Triacylglycerol Is Synthesized in a Specific Subclass of Caveolae in Primary Adipocytes*  

Received for publication, September 13, 2004, and in revised form, October 26, 2004. Published, JBC Papers in Press, November 9, 2004, DOI 10.1074/jbc.C4004292000

Anita Öst‡§, Unn Ortgren‡§, Johanna Gustavsson‡, Fredrik H. Nyström‡§, and Peter Strålfors‡§

From the ‡Department of Cell Biology and Diabetes Research Centre, §Department of Medicine and Care, Linköping University, SE58185 Linköping, Sweden

A principal metabolic function of adipocytes is to synthesize triacylglycerol (TG) from exogenous fatty acids. The level of fatty acids has to be tightly controlled in the adipocyte, as they can act as detergents that rapidly dissolve the plasma membrane, causing cell lysis if allowed to accumulate. Fatty acids therefore have to be efficiently converted to TG and stored in the central lipid droplet. We report that in intact primary adipocytes exogenous oleic acid was taken up and directly converted to TG in the plasma membrane, in a novel subclass of caveolae that specifically contains the protein perilipin. Isolated caveolae catalyzed de novo TG synthesis from oleic acid and glycerol 3-phosphate. Electron microscopy revealed the presence of caveolin and perilipin in caveolae and in lipid-laden bulbs in the plasma membrane, and fluorescence microscopy demonstrated colocalization of fatty acids/TG with caveolin and perilipin at the plasma membrane. A second caveola fraction was isolated, which lacked perilipin and the triacylglycerol synthesizing enzymes. Both caveola fractions contained caveolin-1 and the insulin receptor. The findings demonstrate that specific subclasses of caveolae carry out specific functions in cell metabolism. In particular, triacylglycerol is synthesized at the site of fatty acid entry in one of these caveolae classes.

To gain knowledge of the biochemical pathways of fat metabolism in human physiology is central to understanding the de-regulations and pathology in the adipocyte handling of fatty acids associated with obesity and type 2 diabetes. The adipocyte stores large amounts of triacylglycerol (TG),1 which continually turn over during lipolytic hydrolysis and reacylation of exogenous fatty acids. Human adipocytes do not have the capacity to synthesize significant amounts of fatty acids de novo (1). The large part of triacylglycerol synthesis comes from fatty acids provided by lipoprotein lipase hydrolysis of chylomicron or very low density lipoprotein TG in the capillary blood vessels. Fatty acids act as detergents that rapidly dissolve the plasma membrane, causing cell lysis if allowed to accumulate (2). The flow of fatty acids into the adipocyte can be high and these fatty acids have to be efficiently and rapidly converted to TG and stored away in the central lipid droplet. Several fatty acid-binding proteins that may mediate transfer of fatty acids have been identified (3).

Caveolae invaginations of the plasma membrane are typically 50–100 nm in diameter and are very abundant in adipocytes (4). Caveolae are involved in vesicle-mediated uptake into the cell as well as in organizing signal transduction (5–7). In adipocytes the insulin receptor is localized in caveolae (8, 9) and in response to insulin glucose transporters GLUT4 are translocated to caveolae for glucose uptake (10, 11). Destruction of caveolae causes a reduced uptake of long-chain fatty acids in 3T3-L1 cells (12), and the major structural protein of caveolae, caveolin, and the caveolae-localized protein, CD36, both bind fatty acids (13, 14). We hypothesized that also fatty acids are taken up and released via caveolae of the adipocyte (4). Caveolae membranes are rich in cholesterol and sphingomyelin and are much more resistant to solubilization by detergents than are other parts of the plasma membrane and other cellular membranes. The adipocyte plasma membrane is covered by caveolae invaginations that enlarge the membrane area by 50% and potentially provide relatively detergent-resistant ports for fatty acid entry and exit (4).

Caveolin has been implicated in TG metabolism through its association with so called lipid bodies and with lipid droplets in 3T3-L1 adipocytes and other cell lines (15–18). Caveolin-1 mice that are deficient in caveolin, and caveolae have, moreover, been found to have deranged lipid metabolism and atrophied adipose tissue (19, 20).

We now report that in primary adipocytes exogenous fatty acids are taken up and directly converted to triacylglycerol at the plasma membrane in a novel subclass of caveolae.

EXPERIMENTAL PROCEDURES

Isolation and Incubation of Adipocytes—Adipocytes were isolated by collagenase digestion from Harlan Sprague-Dawley rats (130–160 g, B&K Universal, Sollentuna, Sweden) as described (21). Cells were preincubated with 5 mM insulin for 10 min. [3H]Oleic acid (Amersham Biosciences) or the fluorescent fatty acid analogue C1-BODIPY 500/510 dodecanoic acid (Molecular Probes, Eugene, OR) at 100 μM was added for 40 or 120 s. Incubations were terminated with 2 mM KCN. Cells incubated with [3H]oleic acid were homogenized and caveolae isolated from purified plasma membranes (22).

Isolation of Caveolae—To prepare caveolae fractions without detergent adipocytes were homogenized, and plasma membranes were isolated and disrupted by sonication in 0.5 M Na2CO3, pH 11 (22). This was then adjusted to 45% sucrose in 15 mM Mes, pH 6.5, 100 mM NaCl, 0.3 M Na2CO3 with protease inhibitors, loaded under a continuous 10–40% sucrose density gradient, and centrifuged at 200,000 g for 18 h. Fractions of 1 ml were collected from the bottom of the tube and membranes pelleted by centrifugation. Caveolae purity has been documented (22, 23). A microsomal fraction was obtained as described (22).

For analysis of TG synthesis in isolated membrane fractions, as indicated, isolated caveolae, plasma membranes, or microsomal membrane fractions were transferred to 50 mM Tris-Cl, pH 7.5, 10 mM MgCl2, and 100 μM [3H]oleic acid or 100 μM [14C]glycerol 3-phosphate (Amersham Biosciences) with 200 μM CoA, 5 mM ATP were added. The mixtures were briefly sonicated with a probe-type sonifier and incu-
bated for 30 min at 37 °C. Incubations were terminated by membrane pelleting and lipid extraction.

Lipid Analyses—Membrane fractions were extracted with CHCl3/CH3OH/H2O (85:10:5, by volume) for 2 h. Neutral lipids were applied to thin-layer chromatography (high performance TLC, silicic acid, 10 × 10 cm, Merck). For neutral lipids the plate was developed in CHCl3/CH3OH/H2O (65:35:2.5, by volume) for 2 h and, after drying, in hexane/diethyl ether (65:30:5, by volume) to the top of the plate. Polar lipids were developed in CHCl3/CH3OH/H2O (65:25:4, by volume) (25). Grown marker molecules (from Larodon, Malmö, Sweden) were added as internal standards. Silicic acid was scraped off plates, suspended in 0.2 ml of methanol, and analyzed for radioactivity by liquid scintillation in 2,5-diphenyloxazole/1,4-bis(2-(5-phenyloxazolyl))benzene in toluene.

SDS-PAGE and Immunoblotting—Separated proteins were detected with anti-caveolin-1, anti-insulin receptor β-subunit (Transduction Laboratories, Lexington, KY), anti-perilipin (generous gift of Dr. C. Londos, National Institutes of Health), or anti-fatty acyl-CoA synthetase (generous gift of Dr. J. E. Schaffer, Washington School of Medicine) antibodies. Bound antibodies were detected using ECL-plus horse-radish peroxidase-conjugated anti-IgG as secondary antibody (Amersham Biosciences). Blots were quantitated by chemiluminescence imaging (Las1000, Fuji).

Electron Microscopy—Adipocytes were attached to poly-L-lysine-coated nickel grids, which were flushed with ice-cold 150 mM KCl, 1.9 mM Tris-HCl buffer, pH 7.4. Plasma membranes remaining on the grids were washed, fixed, and treated for immunogold labeling as described (4) using primary antibodies against caveolin-1 or perilipin. Secondary IgG were conjugated with colloidal gold (Aurion, Wageningen, The Netherlands). Grids were lyophilized and coated with 2-nm-thick tungsten in the freeze dryer (26). Transmission electron microscopy was performed with a Jeol EX1200 TEM-SCAN (Jeol, Tokyo, Japan).

Fluorescence Microscopy—Adipocytes were preincubated with 5 nM insulin for 10 min and the fluorescent fatty acid analogue C1-BODIPY 500/510 dodecanoic acid was added for 40 s when incubations were terminated with 2 mM KCN. Cells were rinsed and fixed with 3% paraformaldehyde and attached to poly-L-lysine coated coverslips. Cells were permeabilized with 0.1% saponin, or plasma membrane sheets were prepared by flushing as described above (9). After blocking with bovine serum albumin cells were incubated with indicated primary antibody (anti-caveolin or anti-perilipin) and then fluorescent secondary antibodies (Alexa fluor 594 or 635). Confocal scanning microscopy was performed with a Nikon D-Eclipse CI (Nikon, Tokyo, Japan).

RESULTS AND DISCUSSION

To examine uptake of fatty acids and their conversion to TG by intact cells we incubated primary rat adipocytes with radiolabeled oleic acid, isolated the plasma membranes, and separated caveolae by gradient ultracentrifugation. Caveolar fractions were then analyzed for radiolabel incorporation into TG and other lipids. Caveolae were obtained as two separate milky bands after ultracentrifugation, both of which contained caveolin and the insulin receptor (fractions 6 and 8, Fig. 1a). A shoulder of caveolin suggested also a third caveolar fraction (fraction 4, Fig. 1a), which was not further analyzed herein. The pattern of major proteins was very similar in the two major caveolar fractions (Fig. 1c), demonstrating their close relation. The protein perilipin, which regulates lipolysis (27), and the fatty acyl-CoA synthetase, which catalyzes the first step in the TG synthesis pathway (28), were, however, confined to the higher density caveolar fraction (fraction 6, Fig. 1a). We extracted lipids and analyzed for metabolic conversion products of the [3H]oleic acid by thin-layer chromatography. A substantial fraction of the cellular radioactive fatty acid was recovered in the high density perilipin-containing caveolae as TG but also as diacylglycerol and as fatty acid (Fig. 1b). We did not detect accumulated oleoyl-CoA, phosphatidic acid, or lysophosphatidic acid. It should be noted that larger TG-containing caveolae/lipid bulbs (see below) would have been lost to the large floating lipid fraction during homogenization and the following ultracentrifugation. This fraction contained about 2000- and 3000-fold more radiolabeled TG than the isolated plasma membrane after 40 and 120 s, respectively.

When TG accumulates within the caveolae membrane the membrane has to expand to accommodate increasing amounts of TG and a coordinated synthesis of glycerophospholipids may be required. We analyzed for conversion of the [3H]oleic to glycerophospholipids and found significant amounts of radioactivity as phosphatidylcholine (Fig. 1b) and as an unidentified peak that was neither phosphatidylethanolamine nor phosphatidylsitol in the high density caveolae. Evidently, in intact adipocytes fatty acids are entering the cells and are converted to TG in a specific caveolar fraction.

We also detected some TG, diacylglycerol, fatty acid, and phos-
phatidylincholine in the microsomal fraction, but the total amount of newly synthesized (radioactive) TG in the microsomal fraction was only about half of that in the plasma membrane fraction. The overall rate of [$^3$H]oleic acid conversion to TG in the cells was about 100 nmol/min/ml of cells, which is about one-seventh the maximal rate of catecholamine-stimulated lipolytic hydrolysis of TG (4, 29). It was not possible to directly determine how much of the TG synthesis of the adipocytes is localized to caveolae and therefore the plasma membrane, as newly synthesized TG was continually transferred to the central lipid droplet. We therefore determined the amount of the fatty acyl-CoA synthetase protein present in a purified plasma membrane fraction (including caveolae) compared with a whole-cell fat-free homogenate, using SDS-PAGE and immunoblotting; about 20% of the enzyme protein was recovered in the purified plasma membrane fraction (data not shown), indicating a substantial potential for TG synthesis in the plasma membrane with its caveolae (28).

To ascertain that de novo TG synthesis took place in caveolae and exclude any possibility of TG associating with caveolae during isolation of the organelles or of fatty acid exchange with preexisting TG, we isolated the two caveolae fractions (high and low density caveolae) and examined their ability to synthesize TG (4, 29). It was not possible to directly determine how much of the overall rate of catecholamine-stimulated lipolytic hydrolysis of TG.

![Fig. 2. De novo TG synthesis in isolated caveolae, plasma membrane, and microsomal fractions.](image)

![Fig. 3. Immunogold transmission electron microscopy of plasma membrane sheets.](image)

![Fig. 4. Immunofluorescence confocal microscopy of intact cells and plasma membrane sheets.](image)
as the plasma membrane with its caveolae (Fig. 2b). It should be noted that the TG synthetic capacity was compromised by the necessity to provide reagent access to the inside of plasma membrane vesicles by sonication and by the procedure used to isolate caveolae, which involves sonication in 0.5 M CO$_3^-$ at pH 11. These procedures therefore introduced large variability from preparation to preparation. Recovery of both the oleic acid and glycerol 3-phosphate precursors in TG demonstrated de novo TG synthesis in the isolated caveolae.

The TG-containing caveolae with its caveolin and associated perilipin display an obvious kinship to lipid bodies, which have been reported to contain caveolin and perilipin-related proteins when isolated and characterized from other cell types (15, 16). As described here, in primary adipocytes, they are plasma membrane-associated, caveolae-derived structures and do not originate in the endoplasmic reticulum. It remains to determine whether these TG-containing caveolae should be classified as lipid bodies and if in fact different classes of lipid bodies exist.

By electron microscopy of plasma membrane sheets stained with OsO$_4$, we identified large, variously sized, lipid-laden bulbs scattered over the membrane (Fig. 3). Immunogold labeling showed that both caveolin (Fig. 3, a and b) and perilipin (Fig. 3, c and d) were associated with caveolae and with these lipid bulbs, thus confirming their caveolar nature and relation to the isolated TG-synthesizing caveolae identified above. Examination of plasma membrane sheets by electron microscopy relies on flushing the membranes to wash away cell debris and loosely attached cell material to reveal membrane structures. Too zealous flushing of the membranes will inevitably wash away the larger lipid bulbs, which may explain why they have previously remained uncharacterized.

The actual accumulation of fatty acid in the caveolin/perlipin-associated bulbs was confirmed by immunofluorescence confocal microscopy. In cells stimulated with insulin fluorescent fatty acids accumulated in caveolin-containing spots scattered over the cell periphery (Fig. 4, a and b). These amphiphilic molecules cannot accumulate or dissolve in the large lipid droplet unless they are converted to a non-polar form. They are trapped in the endoplasmic reticulum. It remains to determine whether these TG-containing caveolae should be classified as lipid bodies and if in fact different classes of lipid bodies exist.

This report shows that caveolae are sites of fatty acid entry and sites of TG synthesis and may suggest a novel non-vesicular transport mechanism for TG transfer to the central lipid storage droplet in primary adipocytes. Triacylglycerol synthesis has been considered to take place in the endoplasmic reticulum (32), which is appropriate for TG synthesis in enterocytes and hepatocytes where TG synthesis is coordinated with production of chylomicron and very low density lipoprotein particles, respectively. Adipocytes, on the other hand, are faced with a massive influx of potentially lethal fatty acids from lipoprotein lipase catalyzed hydrolysis of lipoprotein TG in the circulation. If these fatty acids are not immediately reacylated they will cause cell lysis (2). In this context it is interesting that caveolin-1$^{-/-}$ mice that lack caveolae exhibit a general adipose tissue derangement and atrophy, which increase with age (20). Our findings now demonstrate that all enzymes involved in the de novo synthesis of triacylglycerol from fatty acids and glycerol 3-phosphate are localized and functional in a novel specific subclass of caveolae in the plasma membrane of primary adipocytes.

Acknowledgments—We are grateful to Dr. C. Londos and Dr. J. E. Schaffer for their generous sharing of antibodies.

REFERENCES
1. Goldrick, R. B. (1967) J. Lipid Res. 8, 581–588
2. Sträflors, P. (1990) FASEB J. 4, 1535–1543
3. Czech, M. P. (2002) Mol. Cell 9, 695–696
4. Thorp, H., Stenbula, K. G., Karlsson, M., Orten, U., Nyström, P. G., Gustafsson, J., and Sträflors, P. (2000) Mol. Biol. Cell 14, 3967–3976
5. Kazanis, B., Woodman, S. E., and Lisanti, M. P. (2002) Pharmacol. Rev. 54, 431–467
6. Ishikawa, M., and Andersson, R. G. (2003) Traffic 4, 717–723
7. Simons, K., and Tromp, D. (2000) Nat. Rev. Mol. Cell. Biol. 1, 31–40
8. Gustafsson, J., Parpal, S., Karlsson, M., Rasmussen, C., Thorn, H., Borg, M., Lindroth, M., Peterson, K. H., Magnusson, K.-E., and Sträflors, P. (1999) FASEB J. 13, 1961–1971
9. Karlsson, M., Thorn, H., Danielsson, A., Stenbula, K. G., Ost, A., Gustafsson, J., Nyström, F. H., and Sträflors, P. (2004) Eur. J. Biochem. 271, 2471–2479
10. Gustafsson, J., Parpal, S., and Sträflors, P. (1996) Mol. Med. 2, 367–372
11. Karlsson, M., Thorn, H., Parpal, S., Sträflors, P., and Gustafsson, J. (2001) FASEB J. 16, 249–251
12. Pohl, J., Ring, A., Ekhult, R., Schulze-Bergkamen, H., Schad, A., Verkade, P., and Stremmel, W. (2004) Biochemistry 43, 4179–4187
13. Trigatti, B. L., Anderson, R. G., and Gerber, G. E. (1999) Biochem. Biophys. Res. Comm. 255, 34–39
14. Darbysh, D. J., Linz, F. L., Meldrum, C. J., and Burns, G. F. (1996) Biochem. J. 319, 67–72
15. Liu, P., Ying, Y., Zhao, Y., and Mundy, D. I., Zhu, M., and Andersson, R. G. W. (2004) J. Biol. Chem. 279, 3787–3792
16. Pol, A., Martin, S., Fernandez, M. A., Ferguson, C., Carozzi, A., Luster Forste, R., Enrich, C., and Parson, R. G. (2004) Mol. Biol. Cell 15, 99–110
17. Brassemsle, D., Dolos, G., Shapiro, L., and Wang, R. (2004) J. Biol. Chem. 279, 46835–46842
18. Marchess, E., Rutberg, M., Anderson, R. J., Asp, L., Larsson, T., Boren, J., Johansson, B. R., and Olofsson, S. O. (2003) J. Biol. Chem. 278, 27283–27300
19. Cohen, A. W., Razani, B., Wang, X. B., Combs, T. P., Williams, T. M., Scherer, P. E., and Lisanti, M. P. (2003) Am. J. Physiol. 285, C222–C235
20. Razani, B., Combs, T. P., Wang, X. B., Frank, P. G., Park, D. S., Ruse, R. G., Li, M., Tang, B., Jelicz, L. A., Scherer, P. E., and Lisanti, M. P. (2002) J. Biol. Chem. 277, 8635–8647
21. Sträflors, P., and Honnor, R. C. (1989) Eur. J. Biochem. 182, 379–385
22. Orten, U., Karlsson, M., Blazic, N., Bismarck, M., Nyström, F. H., Gustafsson, J., Fredman, P., and Sträflors, P. (2004) Eur. J. Biochem. 271, 2028–2036
23. Aboulach, N., Vainonnen, J., Sträflors, P., and Vener, A. V. (2004) Biochem. J. 383, 237–248
24. Gustafsson, J., Parpal, S., and Sträflors, P. (1995) Exp. Cell Res. 211, 443–447
25. Kates, M. (1986) in Laboratory Techniques in Biochemistry and Molecular Biology (Burdon, R. H., and Kuppenberg, P. H. v., ed.) Vol. 3, pp. 526–425, Elsevier Science Publishers B. V., Amsterdam
26. Lindroth, M., Fredriksson, B. A., and Bell, P. B. (1991) J. Microsc. (Oxf.) 161, 229–239
27. Clifford, G. M., Londos, C., Kraemer, F. B., Vernon, R. G., and Yeaman, S. J. (2000) J. Biol. Chem. 275, 5011–5015
28. Gargiulo, C. E., Stuhlsatz-Krouper, S. M., and Schaffer, J. E. (1999) J. Lipid Res. 40, 881–892
29. Belfrage, P., Fredriksson, G., Sträflors, P., and Tornqvist, H. (1984) in Lipases (Borgström, B., and Brockman, H. L., ed.) pp. 365–416, Elsevier Science Publishers B. V., Amsterdam
30. Kasprzakinen, J. (1992) Biochem. Biophys. Res. Commun. 187, 1594–1601
31. Cohen, A. W., Razani, B., Schubert, W., Williams, T. M., Wang, X. B., Iyengar, P., Brasamend, D. L., Scherer, P. E., and Lisanti, M. P. (2004) Diabetes 53, 1261–1270
32. Wilgram, G. F., and Kennedy, E. P. (1963) J. Biol. Chem. 238, 2615–2619
Triacylglycerol Is Synthesized in a Specific Subclass of Caveolae in Primary Adipocytes
Anita Öst, Unn Örtegren, Johanna Gustavsson, Fredrik H. Nystrom and Peter Strålfors

J. Biol. Chem. 2005, 280:5-8.
doi: 10.1074/jbc.C400429200 originally published online November 9, 2004

Access the most updated version of this article at doi: 10.1074/jbc.C400429200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 30 references, 12 of which can be accessed free at http://www.jbc.org/content/280/1/5.full.html#ref-list-1