Control of glyceroneogenic activity in rat brown adipose tissue


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Festuccia, W. T. L., N. H. Kawashita, M. A. R. Garofalo, M. A. F. Moura, S. R. C. Brito, I. C. Kettelhut, and R. H. Migliorini. Control of glyceroneogenic activity in rat brown adipose tissue. Am J Physiol Regul Integr Comp Physiol 285: R177–R182, 2003; 10.1152/ajpregu.00713.2002.—Brown adipose tissue (BAT) glyceroneogenesis was evaluated in rats either fasted for 48 h or with streptozocin-diabetes induced 3 days previously or adapted for 20 days to a high-protein, carbohydrate-free (HP) diet, conditions in which BAT glucose utilization is reduced. The three treatments induced an increase in BAT glyceroneogenic activity, evidenced by increased rates of incorporation of [1-14C]pyruvate into triacylglycerol (TAG)-glycerol in vitro and a marked, threefold increase in the activity of BAT phosphoenolpyruvate carboxykinase (PEPCK). BAT glycerokinase activity was not significantly affected by fasting or diabetes. After unilateral BAT denervation of rats fed either the HP or a balanced diet, glyceroneogenesis activity increased in denervated pads, evidenced by increased rates of nonglucose carbon incorporation into TAG-glycerol in vivo (difference between 3H2O and [14C]glucose incorporations) and of [1-14C]pyruvate in vitro. PEPCK activity was not significantly affected by denervation. The data suggest that BAT glyceroneogenesis is not under sympathetic control but is sensitive to hormonal/metabolic factors. In situations of reduced glucose use there is an increase in BAT glyceroneogenesis that may compensate the decreased generation of glycerol-3-phosphate from the hexose.

HYDROLYSIS OF STORED TRIACYLGLYCEROL (TAG) to produce fatty acids (FA), which are both substrates and uncoupler messengers for brown adipose tissue (BAT) mitochondria, is an obligatory step in the process of activation of heat production in BAT in both diet-induced and nonshivering thermogenesis (13). Therefore, maintenance of adequate stores of TAG, through esterification, via glycerol-3-phosphate (G3P), of newly synthesized or preformed FA (taken up from the circulation or recycled after hydrolysis of endogenous TAG) seems to be essential for the normal functioning of BAT. There are three possible sources of G3P for acylation and TAG formation (Fig. 1): 1) glucose, via dihydroxyacetone-P in the glycolytic pathway and conversion to G3P by glycerophosphate dehydrogenase; 2) glycerol, produced by hydrolysis of stored TAG or taken up by the tissue from the circulation, that is directly converted to G3P by glycerokinase (GyK); and 3) three carbon intermediates (such as lactate and pyruvate), via glyceroenogenesis, forming phosphoenolpyruvate via the dicarboxylic shuttle and subsequent formation of G3P by a partial reversion of glycolysis. We recently provided strong evidence indicating that BAT GyK is under direct sympathetic control, its activity changing in parallel to increases or decreases in sympathetic flow (16). With respect to glyceroneogenesis, although it has been known for many years that white adipose tissue can generate G3P by this pathway (3, 20), its presence in BAT had not been documented in the literature until our recent work (6) showing that glyceroneogenesis is not only present in BAT but is very active, representing the major source of G3P used for acylation and TAG synthesis. In this same work (6) we found that glyceroenogenic activity, estimated by the incorporation of 14C from pyruvate into TAG-glycerol and the activity of phosphoenolpyruvate carboxykinase (PEPCK), is even higher in rats adapted to a high-protein, carbohydrate-free (HP) diet, a preparation that has been used in our laboratory to investigate the control of energy-linked metabolic processes. It has been demonstrated in several studies that the use of glucose by BAT is increased not only by insulin (13) but also by electrical stimulation of sympathetic nerves (21) or norepinephrine administration, by a mechanism independent of insulin (9, 22). Rats adapted to the HP diet have, in addition to low levels of BAT GyK (4, 16), a reduced uptake and utilization of glucose by BAT, which we recently showed (15) to be due to the combined effects of the low levels of plasma insulin (10, 17) and the reduced BAT sympathetic activity (7) of these animals. Because of the prolonged absence of carbohydrate in the diet of HP diet-fed rats, the increase in glyceroneogenic activity appeared to represent a compensatory mechanism for a reduced production of G3P from glucose and glycerol, caused by the...
decreases in glycolysis and GyK activity, respectively. Therefore, one of the objectives of the present work was to investigate the changes in BAT glyceroneogenesis and GyK activity produced by a short period of carbohydrate restriction during fasting or by diabetes, a situation in which the utilization of glucose is impaired. To this end, rates of synthesis of TAG-glycerol from [14C]pyruvate in vitro and the activities of PEPCK and GyK were determined in BAT from rats fed a balanced diet, previously fasted for 48 h, or 3 days after diabetes induction by streptozotocin. Another objective of this study was to investigate, using the technique of BAT hemidegeneration, the participation of the sympathetic nervous system in the control of BAT glyceroneogenesis. The specific objectives of the experiments were to estimate in intact and denervated BAT pads from rats fed a balanced diet or adapted to the HP diet 1) the contribution of glucose carbon and of carbon from other sources to the synthesis of TAG-glycerol in vivo and 2) the synthesis of TAG-glycerol from [14C]pyruvate in vitro and in the activity of PEPCK. The effect of BAT denervation on PEPCK activity after reversion of the HP diet to a balanced diet was also examined.

MATERIAL AND METHODS

Male Wistar rats weighing initially 110–120 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 a purified (HP) diet containing 70% casein, no carbohydrate, and 8% corn oil or to a balanced 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 (5). As in previous studies (18), after an initial period of adaptation of a few days, food ingestion and the rate of body weight gain were similar for the two groups of rats. The animals weighed 180–220 g when used for the experiments. In the experiments of diet reversion, the diet of the rats adapted to the high-protein diet was replaced by the control diet at 7:30 PM, and PEPCK activity was measured 6 and 12 h later. For diabetes induction, streptozotocin (40 mg/kg body wt, dissolved in citrate buffer, pH 4.5) was injected into the dorsal vein of the penis of rats fed the balanced diet, previously fasted for 12 h. Controls were injected with saline. When used for the experiments, 3 days after streptozotocin injection, the animals had plasma glucose levels between 350 and 450 mg/dl. For the fasting experiments, rats fed the balanced diet were left without food but had access to water ad libitum for 48 h. Care and treatment of animals received prior institutional approval.

Unilateral Denervation of BAT

Under ether anesthesia, five branches of the right intercostal nerve bundles that contain sympathetic fibers entering the right side of the interscapular BAT were isolated and a section of ~5 mm was removed from these nerves. Surgical hemidegeneration was performed 6 days before the use of the animals for the experiments. After this period, the norepinephrine content of the denervated pads, measured as described (11), was reduced to <2% of values in the innervated side.

TAG-Glycerol Synthesis in Vivo

Experimental approach. The contribution of glucose carbon and of carbon from other sources to the synthesis of TAG-glycerol was evaluated by determining simultaneously in the same animal the rate of incorporation of tritiated water, which estimates total synthesis (from all carbon sources), and of 14C from glucose into BAT TAG-glycerol. The assumptions and supportive arguments for the adequacy of 3H2O for measurement of lipid synthesis have been presented by Windmueller and Spaeth (24) and Jungas (14). The flux of glucose carbon to TAG-glycerol was estimated using the semicompartmental approach of Baker et al. (1), which is a modification of the noncompartmental approach of Shipley et al. (23) and combines features of both noncompartmental and compartmental analyses. The 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. (23)] 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. 1. 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-glycerol. [U-14C]glucose (10 μCi) and 3H2O (5 mCi) dissolved in 0.5 ml saline were injected into fed, nonanesthetized rats through a catheter inserted into the right jugular vein 2 days before the experiments. After the catheter was flushed with saline, 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 for determination of [14C]glucose specific activity. Immediately after obtaining the 60-min blood sample, which was also used for determination of plasma water specific activity, the animals were killed by cervical dislocation and the interscapular BAT was rapidly removed and cleaned of adhering muscle and white adipose tissue. Tissue TAG-glycerol was isolated as previously described (6) and dissolved in toluene-triton-POPOP for 3H and 14C measurement.
Plasma $^{14}$C-glucose was isolated by thin-layer chromatography (2), and the concentration of the hexose in plasma was determined with glucose oxidase in a Beckman (Fullerton, CA) glucose analyzer. Body water specific activity was determined directly on aliquots of diluted plasma dissolved in toluene-triton-PPO-POPOP. For radioactivity measurements, simultaneous liquid scintillation counting of the $^3$H and $^{14}$C of glycerol was performed using a channels ratio method (12). Calculations were made as detailed in Ref. 1 through steps summarized in Ref. 6.

In Vitro Experiments

The rats were killed and the interscapular BAT was removed and cleaned free of adhering muscle and fat as described above. Portions of 100 mg of the tissue were cut in small pieces of $\frac{1}{10}$ mg and incubated in 5 ml of Krebs-Henseleit bicarbonate buffer, pH 7.4, containing [1-$^{14}$C]pyruvate (0.5 mM, 1 $\mu$Ci). Incubations were carried out at 37°C with constant shaking for 2 h. The procedure used for isolation and counting of [14C]TAG-glycerol was the same as that described for the in vivo experiments.

Measurement of Enzyme Activity

PEPCK was assayed by the method of Chang and Lane (8) in 100,000-g supernatants obtained after homogenization of BAT in 20 mM triethanolamine buffer, pH 7.5, containing 0.2 M sucrose, 5 mM mercaptoethanol, and 1 mM EDTA. The incorporation of [3$^{14}$C]bicarbonate (2 $\mu$Ci) into acid-stable product was determined in an assay mixture of identical composition as that used in a previous study (6). GyK activity was measured following the recommendations of Newsholme et al. (19) in 2,000-g supernatants obtained after homogenization of BAT in ice-cold 1% KCl in 1 mM EDTA. The composition of the assay mixture, which contained [U-14C]glycerol, and the isolation of labeled glycerol phosphate were previously described in detail (16).

Statistical Methods

Data are expressed as means $\pm$ SE and differences between groups were analyzed using ANOVA, with $P < 0.05$ as the criterion of significance.

RESULTS

The data in Fig. 2 show that the rate of incorporation of [1-$^{14}$C]pyruvate into TAG-glycerol by incubated fragments of BAT was significantly (40%) higher in tissues obtained from rats fasted for 48 h than in fed controls. A significant 25% increase in the rate of incorporation of $^{[14}$C]pyruvate into TAG-glycerol was also observed in BAT fragments obtained 3 days after diabetes induction by streptozotocin (Fig. 2).

In agreement with the results of the experiments with labeled pyruvate, the data in Fig. 3 show that both fasting for 48 h and diabetes for 3 days induced a marked, threefold increase in the activity of BAT PEPCK. On the other hand, Fig. 4 shows that the activity of BAT GyK was not significantly affected by fasting or diabetes. As in our previous work (16), the activity of the enzyme was reduced by 50% in rats adapted to the HP diet (Fig. 4).

Fig. 2. Effect of fasting for 48 h or of a short period (3 days) of streptozotocin-induced diabetes on the rate of incorporation of [1-$^{14}$C]pyruvate into TAG-glycerol by BAT fragments in vitro. Data are means $\pm$ SE from 8 rats. *$P < 0.05$ vs. controls.

Fig. 3. Effect of fasting for 48 h or of a short period (3 days) of streptozotocin-induced diabetes on the activity of BAT PEPCK. Data are means $\pm$ SE from 8 rats. *$P < 0.01$ vs. controls.

Fig. 4. Effect of fasting for 48 h or of a short period (3 days) of streptozotocin-induced diabetes or of adaptation to a high-protein, carbohydrate-free (HP) diet for 20 days on the activity of BAT GyK. Data are means $\pm$ SE from 8 animals. *$P < 0.05$ vs. controls.
Table 1. In vivo incorporation of $^3$H$_2$O and [U-14C]glucose into TAG-glycerol of innervated and denervated IBAT pads from rats adapted to HP diet or a balanced diet

<table>
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<tr>
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<th>Balanced Diet</th>
<th>HP Diet</th>
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<tr>
<td></td>
<td>Innervated</td>
<td>Denervated</td>
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<tr>
<td>$^3$H$_2$O</td>
<td>187 ± 12</td>
<td>243 ± 16*</td>
</tr>
<tr>
<td>[14C]glucose</td>
<td>28 ± 10</td>
<td>14 ± 7</td>
</tr>
<tr>
<td>Nonglucose carbon</td>
<td>159 ± 10</td>
<td>229 ± 13*</td>
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Values are means ± SE in nmol · g$^{-1}$·min$^{-1}$; n = 8 rats. Nonglucose carbon incorporation into triacylglycerol (TAG)-glycerol was estimated by the difference between [14C]glucose and $^3$H$_2$O incorporations. IBAT, interscapular brown adipose tissue; HP, high-protein, carbohydrate free. *P < 0.05 vs. innervated pad; †P < 0.05 vs. balanced, innervated pad.

The results of the experiments with BAT hemide-
ervation are shown in Table 1 and Figs. 5 and 6. Rates of in vivo incorporation of $^3$H and 14C into TAG-glycerol are given in Table 1. The data show that in both rats fed the balanced diet and in rats adapted to the HP diet, rates of incorporation of $^3$H$_2$O into BAT TAG-glycerol, as well as rates of incorporation of nonglucose carbon into TAG-glycerol (glyceroneogenesis, estimated as indicated in the legend of Table 1), were significantly higher in denervated than in intact pads. The data in Table 1 also show that in all conditions rates of incorporation of 14C from glucose into TAG-glycerol were much lower than those obtained with $^3$H$_2$O.

Confirming previous results (6), adaptation to the HP diet resulted in an increase of ~60% in the rate of incorporation of [1-14C]pyruvate into TAG-glycerol by incubated BAT fragments (Fig. 5). The data in Fig. 5 also show that, independent of the type of diet, BAT denervation induced a significant increase in the rate of in vitro synthesis of TAG-glycerol from pyruvate. The increase was more pronounced in HP diet-adapted rats than in rats fed the balanced diet.

As in our previous study (6), adaptation to the HP diet induced a marked increase in the activity of BAT PEPCK (Fig. 6). The data in Fig. 6 show that the activity of the enzyme was not significantly affected by BAT denervation in either HP diet-fed rats or in rats on the balanced diet. As indicated in the legend of Fig. 6, reversion of the diet of HP diet-fed rats to the balanced diet resulted in increases in both plasma glucose and insulin levels, which attained values above those of controls. Figure 6 also shows that BAT denervation did not interfere with the decrease of PEPCK activity induced by replacement of the diet of the HP rats by the balanced diet. After 12 h of diet reversion, BAT PEPCK returned to levels comparable to those in controls in both denervated and intact pads, with values even somewhat lower in the denervated side (Fig. 6).

DISCUSSION

The data of the present work show that fasting for 48 h or a short (3 days) period of insulin deficiency induces an increase in BAT glyceroneogenesis, evidenced by a marked increase in the activity of BAT PEPCK (Fig. 3), which is accompanied by an increased capacity of the tissue to incorporate [14C]pyruvate into TAG-glycerol (Fig. 2). Confirming previous results (6),
similar effects on BAT glyceroneogenesis were induced by adaptation of rats to an HP diet (Figs. 5 and 6). Because the levels of the two direct activators of BAT glucose uptake, plasma insulin (13) and BAT sympathetic activity (21), are low in fasted, diabetic as well as in HP diet-adapted rats (7, 13, 17), the utilization of the hexose is reduced in these animals. The finding in the present study of an increased glyceroneogenic activity in three situations in which the utilization of glucose is reduced supports the contention that this process is activated to compensate for a reduction in the generation of G3P from the hexose via dihydroxyacetone-P in the glycolytic pathway.

In a recent study (16), we provided strong evidence indicating that BAT GyK and, therefore, the generation of G3P by this pathway, is under direct sympathetic control (Fig. 1), its activity changing in parallel to changes in sympathetic flow. It was also found (16) that the response of the enzyme to both decreases and increases in BAT sympathetic flow is not rapid, but gradual and time dependent. Hence, the lack of effect of 48 h of fasting or 3 days of diabetes on the activity of BAT GyK (Fig. 4) can be interpreted as an indication that the reduction of sympathetic activity in these conditions was relatively small and/or of too short duration.

Taking into account the activity of GyK in each situation, other findings of the present work can also be explained if it is assumed that BAT glyceroneogenesis increases to compensate for the generation of G3P by other pathways. Thus the fact that, as judged by the rates of incorporation of [14C]pyruvate into TAG-glycerol, the increase in glyceroneogenesis induced by fasting or diabetes (Fig. 2) was smaller than that induced by adaptation to the HP diet (Fig. 5) can be explained by the fact that BAT GyK activity was reduced only in HP diet-fed rats (Fig. 4), with the consequent greater decrease in G3P production requiring a greater compensatory increase in glyceroneogenesis. A similar explanation could be given to the finding that denervation induced an increase in glyceroneogenic flux both in the in vivo (Table 1) and in the in vitro experiments (Fig. 5). Denervation induces a 50% reduction in the activity of BAT GyK in both normally fed and HP diet-fed rats (16), with a resulting decrease in the production of G3P from glycerol and a greater demand on glyceroneogenesis. As expected from the fact that the activity of GyK in HP diet-adapted rats was already reduced before surgery, this effect of denervation was more pronounced than in rats fed the balanced diet (Fig. 5). As judged by the rates obtained in the experiments in vivo, the activity of PEPCk in rats fed the balanced and HP diet, which did not change after denervation, was more than sufficient to accommodate the increased flux in the glyceroneogenic pathway.

One of the difficulties of understanding the functioning of BAT is the complex interplay of neural and hormonal/metabolic factors that controls the different aspects of BAT metabolism. Several results of the present experiments suggest that, in contrast to BAT GyK (16), glyceroneogenesis is not under control of the sympathetic nervous system. Thus, although the reduction of sympathetic activity in fasted and diabetic rats was relatively small or of short duration, as judged by unchanged levels of GyK, (Fig. 4) the threefold increase in the activity of BAT PEPCk in these conditions (Fig. 3) was similar to that in HP rats (Fig. 6), in which the reduction of sympathetic flow was more marked and prolonged, with a 50% decrease of GyK levels (Fig. 4). Also, BAT denervation did not affect the activity of PEPCk in rats fed either the balanced diet or the HP diet, which have high levels of enzyme activity (Fig. 6). As mentioned above, glyceroneogenic flux actually increased after denervation (Table 1 and Fig. 5). Moreover, BAT hemidenervation did not interfere with the restoration of BAT PEPCk activity in HP diet-adapted rats to control levels after their diet was replaced by the balanced diet (Fig. 6). This contrasts with the results of our experiments with BAT lipogenesis, which is markedly reduced in HP diet-fed rats (6, 17). Although BAT lipogenesis cannot be maintained in the absence of insulin (17), restoration of this process and recovery of the reduced levels of lipogenic enzymes after reversion of the diet of HP diet-fed rats was clearly impaired, even in the presence of high levels of the hormone (17). It would thus appear that BAT glyceroneogenesis is more sensitive to hormonal/metabolic factors, such as plasma insulin levels and glucose availability, than to neural factors.

The data of the present work, together with those of our previous studies (6, 16), clearly illustrate the importance of an active production of G3P to ensure adequate stores of TAG necessary for normal BAT functioning. We showed (16) that in situations of sustained increase in BAT sympathetic activity (and, therefore, in TAG hydrolysis), such as during prolonged cold exposure, there is a stimulation of the activity of BAT GyK, the increased production of G3P contributing to maintain lipid stores. The present data suggest that BAT glyceroneogenesis has an important role in the maintenance of an adequate supply of G3P for TAG synthesis in situations in which there is a reduction in the generation of G3P from glucose, via dihydroxyacetone-P or from glycerol via GyK. In these situations, glyceroneogenesis, in contrast to GyK, seems to be stimulated not by neural, but by hormonal/metabolic, factors that induce a marked increase in the capacity of the pathway, enabling the glyceroneogenic flux to be adjusted to the reduced production of G3P. The biochemical mechanisms through which these adjustments are achieved remain to be elucidated.

**Perspectives**

In this and in a preceding work (16) we report the results of experiments designed to investigate the control of supply of G3P needed for TAG formation in BAT, with special focus on glyceroneogenesis and glycerol phosphorylation by GyK. Despite their importance for the maintenance of adequate TAG stores and normal BAT function, these processes have been little studied. In the present work, only situations in which BAT
thermogenesis is reduced were investigated. For a better, more complete understanding of the control of G3P production, studies in increased BAT thermogenesis are needed. It is well known that during thermogenesis the increased TAG lipolysis and oxidation of FAs are accompanied by increased rates of TAG synthesis, and both glyceroneogenesis and glycerol phosphorylation by GyK could provide G3P for TAG formation. This would enable glycolysis to generate ATP by substrate level phosphorylation at a time when the mitochondrial capacity for ATP synthesis is compromised by uncoupling and heat production. Equally important are studies, in different physiological situations, on the activities of cytosolic (NAD dependent) and mitochondrial (FAD dependent) glycerol-3-phosphate dehydrogenases, which are very active in BAT. The possibility exists that, together with their role in the control of cytoplasmic-reducing equivalents, these enzymes may also have a regulatory role in the production of G3P, acting independently but in concert with G3P-generating pathways.

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