Brown adipose tissue triacylglycerol synthesis in rats adapted to a high-protein, carbohydrate-free diet


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Brown adipose tissue triacylglycerol synthesis in rats adapted to a high-protein, carbohydrate-free diet (HP) diet have been used for many years in our laboratory as a model system to investigate adaptive mechanisms in energy-linked metabolic processes. In previous studies, we have shown that despite a markedly reduced lipogenesis in both liver and adipose tissue, assessed in vivo by the rate of incorporation of tritium from \( 3H_2O \) into fatty acids (FAs) (4, 27), rats adapted to an HP diet are able to maintain considerable reserves of body fat. Thus the effects of the HP diet on the activity of enzymes involved in lipid synthesis and on the relative contribution of glucose carbon and carbon from other sources to the in vivo synthesis of FA and glycerol moieties of triacylglycerols (TAGs) are here reported. In the course of these experiments it was found that both in control and HP rats the rate of label incorporation into glyceride-glycerol was several times higher than into FAs. This prompted us, especially in view of the lack of carbohydrate in the diet of HP rats, to examine more closely BAT glyceroneogenesis, a process that has been little investigated in this tissue. To this end, the rate of in vitro incorporation of several labeled glyceroneogenic substrates into glyceride-glycerol, as well as the activity of phosphoenolpyruvate carboxykinase, a key glyceroneogenic enzyme, were determined in IBAT from control and HP-adapted rats.

**MATERIALS AND METHODS**

**Animals and Treatment**

Male Wistar rats weighing initially 60–90 g were housed in suspended, wire-bottom cages, with water ad libitum, in a room kept at 25 ± 2°C with a 12:12-h light-dark cycle. The animals were adapted for 20 days to an HP-purified diet containing 70% casein, no carbohydrate, and 8% corn oil or to a balanced, control diet, containing 17% casein, 66% carbohydrate, and 8% corn oil. The two diets, which were approximately isocaloric and contained equal amounts of vitamins and minerals, have been described in detail (6). As in previous studies with the same diet, after an initial period of adaptation of a few days, food ingestion and the rate of body weight increase were similar for the two groups of rats (17). The animals weighed 180–200 g when used for the experiments, which were performed between 8:00 and 10:00 AM.

**In Vivo Lipogenesis Studies**

Experimental approach. The glucose contribution to the synthesis of glycerol and fatty acid moieties of TAG was evaluated by determining simultaneously in the same animal the rate of incorporation from tritiated water, which estimates total synthesis (from all carbon sources), and of \( 14C \) from glucose into two TAG moieties of IBAT. The assumptions and supportive arguments for the adequacy of \( 3H_2O \) for...
measurement of lipid synthesis from all carbon sources have been presented by Windmuller and Spaeth (31) and Ungers (15). The flux of glucose carbon to IBAT FA or glycerol was estimated using the semicompartimental approach described by Baker and Huebotter (2), which is a modification of the noncompartimental approach of Shipley et al. (29) and combines features of both noncompartimental and compartmental analysis. The use of compartmental analysis requires measurement of the specific activity-time curve of the precursor after a single injection of a radioactive tracer [as in the method of Shipley et al. (29)] and the measurement of the radioactivity in an “end product” at any point in time (60 min in the present study). The technique’s assumptions and supportive arguments are described in Ref. 2. It was assumed that no appreciable turnover of 3H- or 14C-labeled product occurred during the experimental period, so the rates obtained are minimal values.

Label injection and isolation of tissue TAG-FAs and glycerol. [U-14C]Glucose (10 µCi) and 3H2O (5 mCi) dissolved in 0.5 ml saline were injected into fed, nonanesthetized rats through a Silastic (Dow Corning, Midland, MI) catheter inserted into the right jugular vein 2 days before the experiment. After the catheter was flushed with saline, and with the rat free in its cage, blood samples of 0.2 ml were taken 1, 5, 15, 30, and 60 min after labels injection for determination of [14C]glucose specific activity. Immediately after obtaining of the 60-min sample, which was also used for determination of plasma water specific activity, the animals were killed by cervical dislocation. The IBAT was rapidly removed, carefully cleaned free of adhering fat and muscle, and weighed. IBAT total lipids were extracted with 2:1 chloroform-methanol by the procedure of Folch et al. (9). 3H2O was removed from the lower phase (predominantly chloroform) by washing three times with an upper phase mixture (9). After each shaking, the tubes were briefly centrifuged to sharpen the phase boundary and the upper phase was aspirated and discarded. The chloroform phase was evaporated to dryness under N2, and the TAGs were hydrolyzed with ethanolic KOH for 1 h at 70°C. After extraction of nonvolatile lipids and acidification with 6 N H2SO4, the 14C- and 3H-labeled FA was extracted with petroleum ether, and the extract was evaporated to dryness in a scintillation vial and dissolved in toluene-2,5-diphenyloxazone (PPO). A volume of the aqueous hydrolysate containing 14C- and 3H-labeled glycerol was dissolved in toluene-triton-PPO-POPOP.

Determination of plasma glucose and body water specific radioactivity. Plasma [14C]glucose was isolated by thin-layer chromatography and its radioactivity measured as described by Baker et al. (3). The concentration of plasma glucose was determined with glucose oxidase in a Beckman (Fullerton, CA) glucose analyzer. Body water specific activity was determined directly on aliquots of diluted (20 times) plasma dissolved in toluene-triton-PPO-POPOP.

Radioactivity measurements. The degree of quenching in each sample was obtained to enable calculation of radioactivity in disintegrations per minute. Simultaneous liquid scintillation counting (LS 7600 Beckman spectrometer) of the 3H and 14C content of FA or glycerol was performed using a channels ratio method (12).

Calculations. Plasma glucose levels of HP and control rats 1 min postinjection were (in mg/dl) 117 ± 3 (6 rats) and 125 ± 4 (6 rats), respectively. In the two groups, plasma glucose concentration of each animal did not change significantly during the experimental period (data not shown), a requirement of the technique used. The curves of plasma glucose specific activity were fitted to two terms exponential equations, whose parameters were used in the calculations (2). For the calculations of lipid synthesis in the experiments with 3H2O, it was assumed that the specific activity of intracellular water was identical to that of plasma water. Rates of lipid synthesis were calculated assuming that each glycerol and each FA incorporated into TAG contained 3.3 and 13.3 atoms of tritium, respectively (15, 31).

In Vivo Experiments

The rats were killed by cervical dislocation. The IBAT was removed, cleaned free of fat and muscle, and cut into small pieces of ~5 mg. Portions of 100 mg were incubated in 5 ml of Krebs-Henseleit bicarbonate buffer, pH 7.4, in which appropriate substrates were dissolved, as indicated in Results. Incubation was carried out at 37°C with constant shaking for 2 h. The tissue fragments were then rinsed three times with 0.9% NaCl and transferred to 2:1 chloroform-methanol. The procedure used for isolation and determination of 14C in final products was the same as that described for the in vivo experiments.

Measurement of Enzyme Activities

For determination of glucose-6-phosphate dehydrogenase (G6PDH), malic enzyme (ME), and citrate lyase (CLY) activity, IBAT was homogenized in ice-cold 10 mM Tris buffer, pH 7.4, containing 0.32 M sucrose, 2 mM EDTA, and 5 mM mercaptoethanol. After centrifugation at 10,000 g for 10 min and removal of the top fat layer, the supernatant was centrifuged for 1 h at 100,000 g to obtain a new supernatant, which was used to determine the activity of the enzymes. G6PDH was assayed as described by Lee (18), and ME was assayed by the method of Ochoa (23), with the modifications proposed by Hsu and Lardy (14). Both assays were performed by measuring the rate of formation of NADPH in an assay mixture containing 50 mM triethanolamine, pH 7.7, 7 mM ATP-Mg, 10 mM potassium citrate, 0.1 mM NADH, 10 mM mercaptoethanol, and 0.24 mM CoA, NAD-malate dehydrogenase (1 unit/ml), and aliquots of the 100,000 g supernatant. Phosphoenolpyruvate carboxykinase was assayed by the method of Chang and Lane (7) in 100,000 g supernatants obtained after homogenization of IBAT in 20 mM triethanolamine buffer, pH 7.5, containing 0.2 M sucrose, 5 mM mercaptoethanol, and 1 mM EDTA. The incorporation of 14Cbicarbonate (2 µCi, Amersham) into acid-stable product was determined in an assay mixture (final pH 6.9–7.1) containing 100 mM imidazole, pH 6.6, 2 mM MnCl2, 1 mM glutathione (GSH), 1.25 mM IDP, 2.5 mM NADH, 50 mM KHCO3, 1.5 mM phosphoenolpyruvate, malate dehydrogenase (2 units/ml), and supernatant. For determination of acetyl-CoA carboxylase activity, IBAT was homogenized in 50 mM potassium phosphate buffer, pH 7.3, containing 2 mM EDTA, 4 mM GSH, and albumin (10 mg/ml). The assay was carried out as described by Halestrap and Denton (11) by measuring the incorporation of [14C]bicarbonate (3 µCi) into acid-stable material after incubation of 1,500 g supernatants of whole homogenate with citrate. The assay mixture contained 100 mM Tris-HCl, pH 7.4, 5 mM ATP, 10 mM MgCl2, 1 mM GSH, 15 mM NaHCO3, 0.15 mM acetyl-CoA, and 5 mg/ml albumin. Protein concentration was determined as described by Lowry et al. (19).
HIGH-PROTEIN DIET AND TRIACYLGLYCEROL SYNTHESIS IN BAT

Differences between groups were analyzed using ANOVA, with P < 0.05 as the criterion of significance.

RESULTS

In Vivo Lipogenesis

The data in Table 1 show that adaptation to the HP diet resulted in a marked reduction in the rate of incorporation of $^3$H from tritiated water into IBAT TAG-FA. In both HP and control rats the rate of incorporation of [14C]glucose into BAT TAG-FA was a small fraction of the rate obtained with $^3$H2O, which estimates synthesis from all carbon sources. Also, incorporation rate of hexose carbon into IBAT TAG-FA of rats adapted to the HP diet was reduced to ~25% of that in rats fed the balanced diet. In both experimental groups the rates of incorporation of $^3$H2O or [14C]glucose into IBAT TAG-glycerol were much higher than into tissue TAG-FAs (Table 1). In contrast to the reduction in FA synthesis, adaptation to the diet did not affect TAG-glycerol synthesis from either $^3$H2O or [14C]glucose. However, the proportion of label incorporated into IBAT TAG-glycerol was much higher in HP-adapted rats than in controls. Thus, whereas in control animals the ratio of label incorporation into glycerol over incorporation into fatty acid was ~3 for both $^3$H and 14C, in HP rats this ratio was 8 and 13 for $^3$H and 14C, respectively.

Lipogenic Enzymes

In agreement with the reduction in BAT TAG-FA synthesis associated with the HP diet, the activity of four enzymes associated with FA synthesis was markedly reduced in IBAT from rats adapted to the HP diet (Fig. 1). The activity of ME and of CLY was reduced to only 8% of values in animals fed the control diet. G6PDH and acetyl-CoA carboxylase activities were reduced to 18 and 33%, respectively, of control values.

In Vitro Experiments With Glyceroneogenic Substrates

The results obtained by incubating fragments of IBAT with several concentrations of [2-14C]pyruvate are shown in Fig. 2. The rate of incorporation of [2-14C]pyruvate into TAG-FA was directly related to the substrate concentration in both HP and control rats, but was always smaller in IBAT from HP rats (Fig. 2A). On the other hand, the rate of [2-14C]pyruvate incorporation into TAG-glycerol was not significantly affected by the diet at any of the concentrations of substrate used (Fig. 2B). The proportion of 14C from pyruvate incorporated into TAG-glycerol, expressed in Fig. 2C as percentage of total label incorporation into TAGs ([14C]glycerol × 100/[14C]glycerol + [14C]FA) was much higher in IBAT from HP rats. Figures 3 and 4 show the results of experiments similar to those carried out with [2-14C]pyruvate, but using as substrates several concentrations of [U-14C]lactate (Fig. 3) or [U-14C]alanine (Fig. 4). Although the rate of incorporation of 14C from these two substrates, especially from alanine, into BAT TAG was lower than that obtained with pyruvate, the effects of the HP diet were qualitatively similar to those of the experiments with [2-14C]pyruvate. Thus adaptation to the HP diet induced a reduction in IBAT TAG-FA synthesis from both lactate (Fig. 3A) and alanine (Fig. 4A) and an increase in the proportion of these metabolites incorporated into TAG-glycerol (Figs. 3C and 4C).

Table 1. In vivo incorporation of $^3$H2O and [U-14C]glucose into glyceride-fatty acid and glycerol of IBAT from rats adapted to HP or control diet

<table>
<thead>
<tr>
<th>Glyceride-Fatty Acid</th>
<th>Glyceride-Glycerol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>(nmol · g$^{-1}$ · min$^{-1}$)</td>
</tr>
<tr>
<td>$^3$H2O</td>
<td>37.8 ± 2.3</td>
</tr>
<tr>
<td></td>
<td>(n = 10)</td>
</tr>
<tr>
<td>[U-14C]glucose</td>
<td>7.0 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>(n = 9)</td>
</tr>
</tbody>
</table>

Values are means ± SE in nmol · g$^{-1}$ · min$^{-1}$; n = no. of rats; IBAT, interscapular brown adipose tissue; HP, high protein, carbohydrate free. *P < 0.05 vs. control. †P < 0.01 vs. control.
that glucose does not constitute a preferential substrate for FA formation in BAT but is a major contributor to glyceride-glycerol synthesis, similar to what has been found for white adipose tissue (10, 16, 24). Indeed, even in rats fed the balanced, carbohydrate-rich diet, TAG-FA synthesis from glucose constituted only ~18% of IBAT total FA synthesis, while the rate of hexose incorporation into glyceride-glycerol was three times higher than that into FA (Table 1). It can also be inferred from these experiments that most of the glyceride-glycerol synthesized by BAT is utilized for esterification of preformed FA, which include, in addition to FAs recycled after hydrolysis of stored TAGs, FAs taken up by the tissue from the circulation (FA...
produced by breakdown of lipoproteins and albumin-bound free FAs). In fact, the portion of glycerol that is utilized for esterification of preformed FA can be obtained by discounting the glycerol used to esterify FA synthesized de novo, which corresponds to one-third of the glyceride-FAs synthesized from $^3$H$_2$O (Table 1), if it is assumed that 3 mol of FA are esterified by 1 mol of glycerol. Thus it can be estimated $[(^3$H$)\text{glycerol} - \frac{1}{3}(^3$H$)\text{FA}] \times 100/[^3$H$]\text{glycerol}]$ that ~89% of total glycerol synthesized in BAT of rats fed the HP diet was utilized to esterify preformed FA. For HP-fed rats this percentage is even higher: 96%.

We have previously reported that BAT of rats adapted to the HP diet has a reduced thermogenic capacity that is accompanied by a decreased lipogenic activity, estimated by the incorporation of $^3$H$_2$O into tissue FAs (6). The present data show that FA synthesis from glucose in vivo is also reduced and that the activities of G6PDH and ME, generators of NADPH for lipid synthesis, as well as of CLY and acetyl-CoA carboxylase, which participate in the FA synthesis pathway, are markedly reduced in BAT from rats fed the HP diet. The available evidence suggests that the marked reduction in BAT lipogenesis in HP-adapted rats is probably due to a combination of neural and hormonal factors. Numerous studies, reviewed by Himms-Hagen (13), suggest that chemical signs elicited by qualitative and quantitative changes in the diet modulate the activity of sympathetic neurons in the ventromedial and other hypothalamic areas that control BAT thermogenesis. It has been found that electrical stimulation of the ventromedial hypothalamus markedly increases lipid synthesis in BAT (28) and that this effect is almost completely abolished after sympathetic denervation of the tissue (22). We have recently shown (5) that norepinephrine turnover rate, which is mainly dependent on sympathetic impulse traffic, is greatly reduced in BAT from HP-fed rats, suggesting that the decrease in lipogenesis may be due to a hypothalamic-mediated suppression of BAT sympathetic activity. On the other hand, rats fed high-protein diets have low levels of circulating insulin and high levels of glucagon (8, 25), and it has been found that insulin stimulates BAT lipogenesis, both in vivo and in brown adipocytes in vitro (20, 21, 26), raising the possibility that a low insulin-to-glucagon ratio at the tissue level contributes to the reduction in FA synthesis. Plasma insulin levels in rats of the present work, determined by radioimmunoassay, were (in µU/ml) 29 ± 5 for HP (5 animals) and 46 ± 8 for controls (5 animals). Further experiments are needed to determine the relative importance of neural and hormonal factors.

The data of the present work indicate that glyceroneogenesis is very active in BAT, enabling the tissue to synthesize glycerol from nonglucose sources by forming phosphoenolpyruvate from pyruvate via the dicarboxylic shuttle and subsequent production of glycerol phosphate by a partial reversal of glycolysis. The data in Table 1 show that even in rats fed the balanced diet, glycerol synthesis from noncarbohydrate sources, estimated by subtracting synthesis of glycerol from $[14$C$]$glucose from the rates obtained with $^3$H$_2$O, represented

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**Table 2.** In vitro incorporation of $[1-14$C$]$pyruvate and $[2-14$C$]$pyruvate at concentrations of 0.2, 1.0, and 5.0 mM into glyceride-glycerol by fragments of IBAT from rats adapted to HP or control diet

<table>
<thead>
<tr>
<th>Pyruvate, mM</th>
<th>$[1-14$C$]$Pyruvate</th>
<th>$[2-14$C$]$Pyruvate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>HP diet</td>
</tr>
<tr>
<td>0.2</td>
<td>3.1 ± 0.6</td>
<td>6.6 ± 0.8†</td>
</tr>
<tr>
<td>1.0</td>
<td>20.0 ± 2.8</td>
<td>30.3 ± 3.4*</td>
</tr>
<tr>
<td>5.0</td>
<td>99.7 ± 11.1</td>
<td>132.9 ± 11.5</td>
</tr>
</tbody>
</table>

Values are means ± SE in nmol·g$^{-1}$·min$^{-1}$ of 6 rats. *P < 0.05 vs. control. †P < 0.01 vs. control.
The conversion of $[1-14C]$pyruvate into glycerol, as well as the incorporation of $[2-14C]$pyruvate, $[14C]$lactate, and $[14C]$alanine into glycerol compared with FA, by the increased activity of BAT phosphoenolpyruvate carboxykinase, an enzyme that has been shown to play a regulatory role in white adipose tissue glyceroneogenesis.

The results of the present work emphasize the importance of an active production of glycerol phosphate for the normal functioning of BAT. A significant part of this production seems to be effected through glyceroneogenesis, which proceeds at high rates even in animals fed a balanced, carbohydrate-rich diet. Only a relatively small part of the glycerol phosphate produced appears to be used to esterify newly synthesized FAs, the majority being directed to esterification of preformed fatty acids. Because, in contrast to white adipose tissue, BAT has an appreciable glycerokinase activity, it seems reasonable to conclude that a considerable part of these preformed FAs were FAs taken up from the circulation, especially in the fed state, FA produced by hydrolases of chylomicrons. High rates of glycerol synthesis and of uptake and esterification of preformed FAs seem essential to ensure adequate stores of TAGs, necessary for a normal BAT thermogenic activity. Indeed, activation of heat production by BAT in both diet-induced and nonshivering thermogenesis is associated with stimulation of tissue TAG hydrolases to produce FAs, which are both substrates and uncoupling messengers for BAT mitochondria (13).

The control of BAT metabolism is well illustrated by the adaptive changes that occur in rats adapted to the HP diet. We found in a previous work (6) that despite the extremely reduced FA synthesis, the lipid content (mostly TAGs) of IBAT from rats fed the HP diet for 20–30 days amounted to 53% (per whole tissue), or 87% (mostly TAGs) of IBAT from rats fed the HP diet for 20–30 days. This capacity of BAT from HP-adapted rats to store NAFA in TAGs is essential for heat production by sympathetic activation.

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REFERENCES


