhibited by addition of the extracted protein. The extracted proteins also reduced lipolysis when added to whole milk. More protein was needed to inhibit lipolysis of milk fat globules treated with GuHCl compared with globules treated with buffer only. The inhibition by a given amount of protein decreased if more milk fat globules were used. Protein extracted with MgCl2 had similar effects as those extracted with GuHCl. The major components extracted with MgCl2 migrated in the 40 to 50-kdalton region on sodium dodecyl sulfate-polyacylamide gel electrophoresis. By gel filtration chromatography, two protein fractions were obtained, which inhibited lipolysis more efficiently than the total extract. As has previously been found for inhibition of lipolysis by skim milk, the amount of extracted protein needed to inhibit lipolysis varied between preparations of milk fat globules. Milk with propensity to cold-induced ("spontaneous") lipolysis was normalized by addition of extracted proteins.

INTRODUCTION

Lipolysis in normal bovine milk is low despite the presence of large amounts of lipoprotein lipase (LPL). In some milk samples, however, lipolysis is triggered by cooling ["spontaneous lipolysis", reviewed in (8, 18)]. Cooling probably changes the physicochemical arrangement of milk fat globules (MFG) so that LPL gets access to the lipid. Isolated MFG are resistant to lipolysis if kept warm, but are lipolyzed when chilled (25). In addition, there are factors in skim milk that impede lipolysis (25, 26). Some MFG preparations are more readily lipolyzed by LPL and are less sensitive to inhibition by skim milk (26). Some samples of skim milk are less inhibitory than normal skim milk (26). Thus, both the properties of MFG and the properties of skim milk vary. These variations may explain why some milk samples are more prone to lipolysis, but the molecular mechanisms involved remain unknown.

We previously showed that removal of some proteins from MFG made MFG lipids more accessible to LPL (25). In the present paper we have extracted MFG with guanidine hydrochloride (GuHCl) and with MgCl2 and have studied how lipolysis is affected. We have used
the extracted MFG proteins to inhibit lipolysis of isolated MFG and of whole milk. Both normal milk and milk samples prone to cold-storage lipolysis were used.

MATERIALS AND METHODS

Purification and Labeling of Lipoprotein Lipase

Lipoprotein lipase was purified from bovine skim milk by chromatography on heparin-Sepharose and was iodinated as previously described (5, 29).

Isolation and Extraction of Milk Fat Globules

The MFG were isolated by low speed centrifugation (500 x g for 20 min) of fresh uncooled milk within 1 h after milking (25). The cream layer was removed, suspended in .15 M NaCl, and washed once. All procedures were carried out at room temperature.

For extraction of MFG membrane proteins, 5 M GuHCl (Sigma) or 3 M MgCl₂ were added to washed MFG to obtain the indicated concentrations. After 1 h at room temperature, the MFG were floated by centrifugation, resuspended in .15 M NaCl, and washed twice before they were used as substrate for LPL. All solutions were adjusted to pH 6.8. To obtain the extracted protein, the infranatants from the centrifugations were dialyzed against distilled water. After dialysis, a white precipitate formed that was recovered by centrifugation (1000 x g for 1 h). The precipitate was suspended in .1 M Tris/maleate buffer, pH 6.8, and is called water-insoluble protein. The supernatant, containing the soluble proteins, was lyophilized and then suspended in .1 M Tris/maleate buffer. Solubilization of MFG membrane proteins by GuHCl and by MgCl₂ was previously described (13, 16, 21).

Separation of Membrane Proteins

Proteins extracted by GuHCl were separated by gel filtration on a Sephacryl S-300 (5 x 80-cm column, Pharmacia, Uppsala, Sweden) in 6 M GuHCl, 10 mM Tris/HCl, pH 8.2. The flow rate was 60 ml/h. Fractions of 15 ml were collected. The fractionated proteins were dialyzed against distilled water, lyophilized, and then suspended in .1 M Tris/maleate buffer, pH 6.8, before use.

Analytical Procedures

The MFG were incubated with LPL as described previously (25). Membrane proteins were added to the MFG before LPL, and incubation was then for 24 h at 4°C. Released free fatty acids (FFA) were determined by titration (15). Binding studies with 125I-labeled LPL were performed as described previously (24, 25).

Protein was measured by the Bio-Rad Protein Kit using bovine serum albumin as standard. Polyacrylamide gel electrophoresis in SDS was run according to Laemmli (14) with 15% acrylamide in the separating gel. Reference proteins were from Pharmacia, Uppsala, Sweden.

RESULTS

Extraction of MFG membrane proteins by GuHCl increased hydrolysis of MFG lipids by LPL (Figure 1). The increase paralleled the loss of membrane proteins and was continuous up to 2.5 M GuHCl, which was the highest concentration used. About twice as much protein had been extracted from MFG with 2.5 M GuHCl as with buffer without GuHCl. The amount of protein lost was about 50% of total MFG protein. In concert with previous findings.

Figure 1. Effects of extraction of milk fat globules (MFG) with GuHCl on lipid hydrolysis (●) and on binding of 125I-labeled lipoprotein lipase (LPL) (●). The protein loss at each concentration of GuHCl (●) was calculated as the sum of protein in extracts and the two washings of MFG. The MFG centrifuged in buffer without GuHCl lost an equivalent to .09 mg protein/ml (see 0 M GuHCl). Total assay volume was 2 ml. Binding and lipolysis was measured after 24 h at 4°C. FFA = Free fatty acids.
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Figure 2. Effects of addition of membrane proteins to milk fat globules (MFG) extracted by 2.5 M GuHCl (•) or by .01 M Tris-maleate buffer (○) on A) binding of 125I-labeled LPL and B) lipolysis. The protein used was the total protein fraction extracted from MFG by GuHCl (see Materials and Methods). FFA = Free fatty acids.

(25, 26), binding of LPL to isolated, cooled MFG was high. Treatment with 2.5 M GuHCl increased the fraction bound from 84 to 90%. Although this effect was minor, it also paralleled the loss of MFG protein. No oiling out was seen during extraction of MFG, indicating that their structure was largely intact.

Lipolysis of MFG extracted with 2.5 M GuHCl or with buffer only was inhibited by addition of the extracted protein (Figure 2). More protein (.4 mg/ml) was needed to inhibit lipolysis of MFG treated with GuHCl than that of MFG treated with buffer (.2 mg/ml). Binding of LPL was also reduced by the added protein (Figure 2). However, even with the highest amounts of protein, more than 30% of the LPL remained bound. Under these conditions the bound enzyme was apparently unable to catalyze hydrolysis of MFG lipids. Extracted MFG were difficult to handle after cooling. Addition of .1 mg extracted protein/ml made MFG appear normal again, indicating that some protein bound back to the MFG. Further evidence for this was that addition of more MFG relieved the inhibition (Figure 3). Protein extracted with MgCl2 had similar effects but inhibited at lower concentration (Table 1). The SDS-PAGE showed that the major components extracted with MgCl2 migrated in the 40 to 50-kdalton region (Figure 4). These components also were seen in the GuHCl extract together with proteins in the 30 kdalton and in the 15 to 20 kdalton regions.

Protein extracted by GuHCl was separated by gel filtration (Figure 5). Peaks 3 and 4 were present in all experiments run while 1 and 6 varied. Peaks 2 and 5 were often not detected. Protein from peaks 3 and 4 inhibited binding of 125I-labeled LPL to and lipolysis of MFG (Figure 6). Both protein fractions were more efficient than the total extract. Some inhibition by protein from peaks 1 and 2 was also observed (not shown). Peak 3 appeared to contain mainly the two proteins in the 40 to 50 kdalton region, whereas peak 4 contained material of lower molecular weight (Figure 4).

As was previously found with skim milk (26), the amount of extracted protein needed to inhibit lipolysis varied among preparations of MFG (Table 2). The inhibition obtained by the extracted membrane proteins paralleled that obtained by skim milk. Some MFG preparations were easily inhibited while
TABLE 1. Effects of membrane proteins extracted with magnesium chloride on binding of lipoprotein lipase (LPL) to and lipolysis of milk fat globule.

<table>
<thead>
<tr>
<th>Protein added (µg/ml)</th>
<th>Binding of 125I-labeled LPL (% of total)</th>
<th>Lipolysis (µmol FFA/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>68</td>
<td>2.50</td>
</tr>
<tr>
<td>20</td>
<td>39</td>
<td>1.95</td>
</tr>
<tr>
<td>40</td>
<td>35</td>
<td>1.38</td>
</tr>
<tr>
<td>50</td>
<td>25</td>
<td>.98</td>
</tr>
<tr>
<td>60</td>
<td>19</td>
<td>.58</td>
</tr>
<tr>
<td>70</td>
<td>17</td>
<td>.58</td>
</tr>
</tbody>
</table>

1 FFA = Free fatty acids.

others were not. There was no direct correlation with degree of cold-induced lipolysis in the original milk samples.

Extracted MFG proteins reduced lipolysis when added to whole milk (Figure 7). For this experiment, milk samples with high degree of cold-induced lipolysis were selected. A plateau of less than 1 µmol FFA released was reached with similar amounts of protein for all three milk samples. Also, binding of LPL to cream was reduced (not shown).

DISCUSSION

This study demonstrates that when proteins are removed, the propensity of MFG toward lipolysis increases. When the proteins are added back, the resistance to lipolysis is restored. Lipoprotein lipase, and several other lipases, are inhibited by a variety of lipid binding proteins (10, 19). For instance, the activity of pancreatic lipase against synthetic lipid emulsions is inhibited in a competitive manner by MFG membrane proteins (22) and by β-lactoglobulin and lactoferrin (6). Anderson (1) showed that lipolysis in milk is inhibited by the proteosepeptone fraction of skim milk. It is thus unlikely that specific interactions between the lipase and inhibitory proteins are involved. More likely the mechanism of inhibition is that the lipid binding proteins compete with the lipase for binding sites on the MFG.

The MFG are surrounded by a double membrane that contains specific apoproteins but also intracellular proteins from the milk secretory cells and proteins in pieces of plasma
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Figure 6. Comparison of inhibition by proteins from peak 3 (•) and peak 4 (●) (from Figure 5) with the effects of unseparated extracted protein (●) on A) binding of LPL to and B) lipolysis of milk fat globules (24 h at 4°C). FFA = Free fatty acids.

Figure 7. Inhibition of cold-induced lipolysis in three different milk samples by addition of MFG membrane proteins extracted with 2.5 M GuHCl. The indicated amounts of protein were added before cooling. Incubation was then for 24 h at 4°C with the endogenous lipase in milk. FFA = Free fatty acids.

membranes that adhere during the apical secretory process (17). Immunological identity has been demonstrated between MFG membrane proteins and some whey proteins (13). Some proteins are easily extracted (12, 16, 21)

Table 2. Inhibition by membrane proteins and by skim milk of lipolysis of milk fat globules (MFG) from eight different cows.

<table>
<thead>
<tr>
<th>Milk</th>
<th>Lipolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Whole milk</td>
</tr>
<tr>
<td></td>
<td>(μmol free fatty acids/ml)</td>
</tr>
<tr>
<td>1</td>
<td>.88</td>
</tr>
<tr>
<td>2</td>
<td>1.39</td>
</tr>
<tr>
<td>3</td>
<td>.81</td>
</tr>
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<td>4</td>
<td>1.36</td>
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<td>1.87</td>
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<td>6</td>
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</tr>
<tr>
<td>7</td>
<td>.99</td>
</tr>
<tr>
<td>8</td>
<td>1.52</td>
</tr>
</tbody>
</table>

1 Cold storage lipolysis in whole milk by endogenous lipoprotein lipase (24 h, 4°C).
2 Lipolysis of individual MFG preparations incubated as described in Materials and Methods.
3 As footnote 2 but with addition of 70 μg of MFG membrane protein (extracted by 2.5 M GuHCl, water-insoluble fraction) per ml assay mixture.
4 As footnote 2 but with addition of 200 μl heat-inactivated skim milk (pooled from three normal milk samples) per ml assay mixture.

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and probably exist both bound to MFG and in skim milk. Other MFG proteins, e.g., butyrophiline, are not extracted by mild procedures and are not soluble in buffers without detergents (9, 11). Desorption of membrane constituents during cooling has been reported (2, 3, 4, 7, 27) as well as adsorption of skim milk proteins (20). The changes that occur on cooling are time-dependent and probably involve some as yet undefined structural changes of the MFG.

We had previously shown that lipolysis is enhanced by extraction of the MFG proteins by MgCl₂ or by GuHCl (25), two methods described by others (13, 16, 21). The present study demonstrates that lipolysis of MFG can be effectively inhibited by the extracted proteins. The proteins inhibited lipolysis also when added to whole milk. Inhibition was probably accomplished by binding of the protein to MFG, because more protein was needed to inhibit when higher MFG concentrations were used. Furthermore, MFG extracted with GuHCl required more protein for inhibition than did MFG isolated under mild conditions. The major components in MgCl₂ extracts had apparent molecular weights of about 45,000 and are probably the superficially located proteins described by Mather and Keenan (16). These proteins also were present in GuHCl extracts in addition to proteins with lower molecular weights. Two fractions were isolated by gel filtration. On weight basis, both of these proteins were more effective inhibitors than the total extracted protein.

In normal milk most of the lipoprotein lipase is bound to casein. Lesser amounts of the lipase are found in milk serum and a few percent are bound to MFG (24). In some milk samples, cooling induces binding of more LPL to MFG, and thereby, lipolysis (22, 23, 24, 28, 30). The binding is not saturable and the fraction of LPL that binds depends on properties of both MFG and skim milk (24). There are probably a large number of sites for lipid-binding proteins on MFG. Lipoprotein lipase and other proteins compete for these sites. Even in situations when a large fraction of the LPL binds, the enzyme constitutes a very small fraction of total protein bound to the MFG. Isolated MFG are more sensitive to lipolysis than MFG in whole milk. This could be due partly to dissociation of proteins during isolation, which uncovers sites on the MFG where the lipase can bind.

Increased binding of LPL to MFG is probably an important factor in lipolysis, which is induced by cooling of lipolysis-prone milk samples. An additional factor is degree of availability of the milk lipids to lipase that has bound to the MFG. With isolated MFG, a large fraction of added LPL binds, and this fraction is not increased much by extraction of other proteins from the MFG. Nonetheless, extraction increases lipolysis. With increasing concentrations of GuHCl, up to about 50% of the MFG protein were extracted. Lipolysis increased in parallel with the protein loss. The fraction of LPL bound also increased, but only from 80 to 91%. This increased binding cannot in itself explain the markedly increased lipolysis. Furthermore, when proteins were added back to the MFG, lipolysis was impeded more than was binding of LPL. At the highest concentrations of protein used, there was virtually no lipolysis, but 30% of the LPL remained bound to the MFG. It is apparent that the amount of protein on the MFG not only determines how much lipase can bind but also influences the access of lipase to the triglycerides.

Addition of MFG proteins to milk decreased cold-induced lipolysis. In fact, some milk samples prone to cold-induced lipolysis were normalized by addition of MFG proteins. It has previously been shown that addition of skim milk to isolated MFG decreases lipolysis (Sundheim and Bengtsson-Olivecrona, unpublished data). The MFG from individual milk samples were more or less susceptible to inhibition by membrane proteins as was previously found for inhibition with skim milk (26). The basis for the individual variation is not known, but membrane proteins clearly are potent inhibitors of milk lipolysis and their concentrations or exchange after cooling could be important determinants for the degree of cold-induced lipolysis in bovine milk.

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