Unusual Increase of Lipogenesis in Rat White Adipose Tissue After Multiple Cycles of Starvation-Refeeding

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The purpose of the study was to determine the response of liver and brown (BAT) and white (WAT) adipose tissue lipogenesis and total body weight in rats subjected to multiple cycles of 3 days of fasting and 3 days of refeeding. Rats fasted for 3 days showed significant reduction in body weight. These changes were reversed on 3 days' refeeding. Body weight was much higher in rats fed ad libitum than in animals experiencing more than one cycle of 3 days of fasting followed by 3 days of refeeding. Despite the significant body weight reduction, an unusual increase of lipogenesis in WAT was found after multiple cycles of starvation-refeeding of rats on standard laboratory diet. The rate of lipogenesis in the liver and BAT was also elevated but to a much smaller extent. A parallel increase in enzymatic activities related to fatty acid synthesis, ie, fatty acid synthase, acetyl-coenzyme A carboxylase, adenosine triphosphate (ATP)-citrate lyase, NADP-linked malic enzyme, and hexose monophosphate shunt dehydrogenases, suggests that the increased rate of lipogenesis in WAT is a consequence of increased lipogenic enzyme activities. These data suggest that upregulation of WAT lipogenesis occurs after the multiple cycles of the starvation-refeeding protocol. An unusual increase of lipogenesis in rat WAT may have a survival advantage, because starved-refed rats must develop the ability to ingest large amounts of food during a refeeding period to store it in a convenient form than can be used as an oxidizable substrate during a period of starvation. Moreover, these results suggest that it is possible to develop appropriate starvation-refeeding conditions that may inhibit body weight gain.

IT IS WELL ESTABLISHED that starvation followed by refeeding causes an increase in rat liver lipogenic enzyme activity in comparison to the levels obtained with the same diet fed ad libitum.[1-4] The magnitude of the increase is dependent on the diet composition and the species, age, and sex of the animal.[5-8] These changes in enzyme activity are considered to be due to an alteration in the rate of enzyme protein synthesis as a consequence of increased cellular concentration of specific mRNAs.[9-15] Numerous studies showed that the increased lipogenic capacity was not confined to the liver, but occurred also in white adipose tissue (WAT).[16-19] Furthermore, it has been reported that after the second cycle of starvation-refeeding, the increase in liver malic enzyme and hexose monophosphate shunt dehydrogenase activities is even greater than after one starved-refed cycle.[20,21] Multiple cycles of starvation-refeeding in the experimental animal model could mimic the changes of lipogenic enzyme activity and possibly the rate of lipogenesis in human beings exposed to multiple cycles of a prolonged period of starvation or food restriction due to various eating disorders, or during treatment of obesity with restriction of food intake[22-25] followed by uncontrolled refeeding. Although the existence of adaptive responses to prolonged fasting or food restriction is generally accepted, adaptive responses to repeated fasting/refeeding are not well documented. There have been suggestions that repeated dieting may induce a resistance to weight loss and even an increase in body weight gain during refeeding periods.[21,26,27] Thus, the question arises as to what are the effects of dieting on body weight loss/gain and on lipogenesis. A defect in the regulation of lipogenesis, ie, sustained elevation of fatty acid biosynthesis, may be important for the development of obesity,[28,29] a serious chronic disease with medical sequelae including hypertension, cardiovascular and respiratory diseases, non–insulin-dependent diabetes mellitus, and cholelithiasis, resulting in considerable morbidity and mortality.[29] The important medical problem is that humans who are overweight should attempt to lose the excess weight. Most patients in weight-reduction programs begin to regain weight shortly after the end of active therapy,[30] and in some patients regain occurs despite continued therapy.[31] The regain process has not been well studied. Thus, there is a need to understand the process so that appropriate therapies can be developed to help the formerly obese retain lean body mass while resisting the regain of body fat.

Because of the clinical importance of repeated cycles of dieting in humans, we examined the effect of multiple cycles of starvation-refeeding with a standard laboratory diet on the body weight, lipogenic enzyme activity, and rate of lipogenesis in the rat. Finally, we examined whether similar changes occur in all main lipogenic tissues, ie, the liver, WAT, and brown adipose tissue (BAT).

MATERIALS AND METHODS

Animals

Male Wistar rats weighing approximately 230 g at the start of the investigation were maintained in wire-mesh cages at 22°C with an alternating 12-hour light/dark cycle (light from 8 AM to 8 PM) and were allowed free access to food and tap water (control group). Starvation for 72 hours started at 8 AM, and then the animals were fed a finely ground commercial diet (LSM-Centralne Laboratorium Przemyslu Paszowego, Motycz, Poland) for 72 hours (one cycle of starvation-refeeding) (Table 1). This procedure was repeated two, five, eight, or 12 times (for most experiments, one and eight times). After eight cycles of starvation-refeeding, randomly selected animals were continuously fed for up to 80 days. In this subgroup, only body weight was determined. Four rats were killed at the end of the 72-hour period of starvation of cycle 8. Also, after 12 cycles of starvation-refeeding, six rats were fed standard laboratory diet for 6 days and then killed, and the liver, WAT, and BAT were used for malic enzyme assay. All rats were allowed free access to

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Table 1. Composition of the Standard Laboratory Diet

Component	Amount
Nutrients and minerals (mean content in the diet, %)	
Nitrogen-free extract	49.2
Crude protein	23.0
Crude fiber	4.5
Crude fat	2.5
Ash	3.1
Moisture	13.0
Lysine	0.75
Methionine + cystine	0.55
Calcium	1.1
Phosphorus	0.75
Magnesium	0.2
Sodium	0.2
Potassium	1.0
Trace elements (mean content in 1 kg diet, mg)	
Manganese	75.0
Iron	50.0
Copper	5.0
Iodine	0.75
Zinc	25.0
Cobalt	1.5
Vitamins (additive in 1 kg diet)	
A	8,000 IU
D_3	1,000 IU
E	60.0 mg
K	4.0 mg
B_1	5.0 mg
B_2	10.0 mg
B_6	6.0 mg
B_{12}	20.0 μg
C	40.0 mg
Nicotinic acid	40.0 mg
Folic acid	2.0 mg
Pantothenic acid	20.0 mg
Biotin	0.2 mg
Choline	750.0 mg

tap water. At the end of each study, control and treated rats were used for estimation of in vivo fatty acid synthesis or for in vitro enzyme activity studies.

Measurement of Lipogenesis

Fatty acid synthesis in vivo was measured by determining the incorporation of tritium from tritiated water into fatty acids, as described previously.[32-34] Rats (control and treated) were injected intraperitoneally with 5 mCi (0.2 mL) ^3H_2O. After 60 minutes, the animals were killed, the liver, epididymus, and BAT were removed, and samples were taken for extraction of lipids. Tissue samples were saponified, and the fatty acid fraction was extracted.[32] Heparinized blood was collected for measurement of specific radioactivity of the plasma water.[32] It should be noted that animals used for ^3H-incorporation studies did not undergo enzymatic determination, to avoid contamination of the laboratory with tritiated water.

Enzyme Activity Assay

For determination of enzyme activity, animals were decapitated (all experiments were started at 8 AM) and total interscapular BAT or pieces of liver and epididymal WAT were removed and weighed. In some experiments, blood was collected for determination of plasma insulin by standard radioimmunoassay.

One gram of liver, kidney cortex, brain, heart, skeletal muscle, WAT, and total BAT (~250 mg) were placed in 8 mL ice-cold 20-mmol/L Tris hydrochloride buffer (pH 7.8) containing 0.2% Triton X-100. Care was taken to dissect away any WAT adhering to the BAT. The tissues were minced finely with scissors, homogenized manually with a Teflon-pestle homogenizer, and centrifuged at 30,000× *g* for 20 minutes. The resulting supernatant was decanted, and the pellet was resuspended in 5 mL isolation medium, rehomogenized, and centrifuged as above. The resulting supernatant was combined with this after the first centrifugation step and used for enzyme assay.

The fatty acid synthase (EC 2.3.1.85), adenosine triphosphate (ATP)-citrate lyase (EC 4.1.3.8), malic enzyme (EC 1.1.1.40), and hexose monophosphate dehydrogenases (glucose-6-phosphate dehydrogenase, EC 1.1.1.49; and 6-phosphogluconate dehydrogenase, EC 1.1.1.44) were assayed as described previously.[35,36] All assays were performed in duplicate at 37°C using a Beckman DU 68 spectrophotometer (Beckman Instruments, Fullerton, CA). The absorbance change both against time and against enzyme concentration was linear. Acetyl-coenzyme A carboxylase (EC 6.4.1.2) activity was measured by the $H^{14}CO_3^-$ fixation assay according to the method of Salati and Clarke.[37] Protein assays were performed according to the method of Peterson.[38]

Chemicals

All nonradioactive substances used were obtained from Sigma Chemical (St Louis, MO) or Merck (Darmstadt, Germany). ^3H_2O and $NaH^{14}CO_3$ were obtained from Amersham Buchler and Co (Braunschweig, Germany).

RESULTS

Initial body weights were similar both in controls and in the starved-refed group (Fig 1). Control rats gained weight constantly over the period tested. In starved-refed rats, the net loss

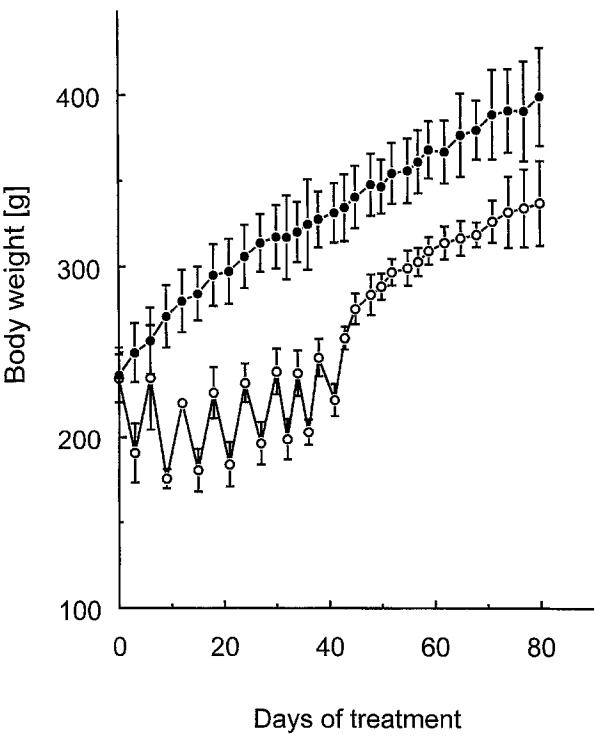

Fig 1. Body weight of rats fed ad libitum (●) or experiencing 8 cycles of starvation-refeeding (○). Each point is the mean ± SD weight of 18 animals.

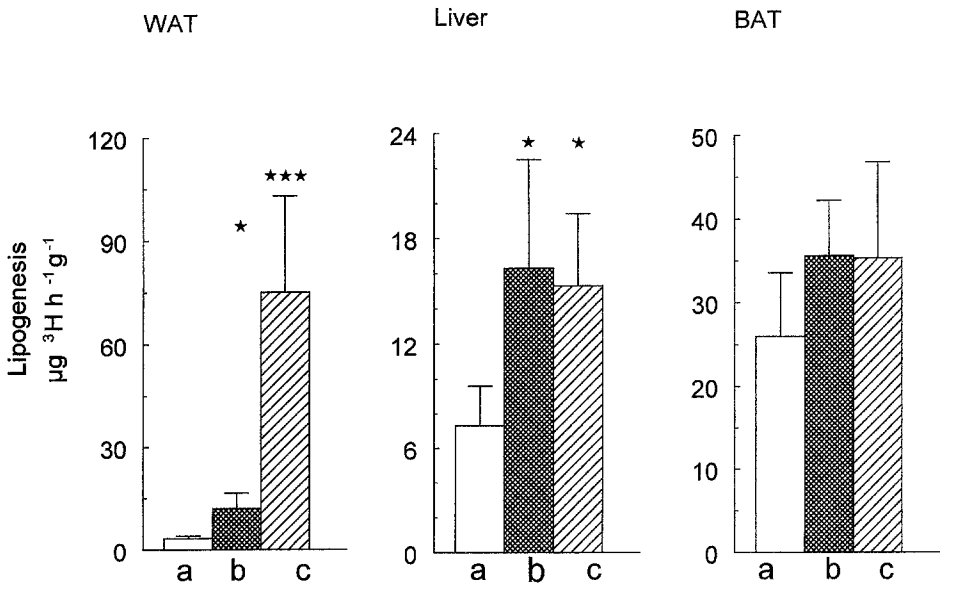

Fig 2. Effect of repeated cycles of starvation-refeeding on lipogenesis in rat WAT, liver, and BAT. a, control; b, 1 cycle; c, 8 cycles. Each bar represents the mean ± SD; n = 8 animals. All values in treated animals were significantly (★$P < .05$, ★★★$P < .001$) different from controls.

of body weight after each 3-day fast remained essentially constant over the course of the experiment. Body weights of starved-refed rats were significantly lower than those of animals fed ad libitum at each point tested (except after one cycle of starvation-refeeding). Body weights after eight cycles of starvation-refeeding were significantly lower (235 ± 13 g) than control weights (315 ± 25 g). Starved-refed rats did not gain weight in comparison to their initial weight. When rats subjected to eight cycles of starvation-refeeding were refed continuously, from 48 to 54 days they gained much more body weight than control animals. However, later (from 54 to 80 days), these rats continued to gain weight at the same rate as control animals. After eight cycles of fasting-refeeding, food intake during 3 days of refeeding was 123 ± 12 g per rat. Rats fed ad libitum had a 3-day food intake of 72 ± 11 g per rat, which remained constant through 48 days of treatment. After one cycle of starvation-refeeding, food intake during 3 days of refeeding was essentially similar to control levels (82 ± 10 g per rat). These data indicate that throughout the entire 48-day "cycling period," total food intake of rats in the experimental group was less (123 g per rat per 6-day cycle) than in the control group (144 g per rat per 6-day cycle). Presumably, this accounts for the substantial differences in body weight of the two groups after 48 days of treatment (Fig 1). When cycling was discontinued (ie, days 48 to 80), food intake gradually increased from 123 g per rat during the refeeding period of the last 6-day cycle to the level of the control group.

To determine whether weight loss resulted in any changes in lipogenic activity, the rate of in vivo fatty acid synthesis was determined. Using liver and adipose tissue from the same animals, it was possible to compare lipogenesis changes in all main lipogenic tissues. After one cycle of starvation-refeeding, lipogenesis increased approximately threefold in WAT compared with the rates in rats fed ad libitum (Fig 2). There was also

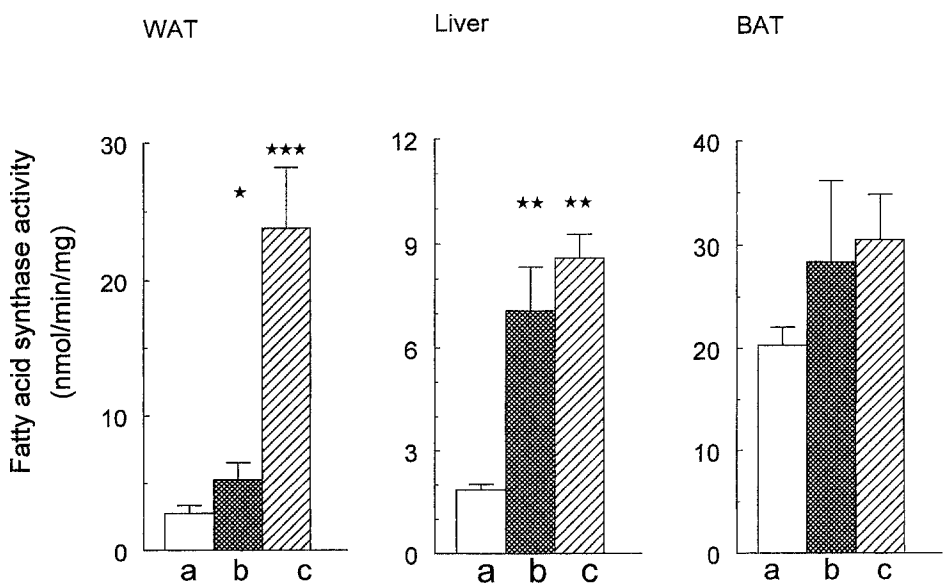

Fig 3. Effect of repeated cycles of starvation-refeeding on fatty acid synthase activity in rat WAT, liver, and BAT. a, control; b, 1 cycle; c, 8 cycles. Each bar represents the mean ± SD; n = 8 animals. All values in treated animals were significantly (★$P < .05$, ★★$P < .01$, ★★★$P < .001$) different from controls.

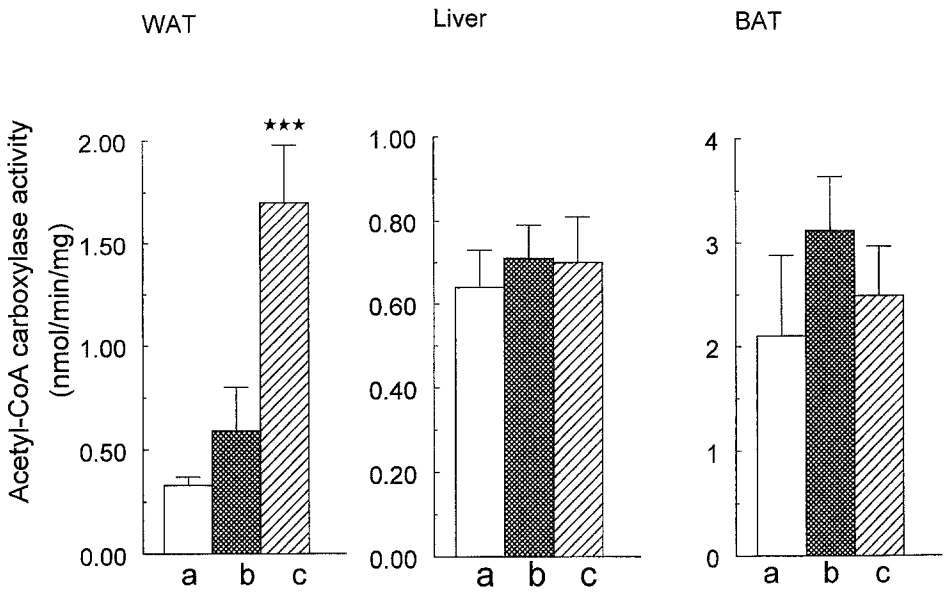

Fig 4. Effect of repeated cycles of starvation-refeeding on acetyl-coenzyme A carboxylase activity in rat WAT, liver, and BAT. a, control; b, 1 cycle; c, 8 cycles. Each bar represents the mean ± SD; n = 8 animals. Values were significantly (★★★$P <$ .001) different in WAT. No significant effects of treatment were found in liver and BAT.

a substantial increase (~twofold) of lipogenesis in the liver, but no significant increase was observed in BAT. The rate of lipogenesis in WAT increased severalfold after eight cycles of starvation-refeeding. In contrast, lipogenesis in the liver and BAT did not increase substantially after eight cycles of starvation-refeeding compared with one cycle (Fig 2). To gain insight into the mechanism of the increase in lipogenesis induced by multiple cycles of starvation-refeeding, we examined the activity of lipogenic enzymes. After one cycle of starvation-refeeding, fatty acid synthase activity increased approximately twofold in WAT and liver, but only a minor effect was observed in BAT. After eight cycles of starvation-refeeding, an unusual increase of fatty acid synthase activity was observed in WAT. No such changes were observed in the liver and BAT (Fig 3).

The effect of multiple cycles of starvation-refeeding on acetyl-coenzyme A carboxylase (Fig 4) and adenosine triphosphate-citrate lyase (Fig 5) activity in WAT was essentially similar to that found for fatty acid synthase. Thus, the rate of lipogenesis and the activities of key lipogenic enzymes are coordinately regulated in WAT by multiple cycles of starvation-refeeding. Furthermore, the enzymes participating in NADPH production required for fatty acid synthesis, ie, hexose monophosphate shunt dehydrogenases (Fig 6) and malic enzyme (Fig 7), were affected to a greater extent in WAT by multiple cycles of starvation-refeeding than the rate of lipogenesis and the activity of key lipogenic enzymes. In the case of malic enzyme, we performed more detailed experiments, checking the enzyme activity after one, two, five, eight, and 12 cycles of starvation-refeeding. After one cycle of starvation-refeeding, malic enzyme activity increased approximately threefold in WAT as compared with levels in rats fed ad libitum (Fig 7A). There was also a substantial increase of enzyme activity in the liver (~threefold; Fig 7B) and in BAT (~twofold; Fig 7C). A second cycle of starvation-refeeding caused approximately a 20-fold

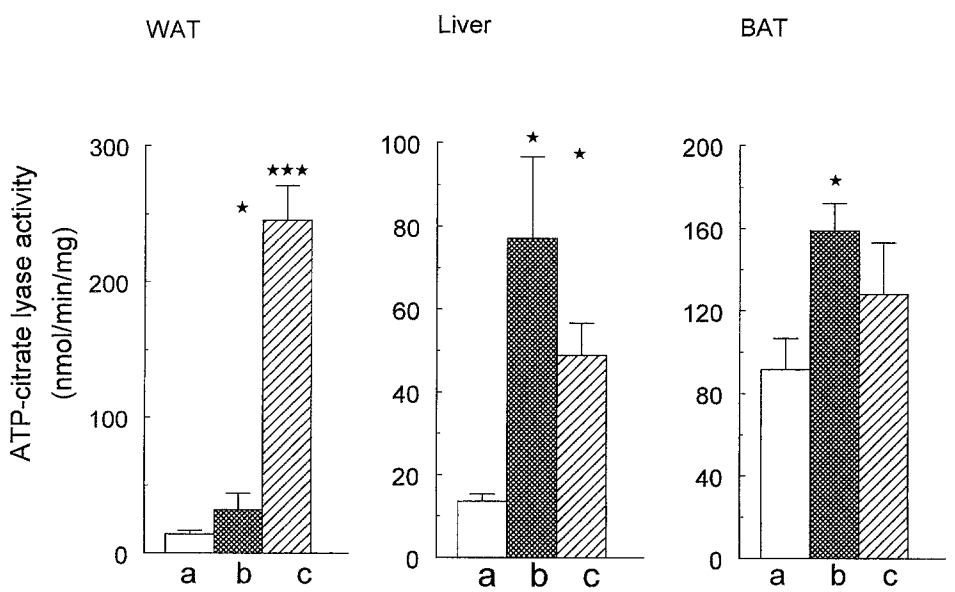

Fig 5. Effect of repeated cycles of starvation-refeeding on ATP-citrate lyase activity in WAT, liver, and BAT. a, control; b, 1 cycle; c, 8 cycles. Each bar represents the mean ± SD; n = 8 animals. All values in treated rats were significantly (★$P <$ .05, ★★★$P <$ .001) different from controls.

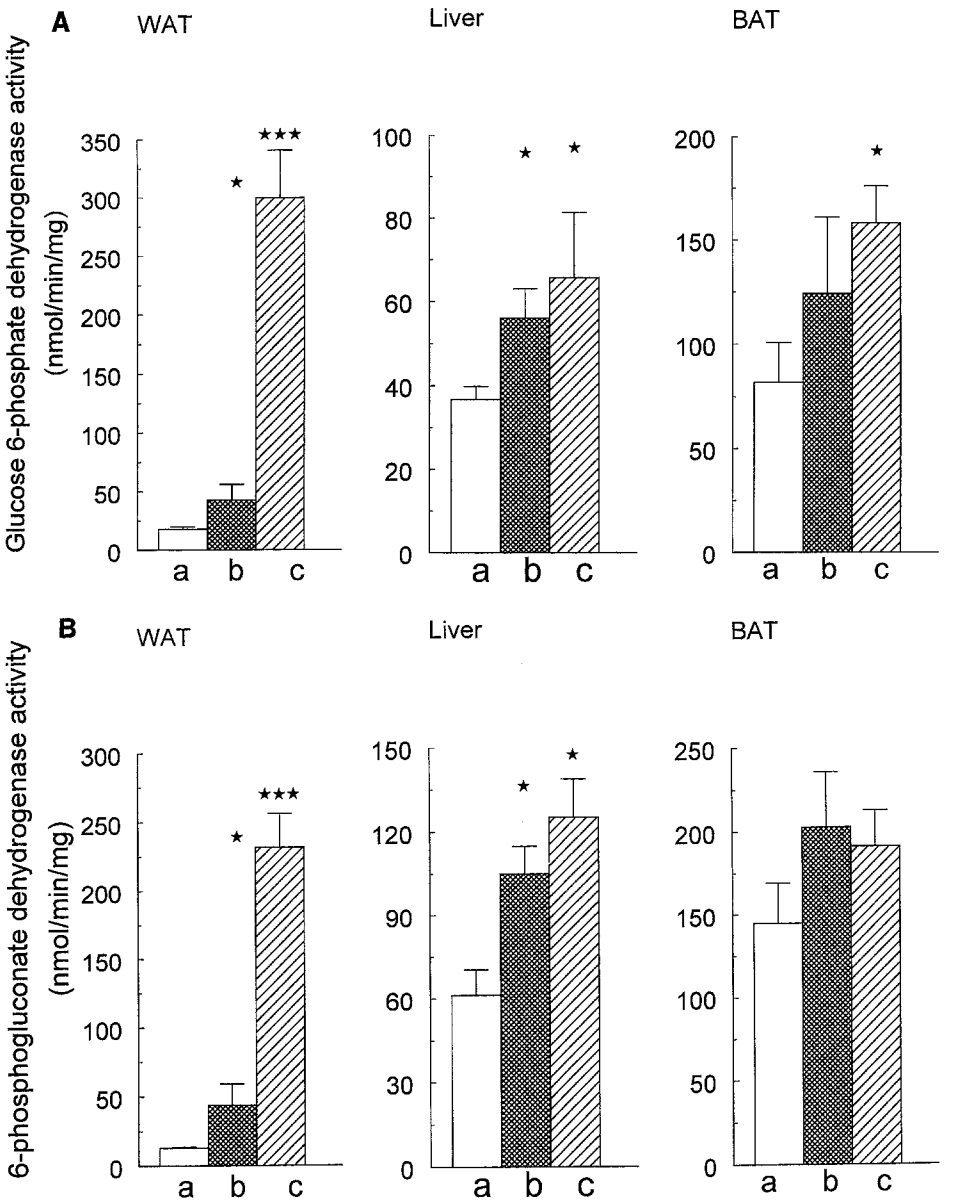

Fig 6. Effect of repeated cycles of starvation-refeeding on (A) glucose-6-phosphate and (B) 6-phosphogluconate dehydrogenase activities in WAT, liver, and BAT. a, control; b, 1 cycle; c, 8 cycles. Each bar represents the mean ± SD; n = 8 animals. All values in treated rats were significantly (★$P < .05$, ★★★$P < .001$) different from controls.

increase of malic enzyme activity in WAT. It also caused a greater response of hepatic malic enzyme than one cycle; however, the induction was much lower than in WAT. Surprisingly, no substantial increase of malic enzyme activity was observed in BAT after a second cycle of starvation-refeeding. After five cycles of 3 days of fasting followed by 3 days of refeeding, malic enzyme activity in WAT increased approximately 30-fold compared with control levels and approximately 10-fold compared with levels obtained with one cycle of fasting-refeeding (Fig 7A). A substantial increase of malic enzyme activity after five cycles of starvation-refeeding was also observed in the liver (~ninefold *v* control or ~threefold *v* one cycle of fasting-refeeding; Fig 7B). However, in BAT, only a twofold increase of malic enzyme activity was observed as compared with control levels, and only a small increase was noted as compared with levels obtained by one cycle of starvation-refeeding (Fig 7C). The activity of WAT malic enzyme reached maximal levels (~40-fold increase *v* control) after eight cycles of starvation-refeeding (Fig 7A). In contrast, liver (Fig 7B) and BAT (Fig 7C) malic enzyme activity reached maximal levels after five cycles and one cycle, respectively. When rats were fed standard laboratory diet ad libitum for 6 days after 12 cycles of starvation-refeeding, malic enzyme activity remained much higher than the control level in all lipogenic tissues studied (Fig 7).

Lipogenesis and lipogenic enzyme activities were measured at the end of the starvation-refeeding cycle, and one may thus conclude that it is not a great surprise that enhanced conversion of carbohydrate through lipogenesis occurred toward the end of this refeeding phase. However, it should be pointed out that fatty acid synthase and malic enzyme activities measured at the end of the 72-hour period of fasting of the eight cycles of starvation-refeeding were significantly higher than after 72-hour fasting of "noncycled" animals (Fig 8).

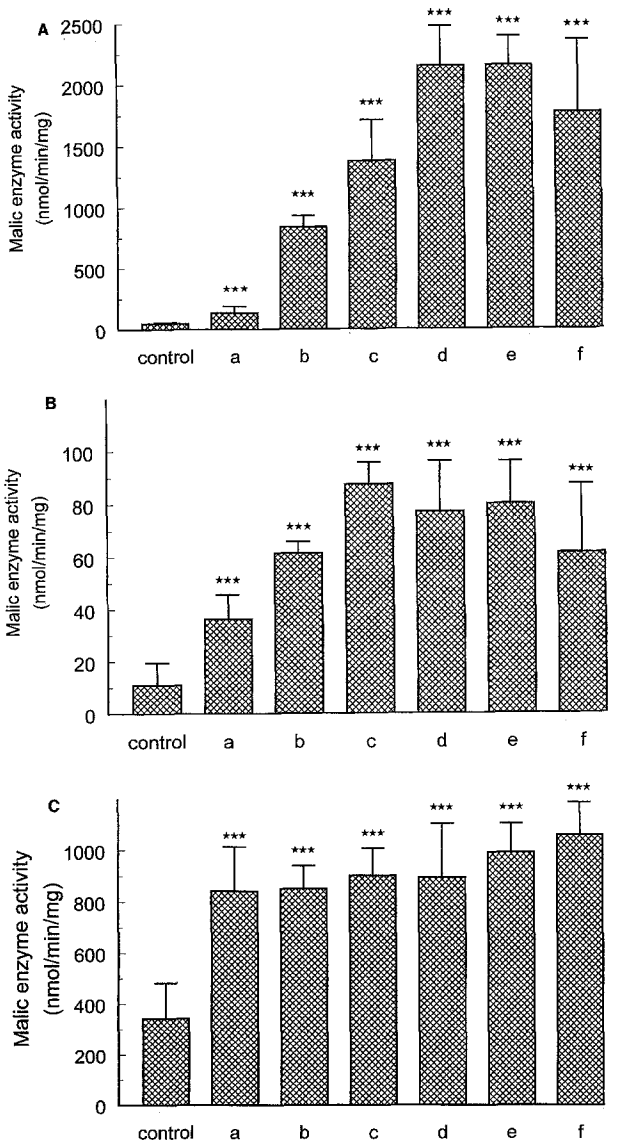

Fig 7. Effect of starvation-refeeding on malic enzyme activity in WAT (A), liver (B), and BAT (C). a, 1 cycle; b, 2 cycles, c, 5 cycles, d, 8 cycles, and e, 12 cycles of starvation-refeeding; f, 12 cycles of starvation-refeeding followed by feeding with standard laboratory diet for 6 days. Values are the mean ± SD (vertical lines) from 6 (a,b,c,e, and f) or 12 (control and d) rats. All values in treated animals were significantly (★★★$P < .001$) different from controls.

To gain more information about tissue specificity of malic enzyme activity regulation by starvation-refeeding conditions, we assessed the enzyme activity in control conditions and after eight cycles of starvation-refeeding in the following tissues: kidney cortex, brain, heart, and skeletal muscle. The ratios of starvation-refeeding versus control samples were as follows: 1.09, 0.93, 1.06, and 1.16, respectively. Hence, no response in malic enzyme activity could be detected in any of the nonlipogenic tissues. The lack of response to multiple cycles of starvation-refeeding exhibited by kidney cortex, brain, heart, and skeletal muscle indicates that malic enzyme in these tissues is not coordinately regulated with the enzyme in lipogenic tissues.

DISCUSSION

One experimental animal model that may be useful in studying the weight loss/regain process is the starvation-refeeding model first described by Tepperman and Tepperman[1] nearly 40 years ago. Szepesi et al,[21] using a two-cycle starvation-refeeding experimental model, showed that starved-refed rats continued to gain weight faster than ad libitum–fed animals. Desautels and Dulos,[39] using 1 day of fasting followed by 2 days of refeeding as the experimental model, showed that the final body weight of mice after 12 cycles of starvation-refeeding was similar to that of animals fed ad libitum. These results agree with data reported by Lizotte et al,[27] who showed no change in body weight and adiposity after three cycles of food restriction in male rats. Using more drastic conditions, ie, 3 days of fasting followed by 3 days of refeeding, we observed a significant decrease of body weight in treated rats compared with animals fed ad libitum (Fig 1). The results suggest that it is possible to develop appropriate starvation-refeeding conditions that would be useful in helping humans retain lean body mass.

Despite a significant reduction of body weight after multiple starvation-refeeding cycles, WAT exhibits a surprisingly high rate of lipogenesis and lipogenic enzyme activity. The liver and especially BAT were much less responsive to multiple cycles of starvation-refeeding compared with WAT. This suggests that the increase of lipogenesis observed in WAT of starved-refed rats upon refeeding is the major determinant in directing dietary carbohydrate for deposition in the form of triacylglycerols. Partial support for this view comes from a study reported by De Bont et al,[40] who showed that incorporation of labeled glucose into WAT lipid in vitro remained higher in meal-fed rats than in rats fed ad libitum. This is also in accordance with the recent report that upon refeeding, WAT of meal-fed rats exhibits higher rates of lipogenesis than the liver or BAT.[19] Furthermore, WAT of dieting men[41] and laboratory animals[42] exhibits a greater

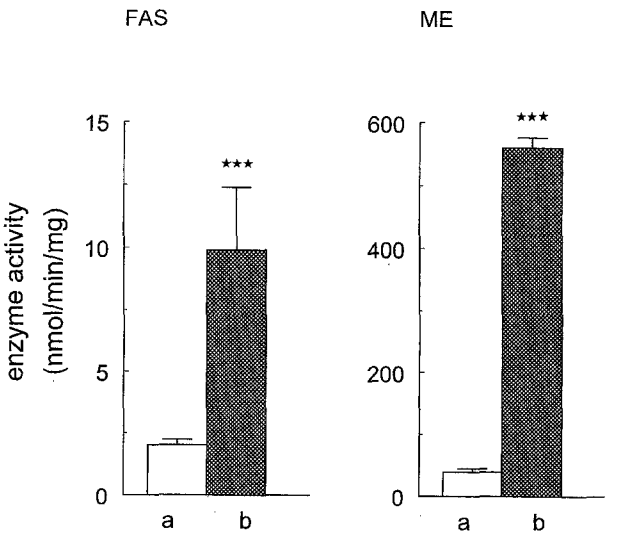

Fig 8. Effect of starvation-refeeding on fatty acid synthase (FAS) and malic enzyme (ME) activity in WAT. a, noncycled rats starved for 72 hours; b, 8 cycles in animals killed at the end of 72 hours of starvation. For experimental conditions, see Figs 3 and 7. All values in cycled animals were significantly (★★★$P < .001$) different from controls.

capacity for deposition of dietary fat, due to increased activity and expression of lipoprotein lipase. Thus, we conclude that starvation-refeeding may lead to an increase of lipogenesis and lipoprotein lipase in WAT, thereby potentially enhancing lipid storage in this tissue.

The increased activities of key enzymes in the lipogenic pathway may play a crucial role in the increased rate of fatty acid synthesis. However, questions as to how multiple cycles of starvation-refeeding produce these changes and why the increase of lipogenic enzymes in WAT was quantitatively much greater than in liver and BAT remain unanswered. One can only speculate that WAT is much more sensitive to an alteration in the hormonal milieu (which takes place during multiple cycles of starvation-refeeding) than the liver or BAT. Some data[43,44] suggest that insulin could play a crucial role in the upregulation of lipogenesis in WAT after the starvation-refeeding situation imposed. However, in our experimental model, we did not observe a statistical difference in serum insulin concentrations in controls and rats subjected to eight cycles of starvation-refeeding (not shown).

Despite the unknown mechanism of the effect of multiple cycles of starvation-refeeding on lipogenic enzyme activity, our data indicate that with repeated starvation-refeeding, enzyme activity levels become chronically elevated, especially in WAT, placing the animals in a metabolic state favoring more efficient lipogenesis. The increased capacity for lipogenesis of WAT in dieting rats after feeding may have a survival advantage, because it redirects glucose for conversion to fatty acids and subsequently to triacylglycerols for storage rather than immediate oxidation, thereby potentially enhancing lipid accumulation in adipocytes. In less drastic conditions, ie, 1 or 2 days of starvation followed by 3 days of feeding, this change would favor a return to obesity in reduced-obese animals.

The starvation-refeeding protocol used in our studies may represent abnormal nutritional conditions that limit extrapolation to humans. However, such conditions may exist in some patients with eating disorders.[22] Furthermore, two important problems must be discussed. Firstly, typical human diets are much higher in fat than diets used in the experiments presented herein. Secondly, lipogenesis in humans likely happens in the liver and not in adipose tissues. However, we do not known what happens with lipogenic enzymes and consequently with lipogenesis in human adipose tissue after multiple cycles of 3 days of fasting and 3 days of refeeding a high-carbohydrate diet. Considering that lipogenic enzymes are present in human adipose tissue,[45] it cannot be excluded that multiple cycles of fasting and refeeding cause a significant increase in their activity. However, it is obvious that further investigation will be necessary to establish whether human lipogenesis is influenced by multiple cycles of fasting-refeeding.

In conclusion, the results indicate that multiple cycles of starvation-refeeding impose a stress on rats that requires special adaptation for survival. Starved-refed animals must develop the ability to ingest large quantities of food during refeeding to store in a convenient form that can be used as an oxidizable fuel during a period of starvation. WAT becomes an organ of great importance as the site of fat synthesis and storage. This may be why WAT is the site of the major enzymatic adaptive changes observed in starved-refed rats.

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