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Increased glyceroneogenesis in adipose tissue from rats adapted to a high-protein, carbohydrate-free diet: role of dietary fatty acids

Salete Cipriano Brito, William Lara Festuccia, Nair Honda Kawashita, Maria Ferreira Moura, Analúcia Rampazzo Xavier, Maria Antonieta Garófalo, Isis Carmo Kettelhut, Renato Hélios Migliorini*

Department of Biochemistry-Immunology, School of Medicine, University of São Paulo, 14049-900 Ribeirão Preto, São Paulo, Brazil
Department of Physiology, School of Medicine, University of São Paulo, 14049-900 Ribeirão Preto, São Paulo, Brazil

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Abstract

We have previously shown in in vivo experiments that adipose tissue glyceroneogenesis is increased in rats adapted to a high-protein, carbohydrate-free (HP) diet. The objectives of the present study were (1) to verify if the increased glyceroneogenic activity is also observed in isolated adipocytes and (2) to investigate the role of preformed fatty acids in the production of the increased adipose tissue glyceroneogenesis. Control rats received a balanced diet, with the same lipid content of the HP diet. Glyceroneogenic activity was found to be higher in adipocytes from HP rats than in controls, as evidenced by increased rates of conversion of pyruvate and lactate to triacylglycerol (TAG)-glycerol. Administration of Triton WR 1339, which blocks the removal of TAG incorporated into circulating lipoproteins, to HP diet–adapted rats caused a significant reduction in the incorporation of ^{14}C-pyruvate into TAG-glycerol by adipose tissue, which was accompanied by a marked inhibition of phosphoenolpyruvate carboxykinase activity, the key enzyme of glyceroneogenesis. The inhibitory effect of Triton on TAG-glycerol synthesis by adipose tissue was also observed in vivo, after administration of $^{3}H_2O$. Adaptation to the HP diet induced a marked increase in the activity of retroperitoneal and epididymal fat LPL, which was restored to control values 24 hours after replacement of the HP diet by the balanced diet. The data suggest that in rats adapted to a carbohydrate-free diet, adipose tissue glyceroneogenesis is activated by an increased use of diet-derived fatty acids.

1. Introduction

The mobilization of fatty acids incorporated into triacylglycerols (TAGs) to attend the energy demands of peripheral tissues is a main function of white adipose tissue (WAT). The maintenance of adequate stores of TAG therefore seems essential for the normal functioning of WAT. The preservation of WAT TAG reserves requires a continuous supply of glycerol-3-phosphate (G3P) to esterify newly synthesized or preformed fatty acids (taken up from the circulation, where they incorporated into TAG of lipoproteins or recycled after hydrolysis of endogenous TAG). Because adipose tissue has relatively low levels of glycerokinase, the use of glycerol as a source of G3P for fatty acid esterification and TAG formation is considered negligible. The generally recognized source of G3P for this purpose has been glucose, via dihydroxyacetone in the glycolytic pathway and conversion to G3P by glycerophosphate dehydrogenase. It was demonstrated more than 30 years ago that pyruvate and glucogenic amino acids can also be converted to TAG-glycerol at appreciable rates in adipose tissue [1-4]. The formation of TAG-glycerol was shown to proceed through a pathway, named glyceroneogenesis [4], that involves the carboxylation of pyruvate to oxaloacetate, decarboxylation of oxaloacetate to phosphoenolpyruvate, and subsequently the production of G3P through a partial reversal of glycolysis. It was also shown in these pioneering studies that the activity of adipose tissue glyceroneogenesis increases in situations of low glucose availability, such as fasting and diabetes [1,4]; but only recently its importance as a supplier of G3P for fatty acid esterification and lipid metabolism has been fully recognized (see Ref [5] for a recent review). The key glyceroneogenic enzyme was

* Corresponding author. Tel.: +55 16 6023113; fax: +55 16 6336840.
E-mail address: rhmiglio@fmrp.usp.br (R.H. Migliorini).

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determined to be phosphoenolpyruvate carboxykinase (PEPCK-C) [1,3]. A large number of studies, recently reviewed [6], have been published on the control of PEPCK-C gene transcription.

The experiments described in the present study were motivated by previous findings [7,8] in rats adapted to a high-protein, carbohydrate-free (HP) diet, a preparation that has been used for many years in this laboratory to investigate the nutritional and hormonal control of energy-linked metabolic processes. In experiments with fragments of epididymal adipose tissue in vitro [7], we showed that adaptation of rats to an HP diet induces an increase in the glyceroneogenic activity of the tissue, evidenced by increased rates of incorporation of ^{14}C-pyruvate into TAG-glycerol and by an increased activity of PEPCK-C. These findings were consistent with the absence of carbohydrate in the HP diet, suggesting that the increased glyceroneogenesis represented a compensatory mechanism for a reduced generation of G3P via glycolysis. Indeed, we have more recently found [9] that both the uptake of glucose and the flux in the glycolytic pathway are markedly reduced in HP rats. In the in vitro experiments [7], it was not taken into account the previous observation that the adipose tissue of rats adapted to the HP diet contains, per unit weight, about 30% more fat cell than tissues from rats fed with a balanced diet [10] Because the data were expressed in tissue weight, the differences in rates of incorporation of ^{14}C-pyruvate could be due, at least in part, to the different number of cells. To circumvent this problem, we investigate in the present work the effect of adaptation to the HP diet on the glyceroneogenic activity of isolated adipocytes.

In in vivo experiments, performed in freely moving rats in the fed state, we showed, by determining simultaneously in the same animal the rate of incorporation of $^{3}H_2O$ and ^{14}C-glucose into the 2 TAG moieties of the epididymal and retroperitoneal adipose tissues, that glyceroneogenesis increases significantly in both tissues in HP diet–adapted rats [8]. This finding constitutes the first clear in vivo evidence of the physiological adaptive role played by adipose tissue glyceroneogenesis in situations of reduced carbohydrate availability in the diet. Of direct interest to the present work was the observation in the in vivo experiments that most of the glycerol synthesized via glyceroneogenesis by both retroperitoneal and epididymal adipose tissue is used to esterify preformed fatty acids, especially in HP rats, in which de novo fatty acid synthesis was markedly reduced. It was suggested [8] that, in rats adapted to the HP diet, the increased adipose tissue glyceroneogenesis is important for esterification of diet-derived fatty acids and preservation of body fatty stores. In the present study, we test this hypothesis by investigating both the effect of HP diet adaptation on the activity of adipose tissue lipoprotein lipase (LPL) and the effect of inhibiting the uptake of fatty acids incorporated into TAG from circulating lipoproteins on adipose tissue glyceroneogenesis.

In summary, the objectives of the present experiments were to investigate in rats adapted to an HP diet (1) the glyceroneogenic activity of isolated adipocytes, assessed by the rate of incorporation of ^{14}C from pyruvate or lactate into TAG-glycerol; (2) the activity of adipose tissue LPL, an enzyme that hydrolyzes TAG incorporated in circulating chylomicrons and very low-density lipoprotein; and (3) the adipose tissue glyceroneogenesis after administration of Triton WR 1339, which blocks the uptake of fatty acids from circulating lipoproteins.

2. Materials and methods

2.1. Animals and treatment

Male Wistar rats initially weighing 100 to 110 g were housed in suspended, wire-bottomed cages and maintained at 25°C ± 2°C on a 12-hour light-dark cycle. Two types of purified diets, previously described in detail [11], were used in this study: an HP diet, containing (wt/wt) 70% protein, no carbohydrate, and 8% corn oil, and a balanced diet, containing 17% protein, 66% carbohydrates, and 8% corn oil. The 2 diets were approximately isoenergetic and contained equal amounts of vitamins and minerals. As in previous studies [12], after an initial period of adaptation of a few days, food ingestion and the rate of body weight gain were similar for the 2 groups of rats. Although HP diet–fed rats have plasma levels of insulin and glucose somewhat lower than controls, their glycemia is very resistant to starvation because of a markedly activated gluconeogenesis [12]. The rats were kept on the diets for 15 days and weighed 180 to 200 g when used in the experiments. All experiments were carried out with the animals in the fed state between 8:00 and 10:00 AM.

In the experiments with Triton WR 1339, rats were killed 1 hour after intravenous injection of this compound (0.5 mL of a 20% solution in saline). The activity of adipose tissue LPL was also measured 24 hours after replacement of the diet of rats previously adapted to the HP diet by the balanced diet. Care and treatment of experimental rats received prior institutional approval by the Ethical Committee of São Paulo State University.

2.2. In vivo lipogenesis measurement

De novo fatty acid synthesis and TAG-glycerol produced via glycolysis plus via glyceroneogenesis were estimated by the incorporation of ^{3}H from tritiated water into the moieties of TAG. There is no incorporation of ^{3}H from tritiated water when glycerol is directly phosphorylated by glycerokinase. $^{3}H_2O$ (3 mCi in 0.5 mL saline) was injected intraperitoneally and the rats killed by cervical dislocation 60 minutes after label injection. The epididymal adipose tissue was removed, carefully dissected, weighed, and the lipids extracted with chloroform-methanol by the procedure of Folch et al [13]. The procedures for isolation and measurement of radioactivity of TAG ^{3}H-fatty acids and

glycerol have been previously described [14]. Rates of lipid synthesis were calculated assuming each fatty acid and each glycerol incorporated into TAG contained 13.3 and 3.3 atoms of tritium, respectively [15,16].

2.3. Adipocytes isolation

After cervical dislocation, the epididymal fat pads from 5 to 7 rats on each diet were removed, pooled together, and disaggregated with collagenase, as described by Rodbell [17], in Krebs-Henseleit bicarbonate buffer (pH 7.4), containing 1% fatty acid–free albumin. After incubation under continuous shaking for 1 hour at 37°C, the adipocytes were filtered through a 300-μm nylon mesh and washed 3 times with the same buffer. All steps of adipocyte isolation were carried out in the absence of glucose.

2.4. In vitro incorporation of ^{14}C-labeled pyruvate and lactate into TAG–fatty acid and glycerol

Approximately 400 000 cells or 200 mg of the distal portion of the epididymal adipose tissue were incubated for 1 hour at 37°C with constant shaking in 1 mL of glucose-free Krebs-Henseleit buffer, pH 7.4 (supplemented with 2% fatty acid–free albumin for adipocytes incubation), and containing [1-^{14}C]pyruvate (1 mmol/L, 1 μCi) or [^{14}C]lactate (1 mmol/L, 1 μCi). The procedures used for lipid extraction, isolation of TAG–fatty acids, and determination of ^{14}C in final products were as previously described [14].

2.5. Measurement of enzyme activity

PEPCK-C was assayed by the method of Chang and Lane [18] in 100 000-g supernatants obtained after homogenization of epididymal fat pad in 20 mmol/L triethanolamine buffer, pH 7.5, containing 0.2 mol/L sucrose, 5 mmol/L mercaptoethanol, and 1 mmol/L EDTA. The incorporation of [^{14}C]bicarbonate (2 μCi) into acid-stable product was determined in an assay mixture of identical composition to that used in a previous study [14]. The concentration of protein in the homogenate was determined by the method of bicinchoninic acid [19].

2.5.1. Lipoprotein lipase

An anhydrous emulsion of tri-^{14}C-oleoylglycerol, stabilized by lecithin, was prepared in glycerol as described by Nilsson-Ehle and Schotz [20]. The assay substrate solution was prepared daily by adding 2 vol of the emulsion, 2 vol of Tris buffer (0.2 mol/L, pH 8.8) containing 6% (wt/vol) bovine serum albumin and 1 vol of 36-hour fasted rat serum. After vigorous shaking in a vortex mixer for 5 seconds, the new emulsion was ready for immediate use. Retroperitoneal and epididymal fat pads were homogenized in 0.25 mol/L sucrose and 1 mmol/L EDTA buffer. Incubations were performed at 37°C in a total volume of 0.2 mL (0.1 mL of assay substrate and 0.1 mL of tissue homogenate and/or buffer). The ^{14}C-fatty acid produced during the incubations was isolated using a modification of the liquid-liquid partition system described by Belfrage and Vaughan [21] and counted. Enzyme activity was expressed as nanomoles of oleic acid per minute per gram of tissue. The concentration of protein in the homogenate was determined by the method of Lowry et al [22].

2.6. Other methods of chemical analysis

Adipocyte DNA was extracted as previously described [23]. Briefly, 0.2 mL of an adipocyte suspension was homogenized with 5 mL of 5% $HClO_4$ and 5 mL of ether and centrifuged for 15 minutes at 2000 rpm. The precipitate was washed with acetone, centrifuged, and resuspended in TE buffer (Tris 10 mmol/L and EDTA 1 mmol/L) containing 1 μg/mL of RNAse. After extraction, the amount of DNA was measured in a spectrophotometer using the Hoescht dye 33258 (bisbenzamide, Hoechst, Frankfurt, Germany) as previously described [24]. For measurement of plasma TAG, after phospholipid removal, TAGs were hydrolyzed, and the glycerol produced was assayed enzymatically using a kit from LABTEST (Lagoa Santa, Brazil).

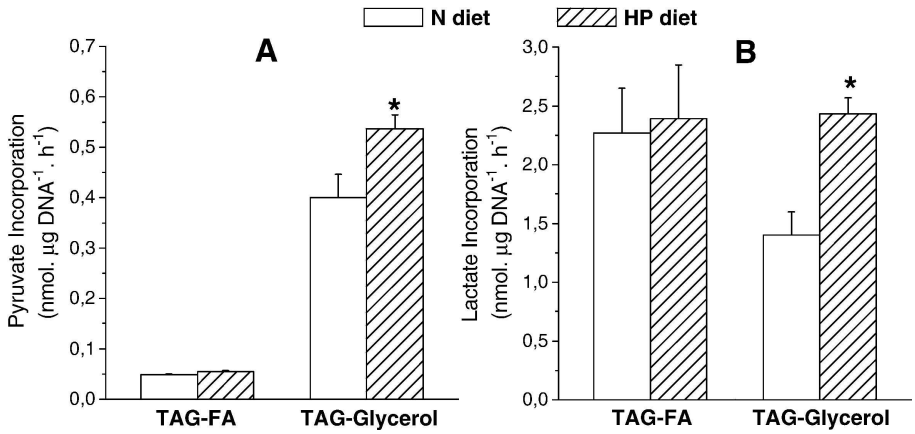

Fig. 1. [1-^{14}C]-Pyruvate (A) and [U-^{14}C]-lactate (B) incorporation into TAG–fatty acids or TAG-glycerol in vitro epididymal adipocytes of rats adapted either to a balanced (N) or an HP diet. Data are means ± SEM of 6 to 8 rats. *P < .05 vs N diet.

2.7. Statistical methods

Data are expressed as mean ± SEM. One- or two-way analysis of variance and, subsequently, the Newman-Keuls multiple range test were used. $P < .05$ was taken as criterion of significance.

3. Results and discussion

Rates of incorporation of ^{14}C from both pyruvate and lactate into TAG–fatty acids did not differ in adipocytes from the 2 experimental groups, but the incorporation into TAG-glycerol was significantly increased in adipocytes from rats adapted to the HP diet (Fig. 1). The finding of an increased glyceroneogenic activity in isolated cells excludes the possibility of attributing the increase previously obtained using adipose tissue fragments from HP rats [7] solely to the higher number of fat cells per unit of tissue weight. In our studies with HP adipocytes, we observed that clear increases in glyceroneogenesis could only be obtained if the cells were prepared in the absence of glucose. Likewise, in the experiments with tissue fragments, significant increases in the conversion of pyruvate into TAG-glycerol in adipose tissue from HP rats occurred only in the absence of glucose. An insignificant contribution of pyruvate to TAG-glycerol synthesis in the presence of glucose has also been observed in adipose tissue from fasted rats [1]. The apparent inconsistency of the inhibitory effects of the hexose in vitro with the increase in adipose tissue glyceroneogenesis observed in HP rats in vivo [8], in the presence of normal levels of blood glucose, can be explained by the marked in vivo reduction in the uptake and use of glucose by adipose tissue [14], induced by the metabolic and hormonal environment prevailing in vivo.

The experiments with Triton WR 1339, an inhibitor of tissue uptake of fatty acids incorporated into TAG of circulating lipoproteins, were motivated by the finding in in vivo studies [8] that most of glycerol synthesized via glyceroneogenesis is used to esterify preformed fatty acids. At the dose used, Triton produced marked increases in the plasma concentration of TAG in both experimental groups (Table 1). Confirming previous finding [7], the incorporation of ^{14}C from pyruvate into TAG-glycerol and the activity of PEPCK-C were markedly higher in fat pads from HP rats than in controls (Figs. 2 and 3). Administration of Triton to HP diet–adapted rats caused a significant reduction in the conversion of ^{14}C from pyruvate to TAG-glycerol by

Table 1
Plasma TAG concentration of rats adapted to a balanced diet or an HP diet injected with Triton WR 1339 or saline

	HP	Control
Saline	0.44 ± 0.05* (6)	1.05 ± 0.37 (6)
Triton WR 1339	3.15 ± 1.00** (6)	4.30 ± 0.87** (6)

Data are presented as means ± SEM (number of animals).
* $P < .05$ vs rats fed with a balanced diet.
** $P < .05$ vs rats treated with saline.

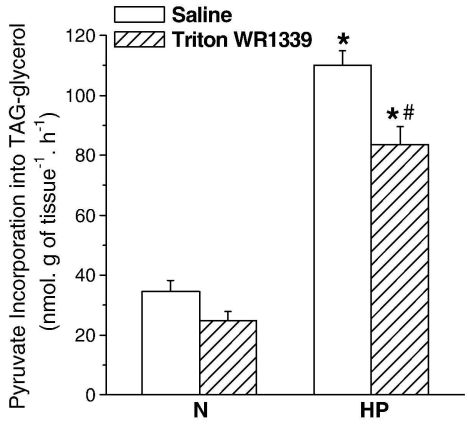

Fig. 2. Effect of intravenous Triton WR 1339 administration on the incorporation of [1-^{14}C]-pyruvate into TAG-glycerol in fragments of epididymal fat pad of rats adapted either to a balanced (N) diet or an HP diet. Data are means ± SEM of 6 to 8 rats. *$P < .05$ vs N diet; $^{\#}P < .05$ vs saline administration.

epididymal fat pad (Fig. 2), which was accompanied by a marked inhibition of PEPCK-C activity, the key enzyme of glyceroneogenesis (Fig. 3). These findings strongly support the hypothesis that fatty acids are potent positive modulators of adipose tissue glyceroneogenesis. In accordance with this idea, it has been found that the expression of PEPCK-C is strongly stimulated by long-chain, unsaturated fatty acids in both 3T3-F442A [25] and normal adipocytes [26]. In addition, the transcription factor peroxisome proliferator–activated receptor γ, which is highly expressed in adipose tissue, can be activated by fatty acids and has been found to have an important role in the control of adipose tissue PEPCK-C [27].

The inhibitory effect of Triton WR 1339 administration on adipose tissue glyceroneogenesis was also observed in experiments (Fig. 4) in which $^{3}H_2O$ and Triton WR 1339 were injected in vivo. Fig. 4 shows that, as in previous studies [28], Triton injection did not affect the marked decrease in adipose tissue fatty acid synthesis induced by

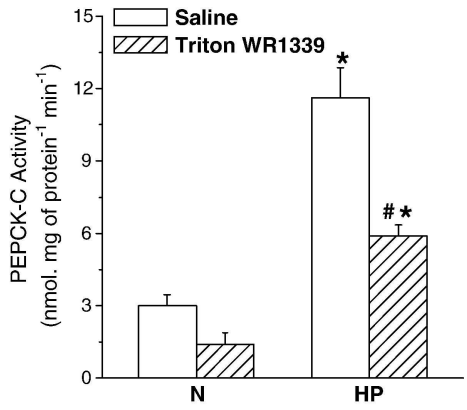

Fig. 3. Effect of intravenous Triton WR 1339 administration on the activity of cytosolic PEPCK-C in epididymal fat pad of rats adapted either to a balanced (N) diet or an HP diet. Data are means ± SEM of 6 to 8 rats. *$P <$ .05 vs N diet; $^{\#}P < .05$ vs saline.

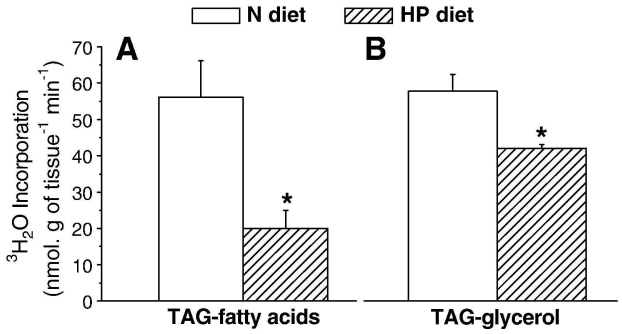

Fig. 4. Incorporation of ^3H_2O into TAG–fatty acids (A) and TAG-glycerol (B) in epididymal fat pad of rats adapted either to a balanced (N) diet or an HP diet and injected with Triton WR 1339. Data are means ± SEM of 6 to 8 rats. *$P < .05$ vs N diet.

the HP diet. Rates of TAG-glycerol synthesis, in contrast to the increase that occurs in rats not injected with Triton, were reduced in epididymal fat pads of HP rats (Fig. 4), as expected from the fact that most of the TAG-glycerol synthesized by the adipose tissue of these animals is used to esterify fatty acids [8]. This finding confirms the results obtained in vitro (Fig. 3) and represents further evidence of the stimulatory role of fatty acids in adipose tissue glyceroneogenesis.

The evidence indicating a stimulatory effect of preformed fatty acids on adipose tissue glyceroneogenesis, together with the previous finding [8] that most of the glycerol synthesized via glyceroneogenesis in that tissue is used to esterify preformed fatty acids, raised the possibility of the increased glyceroneogenic activity in HP diet–adapted rats being due to an increased use of fatty acids derived from dietary lipids. This possibility was investigated by determining in HP rats the activity of adipose tissue LPL, an enzyme that catalyzes the hydrolysis of TAG components of chylomicrons and very low-density lipoprotein. Adaptation to the HP diet induced a marked increase in the activity of epididymal and retroperitoneal fat pads LPL, which was restored to control values 24 hours after replacement of the HP diet by the balanced diet (Fig. 5). The present data do not permit any conclusion about the mechanism of the changes observed. It is almost certain that insulin played no role, as the effect of the HP diet on adipose tissue LPL activity was opposite to that expected from plasma insulin levels in HP rats, which are, in the fed state, significantly lower than those of rats fed with the balanced diet [10]. Whatever the mechanisms involved, the increase in LPL activity in HP rats seems to be essential for the preservation of body fat stores in these animals. De novo lipogenesis in HP rats, assessed by in vivo rates of incorporation of ^3H_2O into TAG–fatty acids, is markedly reduced in whole carcass, liver, and in 4 depots of adipose tissue [28]. Despite this fact, body fat stores of rats fed with the high-protein diet are remarkably well kept, carcass fatty acids amounting to 80% to 90% of values in rats fed with a balanced diet after 30 days on the diet [29]. We have previously shown that the mobilization of FFA during fasting is reduced in rats fed with the HP diet, as indicated by smaller increases in plasma FFA and lower rates of adipose tissue lipolysis after a 24-hour fast [10]. We have recently shown that adipocytes from HP diet–adapted rats have a reduced sensitivity to the lipolytic action of intracellular and extracellular agents and a small (17%) decrease in the activity of hormone-sensitive lipase, with no change in the content of the enzyme [30]. However, it is unlikely that the reduced lipomobilizing response to food restriction may have contributed to a significant degree to the conservation of body fat stores in these animals. Another factor that might contribute to maintain body lipid reserves is a decreased rate of fat oxidation for energy requirements, consequent to a reduced thermogenic capacity of brown adipose tissue, which has an important role in the regulation of diet-related energy dissipation in small rodents. In fact, we have shown that brown adipose tissue sympathetic activity and thermogenic capacity are reduced in rats adapted to the HP diet [11,31]. However, the present findings, particularly the increased adipose tissue LPL activity, strongly support our previous suggestion [8] that the preservation of body fat in rats adapted to the HP diet is mainly due to a more efficient use

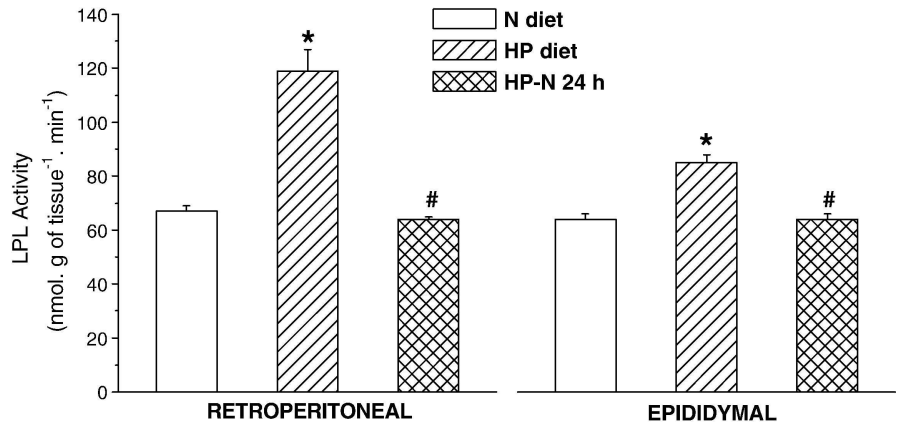

Fig. 5. Lipoprotein lipase activity in retroperitoneal and epididymal fat pads of rats adapted either to a balanced (N) diet or an HP diet, before and after 24 hours of replacement of HP diet by the balanced diet (HP-N, 24 hours). Data are means ± SEM of 6 to 8 rats. *$P < .05$ vs N diet; $^{\#}P < .05$ vs HP diet.

of dietary fatty acids by the adipose tissue. It is interesting to note in this respect that it has been found, both in humans [32] and in rats [33], that in situations where there is a loss of body fat, there is an increase in adipose tissue LPL activity, as if in an attempt to maintain body lipid reserves.

The main conclusions of the present study can be summarized as follows: (1) the increased glyceroneogenic activity in adipose tissue from rats adapted to an HP diet, previously observed in vivo, can be detected in free fat cells prepared and incubated in the absence of glucose; (2) an increased use of diet-derived fatty acids by the adipose tissue seems to be essential for the increase in glyceroneogenesis and the maintenance of body fat stores of rats adapted to the HP diet.

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