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"In Vitro" Utilization of Labelled Esterified Fatty Acids and Glyceride Glycerol from Triglyceride-Rich Lipoproteins in Rat Adipose Tissue

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Summary

Triglyceride-rich lipoproteins (chylomicrons and very low density lipoproteins) were labelled "in vivo" by injecting ($U-^{14}C$)-glycerol and ($9-10(n)-^3H$)-palmitate in female rats. After purification, these lipoproteins contained most of the 3H in esterified fatty acids and the ^{14}C in glyceride glycerol of neutral lipids. This preparation was incubated "in vitro" in the presence of either isolated adipocytes or epididymal fat pad pieces from male rats. With the incubation, a certain proportion of both 3H -esterified fatty acids and ^{14}C -glyceride glycerol disappeared from the medium, the effect being greater when the incubations were performed with adipocytes than with fat pad pieces. Much greater radioactivity appeared in the lipids of adipocytes than in those of fat pad pieces at the end of 60 or 120 min incubation, and the incorporation of 3H being relatively greater than that of ^{14}C . With the latter isotope, the label appeared not only in the glyceride glycerol fraction but also in the free and esterified fatty acids. Although it is known that lipoprotein lipase activity is lower in adipocytes than in fat pad pieces, our results indicate that, in the former preparation, the enzyme may be more accessible for the substrate. These data also demonstrate that glycerol released by the hydrolysis of lipoprotein glycerides may be partially incorporated into lipids by adipose tissue.

Key-Words: Triglyceride-Rich Lipoproteins - Adipose Tissue - Lipoprotein Lipase - Glycerol Utilization

Introduction

It has been proposed that free fatty acids, derived from the hydrolysis of triglycerides, may be taken up directly by

adipose tissue to be esterified and stored as tissue glycerides (Carlson and Walldius 1976; Kerpel, Shafrir and Shapiro 1961; Rubba 1978), but it seems well established that the main exogenous sources of adipose tissue triglycerides are those coming from circulating chylomicrons and very low density lipoproteins (VLDL) (Masoro 1977). Although it was initially proposed that rat adipose tissue could take up intact lipoprotein-borne triglyceride directly (Markscheld and Shafrir 1965), it was later demonstrated that lipoprotein lipase present in the tissue hydrolyzes the plasma lipoprotein triglycerides prior to deposition of fatty acids in the fat cell (Austin and Nestel 1968; Scow, Hamosh, Blanchette-Mackie and Evans 1972; Smith, Pownall and Gotto 1978). The glycerol moiety of lipoprotein triglycerides is not incorporated in adipose tissue when administered "in vivo" (Scow, Chernick and Fleck 1977). This finding could be due to the rapid metabolization of glycerol passing through the liver (Carmaniu 1978) more than to adipose tissue inability to take up glycerol, as it has been well documented that the tissue utilizes a certain proportion of this metabolite in "in vitro" conditions (Chaves and Herrera 1978; Dominguez and Herrera 1976; Herrera and Ayanz 1972). In the present study, "in vitro" rat adipose tissue preparations incubated in the presence of rat plasma triglyceride-rich lipoproteins prelabelled with 3H in the esterified fatty acids and with ^{14}C in the glyceride glycerol fractions were used to study the relations between hydrolysis and the adipose tissue uptake of both fatty acids and glycerol moieties of lipoprotein triglycerides.

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Materials and Methods

Female Wistar rats weighing 160–180 g and fed standard rat chow were maintained in a temperature ($22 \pm 2^\circ\text{C}$) and light cycle (12 h on-off) controlled room. Each animal was injected i.v. in the tail with an 0.5 ml solution of 8% purified (Chen 1967) bovine albumin containing 60 μCi of sodium (9–10 (n)- ^3H)-palmitate (230 mCi/mmol) (from the Radiochemical Center, Amersham), prepared as described elsewhere (Carmaniu and Herrera 1979), and 30 μCi of (U- ^{14}C)-glycerol (46 mCi/mmol) (from the Radiochemical Center, Amersham). On the basis of preliminary experiments, 30 min after the injection, rats were sacrificed under light ether anesthesia and blood was collected from the aorta into a syringe containing 100 mg of $\text{Na}_2\text{-EDTA}$. Plasma obtained after centrifugation at $1000 \times g$ for 30 min at 4°C was centrifuged under 0.15 M NaCl ($d = 1.006$, as tested by using an Abbe refractometer) for 18 h at $143,000 \times g$ in a 40.3 rotor of a Beckman LS-75 preparative ultracentrifuge, at 15°C . The supernatant was recovered by tube slicing and contained both chylomicrons and VLDL, as shown by electron-microscopic study (Lasunción, Llobera and Herrera 1980) which were identified as triglyceride-rich lipoproteins. This supernatant was further purified by dialysis against three passes on 5 l of 0.9% NaCl-1 mM EDTA and a final pass on plain 0.9% NaCl for a total of 18 h at 4°C . Aliquots of the purified triglyceride-rich lipoproteins were used for radioactive determination (total counts), lipid extraction and "in vitro" incubation. Of the triglyceride-rich lipoprotein preparation, 0.5 ml (coming from 0.4 ml of initial plasma) were incubated for 60 or 120 min in a final volume of 1.25 ml of Krebs-Ringer bicarbonate buffer, pH 7.4, with suitable salt content to yield the desired final concentration (Umbreit, Burris and Stauffer 1964), supplemented with glucose and purified (Chen 1967) bovine albumin. Final concentrations of glucose and albumin in the media were 4 mM and 0.8% respectively.

Incubations were performed with or without epididymal fat pad pieces (20 mg/vial) or adipocytes from epididymal fat pads, isolated by a modification (Bellido and Herrera 1978) of the method of Rodbell (1964) in the presence of an ovomucoid trypsin inhibitor (Sigma) (2 mg/ml) and crude collagenase (Worthington Biochemical Co.) (2 mg/ml), obtained from fed 160–180 g male Wistar rats sacrificed by cervical fracture. Incubations were performed at 37°C in sealed siliconized glass vials, gassed for 5 min with O_2/CO_2 (95:5) in a Dubnoff shaking incubator at 100 cycles/min. After centrifugation at 200 rpm for 2 min, incubations were terminated by removing either the adipose tissue pieces or the adipocytes by flotation. Aliquots of the media were used for glycerol determination by an enzymatic method (Garland and Randle 1962) for radioactivity counting and lipid extraction. The tissue pieces and adipocytes were thoroughly washed in plain Krebs Ringer bicarbonate buffer and immediately placed in chloroform-methanol for lipid extraction. Lipids in the initial lipoprotein preparation, in the media aliquots, and in the incubated tissue pieces and adipocytes were extracted (Folch, Lees and Sloane-Stanley 1957), purified and fractionated (Dominguez and Herrera 1976; Herrera and Ayanz 1972; Kerpel, Shafrir and Shapiro 1961). In the fraction containing glycerides, phospholipids were discarded by treatment with activated

silicic acid in a chloroform medium so that values of glycerides corresponded to neutral lipids. Aliquots of lipid fractions from the initial lipoprotein preparation were saponified in ethanolic 2 N NaOH and after suitable neutralization, glycerol was measured in the aqueous layer (Garland and Randle 1962). Aliquots of all lipidic fractions were used for counting their radioactivity in a PPO/POPOP xylene/triton X-100 based scintillation cocktail. Radioactivity values were adjusted to 1×10^4 DPM for the ^3H and ^{14}C -label contained in each vial before the incubation. Proteins were measured (Wang and Smith 1975) in aliquots of the tissue preparations. Statistical analysis of the data was performed by the Student's "t" test.

Results

Prelabelled triglyceride-rich lipoproteins were purified from plasma of rats previously injected with (9–10 (n)- ^3H)-palmitate and (U- ^{14}C)-glycerol. As shown in Table 1, most of the lipidic ^3H of these lipoproteins was present in the esterified fatty acid fraction with only a minor proportion in the free fatty acids, while practically all the lipidic ^{14}C corresponded to glyceride glycerol of neutral lipids (presumably triglycerides). These values were not altered by a 120 min "in vitro" incubation at 37°C in the absence of tissue (Table 1). When incubations were performed in the presence of epididymal fat pad pieces or isolated adipocytes, the disappearance rate of ^3H -esterified fatty acids and ^{14}C -glyceride glycerol of the lipoproteins from the medium were different (see Table 2). This effect was greater in incubations with adipocytes than with fat pad pieces and was progressive during incubation. The difference between disappearance rate values of epididymal fat pad pieces and adipocytes was statistically significant. The ratio between the disappearance of ^3H -esterified fatty acids and ^{14}C -glyceride glycerol was above 1 in all situations and was constant with the incubation time. It was greater when incubations were performed in the presence of fat pad pieces than with adipocytes, although this difference was not statistically significant.

A significant percentage of counts in the labelled triglyceride-rich lipoproteins that disappeared from the medium during incubation was incorporated into the tissue as lipids. As shown in Table 3, much higher radioactivity was found in adipocyte than in fat pad lipids, and the incorporation of ^3H was relatively greater than that of ^{14}C . Most of the label in ^3H lipids appeared in the esterified fatty acid fraction and only a minute amount was found in the free fatty acids. The ratio between the ^3H -esterified fatty acids

Table 1 Percental composition of labelled lipids in the triglyceride-rich lipoproteins of $d < 1.006$ purified from plasma of rats i.v. injected with (9–10 (n)- ^3H)-palmitate and (U- ^{14}C)-glycerol 30 min before sacrifice

| | ^3H | | ^{14}C | |
|--------------------------------------|----------------|--------------------------|-----------------|--------------------------|
| | Basal | After 120 min incubation | Basal | After 120 min incubation |
| Esterified fatty acids | 87.8 \pm 3.1 | 85.6 \pm 4.2 | 2.4 \pm 0.9 | 2.9 \pm 0.9 |
| Non-saponifiable lipids | 1.8 \pm 0.4 | 1.6 \pm 0.5 | 0.1 \pm 0.02 | 0.1 \pm 0.03 |
| Free fatty acids | 10.3 \pm 1.5 | 12.4 \pm 2.3 | 0.5 \pm 0.2 | 0.4 \pm 0.2 |
| Glyceride glycerol of neutral lipids | 0.6 \pm 1.6 | 0.4 \pm 1.8 | 97.0 \pm 1.4 | 96.5 \pm 1.6 |

After purification by ultracentrifugation and dialysis, aliquots of the labelled lipoproteins were directly used for lipid extraction and fractionation while other aliquots were previously incubated in Krebs-Ringer bicarbonate buffer containing 4 mM glucose and 0.8% purified bovine albumin for 120 min at 37°C and shaking. Values are expressed as % of the labelled total lipids and correspond to means \pm S.E.M. of 7 samples per group.

Table 2 Disappearance of labelled glycerides from prelabelled triglyceride-rich lipoproteins incubated "in vitro" with either rat epididymal fat pad pieces or isolated adipocytes

| Incubation time | Adipocytes | | | Epididymal fat pad pieces | | |
|---|-------------|-------------|--------|---------------------------|--------------|------|
| | 60 min | 120 min | P | 60 min | 120 min | P |
| A) Disappearance of ³ H-esterified fatty acids from medium | 878 ± 97 | 1356 ± 79 | < 0.01 | 604 ± 64 * | 733 ± 51 *** | N.S. |
| B) Disappearance of ¹⁴ C-glyceride glycerol from medium | 736 ± 96 | 1219 ± 75 | < 0.01 | 416 ± 87 * | 540 ± 42 *** | N.S. |
| Ratio of A/B | 1.24 ± 0.16 | 1.15 ± 0.12 | N.S. | 1.74 ± 0.30 | 1.39 ± 0.10 | N.S. |

Adipocytes were isolated by treatment with collagenase in the presence of trypsin inhibitor, as described in the Materials and Methods. Incubations were carried out in Krebs-Ringer bicarbonate buffer containing 4 mM glucose and 0.8% purified bovine albumin. Values are dpm/100 µg proteins (initial radioactivity adjusted to 1×10^4 dpm per vial) that disappeared from the medium in the form of either ³H-esterified fatty acids or ¹⁴C-glyceride glycerol during the incubation time, and correspond to means ± S.E.M. (n = 7). P is the statistical comparison between the samples incubated for 60 and 120 min while asterisks correspond to the comparisons between fat pad pieces and adipocytes (* = p < 0.05 and *** = p < 0.001).

Table 3 Incorporation of labelled lipidic products into the tissue after the "in vitro" incubation of either rat epididymal fat pad pieces or isolated adipocytes in the presence of prelabelled triglyceride-rich lipoproteins

| | Adipocytes | | | Fat pad pieces | | |
|---|-------------|-------------|---------|-----------------|------------------|--------|
| | 60 min | 120 min | P | 60 min | 120 min | P |
| ³ H | | | | | | |
| Total lipids | 484 ± 78 | 1005 ± 96 | < 0.01 | 50.2 ± 14.4 *** | 139.2 ± 26.3 *** | < 0.05 |
| Free fatty acids | 47 ± 18 | 89 ± 29 | N.S. | 10.7 ± 3.9 | 27.9 ± 11.6 | N.S. |
| Esterified fatty acids | 427 ± 61 | 911 ± 59 | < 0.001 | 51.2 ± 15.3 *** | 124.9 ± 22.3 *** | < 0.05 |
| Disappearance of ³ H-esterified fatty acids from medium/tissue total lipids ratio | 1.94 ± 0.22 | 1.43 ± 0.16 | N.S. | 16.0 ± 3.1 ** | 8.01 ± 2.70 * | N.S. |
| ¹⁴ C | | | | | | |
| Total lipids | 53.8 ± 8.7 | 93.9 ± 11.4 | < 0.05 | 13.3 ± 3.5 ** | 21.9 ± 2.4 *** | N.S. |
| Free fatty acids | 13.0 ± 3.6 | 19.4 ± 7.0 | N.S. | 3.8 ± 1.7 * | 5.1 ± 1.2 | N.S. |
| Esterified fatty acids | 20.8 ± 3.2 | 37.5 ± 9.7 | N.S. | 5.3 ± 1.4 ** | 11.6 ± 2.2 ** | < 0.05 |
| Glyceride glycerol | 20.3 ± 5.4 | 39.6 ± 8.7 | N.S. | 6.0 ± 4.4 | 5.1 ± 1.2 ** | N.S. |
| Disappearance of ¹⁴ C-glyceride glycerol from medium/tissue ¹⁴ C-total lipids ratio | 15.8 ± 2.8 | 14.5 ± 2.0 | N.S. | 36.4 ± 13.1 | 26.0 ± 2.3 ** | N.S. |

Adipocytes were isolated by treatment with collagenase in the presence of trypsin inhibitor, as described in the Materials and Methods. Incubations were carried out in Krebs-Ringer bicarbonate buffer containing 4 mM glucose and 0.8% purified bovine albumin. Values are expressed as dpm/100 µg protein, the radioactivity values being adjusted to 1×10^4 dpm for the ³H and ¹⁴C-label contained in each vial before incubation, and correspond to means ± S.E.M. (n = 7). P is the statistical comparison between the samples incubated for 60 and 120 min while asterisks correspond to the comparisons between fat pad pieces and adipocytes (* = p < 0.05, ** = p < 0.01 and *** = p < 0.001).

that disappeared from the incubation medium and the ³H lipids that appeared in the adipocytes was always below 2, indicating that most ³H-fatty acids (leaving the medium) were incorporated into the adipocytes. The ratio was much greater with the fat pad pieces, demonstrating their more limited incorporation.

The incorporation of ¹⁴C into tissue lipids was much lower than that of ³H (Table 3) both in absolute counts and in proportion to the amount of label lost from the medium as glyceride glycerol during incubation. Here again, the proportion of ¹⁴C-lipids in the fat pad pieces was lower than in isolated adipocytes (Table 3). The ¹⁴C-label in the tissue preparations appeared not only in the form of glyceride glycerol but also in both the free fatty acid and esterified

fatty acid fractions. The ratio between ¹⁴C-glyceride glycerol disappearance from the medium and tissue ¹⁴C total lipids was much greater than that of ³H, indicating a lower incorporation of the glyceride glycerol moiety than that of esterified fatty acids from the media lipoproteins into the tissue preparations.

Parallel experiments were performed to determine the comparative production of glycerol during the incubation time in adipocytes and fat pad pieces in the presence of the triglyceride-rich lipoprotein preparations, and no differences appeared between these tissue preparations (27.4 ± 4.6 nmoles of glycerol/100 µg protein in the incubation medium of adipocytes incubated for 120 min, and 30.6 ± 5.3 in that of fat pad pieces, n = 5/group, p > 0.05).

Discussion

Results indicate that during the "in vitro" incubation of triglyceride-rich lipoproteins in the presence of adipose tissue preparations, a significant proportion of the glycerides disappears from the incubation medium as a result of their hydrolysis. As suggested by other authors, this process may be the result of lipoprotein lipase activity in fat cells (Austin and Nestel 1968; Scow, Chernick and Fleck 1977). The fact that the ratio between the disappearance of ^3H -esterified fatty acids and ^{14}C -glyceride glycerol is greater than 1 throughout incubation indicates the presence of incompletely hydrolyzed products of triglyceride breakdown (presumable di- and mono-acyl-glycerides). This hypothesis is in agreement with Scow and Olivecrona (1977) who reported the formation of these products after the incubation of chylomicrons with purified lipoprotein lipase. Since the hydrolysis of tri-acyl-glycerol to glycerol and fatty acids by lipoprotein lipase is dependent on the isomerization of 2-mono-acyl-glycerol to 1(3)-mono-acyl-glycerol (El-Maghribi, Waite, Rudel and Sisson 1978; Nilsson-Ehle, Garfinkel and Schotz 1974; Scow and Olivecrona 1977), the transient accumulation of low-esterified glycerides is probably caused by mono-acyl-glycerol undergoing isomerization within the lipoprotein particle before being hydrolyzed by lipoprotein lipase. It is noteworthy that isolated adipocytes hydrolyze a greater proportion of lipoprotein-glycerides than the fat pad pieces and the effect of the incubation period is much greater in the former preparation. These findings are compatible with others showing lower lipoprotein lipase activity in adipocytes than in fat pad pieces (Nilsson-Ehle, Garfinkel and Schotz 1976; Vanhove, Wolf, Breton and Glangeaud 1978). Unlike intact tissue activity during incubation, isolated adipocytes secrete substantial amounts of lipoprotein lipase to the medium (Nilsson-Ehle, Garfinkel and Schotz 1976; Stewart and Schotz 1974), making it more available for the substrate. Although it is known that collagenase inactivates extracellular lipoprotein lipase during the cell preparation procedure (Cunningham and Robinson 1969; Pokrajac, Lossow and Chaikoff 1967), the trypsin inhibitor as well as the special experimental conditions (Nilsson-Ehle, Garfinkel and Schotz 1976) used in the present study may have minimized such inactivation.

Most of the esterified fatty acids that disappeared from the medium during incubation time appeared in the adipocytes in the form of esterified fatty acids. Since the incorporation of ^{14}C -glyceride glycerol into the cell glycerides was much lower than that of fatty acids, labelled fatty acids in the cells may result from the previous hydrolysis of esterified fatty acids in the medium and their later re-esterification with non-labelled α -glycerophosphate into the tissue. The incorporation of fatty acids into the lipids of the fat pad pieces is minimal. The different effect of isolated adipocytes and pieces remains to be explained. On the basis of previous results (Nilsson-Ehle, Garfinkel and Schotz 1976), lipoprotein lipase activity appears greater in fat pad pieces than in adipocytes. However, this is not the first time that inconsistencies have been reported between the activity of lipoprotein lipase in adipose tissue and the uptake of glycerides. For example, rat mesenteric adipose tissue exhibits a higher triglyceride uptake than does epidid-

ymal tissue (Markscheld and Shafrir 1965), while lipoprotein lipase activity is the reverse (Cherkes and Gordon 1959).

Our findings also demonstrate that part of the free labelled glycerol released in the hydrolysis of lipoprotein-triglycerides is taken up by the tissue preparations to be metabolized in free fatty acids, esterified fatty acids and glyceride glycerol. Although the percental amount of labelled metabolized glycerol is small, it is greater if the continuous dilution by the cold glycerol released from the cells is taken into account (Dominguez and Herrera 1976; Herrera and Ayanz 1972). These results contrast with the common contention that the glycerol moiety is not incorporated into lipids by adipose tissue (Scow et al. 1972; Scow, Chernick and Fleck 1977), although this point has never been studied in detail. Schotz, Stewart, Garfinkel, Whelan and Baker (1969) did not find any tritium label recovered in the fatty acid moiety of adipose tissue cells incubated for 30 min in the presence of ^3H -glyceride glycerol in VLDL, however, in spite of the short incubation time, it is known that tritium from other sources (including glucose) is very rarely incorporated into the fatty acids (Shreeve, Lamdin, Oji and Slavinski 1967). It has recently been shown that "in vivo" incorporation of the glycerol moiety of prelabelled VLDL or chylomicrons into adipose tissue lipids is minimal (Carmaniu 1978; Scow, Chernick and Fleck 1977), but in these conditions, the liver rapidly remetabolizes glycerol (Carmaniu 1978) allowing almost no labelled glycerol to become available to the adipose tissue. Thus, in situations of hepatic damage and/or hyperlipidemia, part of the glycerol liberated at the adipose cell level by hydrolysis of triglyceride-rich lipoproteins by the action of lipoprotein lipase may be incorporated into the tissue to contribute to fat deposition. Perhaps this action is only significant when the activity of both glycerokinase and lipoprotein lipase is elevated, as in obesity (Jaillard, Sezille, Fruchart, Dewailly and Romon 1976; Rath, Hems and Beloff-Chain 1974; Trebel and Meyer 1963).

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