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 (HP) diet have been used for many years in our laboratory as a model system to investigate adaptive mechanisms in BAT. We have shown that despite a markedly reduced lipogenic activity, estimated in vivo by the incorporation of $^3$H$_2$O and [14C]glucose into BAT fatty acid (FA) synthesis from both $^3$H$_2$O and [14C]glucose, rats adapted to the HP diet are able to maintain considerable reserves of body fat. Thus, the present experiments were undertaken to further investigate the diet-induced alterations in BAT lipogenesis. The effects of the HP diet on the activity of enzymes involved in lipid synthesis and on relative contributions of glucose carbon and carbon from other sources to the in vivo synthesis of FA and glycerol moieties of BAT 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 the contribution of glucose carbon and carbon from other sources to the in vivo synthesis of FA and glycerol moieties of BAT 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 $^{14}$C from glucose into two TAG moieties of IBAT. The assumptions and supportive arguments for the adequacy of $^3$H$_2$O for this purpose are described in detail (6).
measurement of lipid synthesis from all carbon sources have been presented by Windmuller and Spaeth (31) and Jungas (15). The flux of glucose carbon to IBAT FA or glycerol was estimated using the semicompartmental approach described by Baker and Huebner (2), which is a modification of the noncompartmental approach of Shipley et al. (29) and combines features of both noncompartmental and compartmental analyses. This semicompartmental 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 $^3$H- or $^{14}$C-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-13C]glucose (10 µCi) and $^3$H$_2$O (5 mCi) dissolved in 0.5 ml saline was 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 label injection. TAG was isolated as described by Lowry et al. (19). 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 $^{14}$C in final products was the same as that described for the in vivo experiments.

In Vitro 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-Henselit 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 $^{14}$C 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 the assay mixture containing 50 mM trithiamine, pH 7.7, 7 mM ATP-Mg, 10 mM potassium citrate, 0.1 mM NAD+, and 0.2 mM NADPH. CLY was assayed as described by Srere (30), measuring the rate of oxidation of NADH in a assay mixture containing 50 mM trithiamine, pH 7.7, 7 mM ATP-Mg, 10 mM potassium citrate, 0.1 mM NADH, 10 mM mercaptoethanol, 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 $^{14}$C]bicarbonate (2 µCi, Amersham) into acid-stable product was determined in an assay mixture containing 100 mM imidazole, pH 6.8, 2 mM MnCl$_2$, 1 mM glutathione (GSH), 1.25 mM IDP, 2.5 mM NADH, 50 mM KHCO$_3$, 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 $^{14}$C]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 MgCl$_2$, 1 mM GSH, 15 mM NaHCO$_3$, 0.15 mM acetyl-CoA, and 5 mg/ml albumin. Protein concentration was determined as described by Lowry et al. (19).
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-FAs. In both HP and control rats the rate of incorporation of [$^{14}$C]glucose into BAT TAG-FA was a small fraction of the rate obtained with $^3$H$_2$O, 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 $^3$H$_2$O or [$^{14}$C]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 $^3$H$_2$O or [$^{14}$C]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 $^{14}$C, in HP rats this ratio was 8 and 13 for $^3$H and $^{14}$C, respectively.

Lipogenic Enzymes

In agreement with the reduction in BAT TAG-FA synthesis induced by 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-$^{14}$C]pyruvate are shown in Fig. 2. The rate of incorporation of [2-$^{14}$C]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-$^{14}$C]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 $^{14}$C from pyruvate incorporated into TAG-glycerol, expressed in Fig. 2C as percentage of total label incorporation into TAGs ($[^{14}$C]glycerol x 100/[4C]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-$^{14}$C]pyruvate, but using as substrates several concentrations of [U-$^{14}$C]lactate (Fig. 3) or [U-$^{14}$C]alanine (Fig. 4). Although the rate of incorporation of $^{14}$C 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-$^{14}$C]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 2 show the results of experiments in which the rate of incorporation of carbon-1 from pyruvate into IBAT glyceride-glycerol was compared with the rate of incorporation of carbon-2. As anticipated, incorporation of [1-$^{14}$C]pyruvate into TAG-FA was negligible (data not shown), because carbon-1 is lost in the formation of acetyl-CoA. In IBAT from both HP-adapted and control rats, the rate of incorporation of [1-$^{14}$C]pyruvate into glyceride-glycerol was ~40–50% of rates obtained with [2-$^{14}$C]pyruvate (Table 2). These data are consistent with the presence of a functioning dicarboxylic acid shuttle in BAT. In the experiments with [1-$^{14}$C]pyruvate, when label incorporation into FA was negligible, incorporation into glyceride-glycerol was significantly higher in IBAT from HP-adapted rats than in controls in tissues incubated with 0.2 and 1.0 mM of [1-$^{14}$C]pyruvate (Table 2). With 5.0 mM pyruvate, statistical significance was not attained, but there was a clear tendency for higher values (P < 0.10) in HP-fed rats (Table 2).
Phosphoenolpyruvate Carboxykinase Activity

In agreement with the data above, indicative of an increased BAT glyceroneogenesis in HP-adapted rats, the activity of phosphoenolpyruvate carboxykinase activity (in nmol·mg protein⁻¹·min⁻¹) was about four times higher in IBAT from these animals (28.4 ± 4.2, 7 rats) than in controls fed the balanced diet (6.8 ± 0.6, 9 rats).

DISCUSSION

Before discussing the changes in lipogenic activity induced by the HP diet, it is interesting to note that the results of the in vivo experiments (Table 1) indicate 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

Fig. 2. In vitro incorporation of [2-¹⁴C]pyruvate at concentrations of 0.2, 1.0, and 5.0 mM into triacylglycerol (TAG)-fatty acids (FAs) (A) and into glyceride-glycerol (B) of IBAT from rats adapted to HP or control diet. C: percentage of total label incorporation into TAG incorporated into TAG-glycerol (see text). *P < 0.05. **P < 0.01.

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Fig. 3. In vitro incorporation of [U-¹⁴C]lactate at concentrations of 0.2, 1.0, and 5.0 mM into TAG-FAs (A) and into glyceride-glycerol (B) of IBAT from rats adapted to HP or control diet. C: percentage of total label incorporation into TAG incorporated into TAG-glycerol (see text). *P < 0.05. **P < 0.01.
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 that 89% of total glycerol synthesized in BAT of rats fed the balanced 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 noncarbohydrate 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

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>[1-14C]Pyruvate</th>
<th>[2-14C]Pyruvate</th>
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<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>
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Values are means ± SE in nmol · g$^{-1}$·min$^{-1}$ of 6 rats. *P < 0.05 vs. control. †P < 0.01 vs. control.
~81% of total glycerol production. The present study also shows that the glyceroneogenic activity is increased in BAT from rats adapted to the HP diet, as evidenced by the markedly higher proportion of [2-14C]pyruvate, [14C]lactate, and [14C]alanine incorporated into glycerol compared with FA, by the increased conversion of [1-14C]pyruvate into glycerol, as well as by the fourfold increase in the 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 hydrolysis 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 hydrolysis 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% (per gram tissue) of values in animals fed the control diet. This capacity of BAT from HP-adapted rats to maintain substantial reserves of lipid can be explained by the high rate of glyceroneogenesis and esterification of preformed fatty acids suggested by the present data. Lipid stores are necessary for an adequate response of BAT when the tissue is recruited in nonshivering thermogenesis. We have recently shown (5) that the depressed sympathetic activity in IBAT from HP rats is rapidly restored after acute cold exposure (4°C), with no change in the body (colonic) temperature, despite the initially reduced thermogenic capacity. There can be little doubt that the lipid stored in BAT from HP rats was essential for heat production by sympathetic activation. The results of the cold exposure experiments (5) also illustrate the prevalence of heat generation for thermal protection in a situation where BAT thermogenesis is reduced to increase the metabolic efficiency of the diet.

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