Relative Importance of Sympathetic Outflow and Insulin in the Reactivation of Brown Adipose Tissue Lipogenesis in Rats Adapted to a High-Protein Diet

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The effect of denervation or acute insulin deficiency on brown adipose tissue lipogenesis was investigated in rats adapted to a high-protein diet before and after diet reversion to a balanced diet. Denervation of rats fed the balanced diet induced a 50% reduction in in vivo rates of brown adipose tissue fatty acid synthesis, with decreased activities of acetyl-CoA carboxylase and adenosine triphosphate (ATP)-citrate lyase. The markedly (80%) reduced fatty acid synthesis and enzyme activities in brown adipose tissue from rats adapted to the high-protein diet were not affected by denervation. Replacement of the high-protein diet by the balanced diet for 24 hours restored fatty acid synthesis to normal levels, but recovery of enzyme activities was only partial. Lipogenesis restoration and partial recovery of enzyme activities were impaired in denervated tissue from high-protein diet-fed rats. In all experimental conditions, the activities of acetyl-CoA carboxylase and ATP-citrate lyase showed a better correlation with brown adipose tissue lipogenesis than the generators of H^+, glucose-6-P dehydrogenase, and malic enzyme. Anti-insulin serum administration during the 12- to 24-hour period after diet reversion completely blocked lipogenesis recovery in innervated and denervated tissues and drastically reduced brown adipose tissue lipogenesis of concomitantly injected rats fed the balanced diet. The data suggest that efficient and rapid adjustments of brown adipose tissue lipogenesis require sympathetic activation, and that this tissue can maintain significant, albeit reduced, rates of lipogenesis in the absence of sympathetic activation, but not in the absence of insulin.

BROWN ADIPOSE TISSUE is richly innervated by the sympathetic nervous system, and numerous studies have shown that sympathetic activation of triacylglycerol hydrolysis is an essential element in the sequence of events leading to fatty acid oxidation and heat production in both nonshivering and diet-induced thermogenesis (DIT), the 2 kinds of facultative thermogenesis known to be under brown adipose tissue control in small rodents.[1,2] Although one would expect fatty acid oxidation and fatty acid synthesis to be mutually exclusive events, it has been found that increases in brown adipose tissue thermogenic activity and increased fatty acid oxidation induced in situations of nonshivering (eg, cold exposure) or diet-induced (eg, overfeeding) thermogenesis are accompanied by simultaneous increases in fatty acid synthesis. Conversely, fatty acid synthesis decreases in situations of reduced brown adipose tissue thermogenesis (eg, starvation).[1,2] The biochemical mechanism of this parallelism between brown adipose tissue thermogenesis and lipogenesis is not completely understood. A direct control of brown adipose tissue lipogenesis by the sympathetic nervous system has been suggested by experiments showing that fatty acid synthesis is rapidly increased by stimulation of the ventromedial hypothalamic nucleus[3,4] or by intermittent electrical stimulation of brown adipose tissue sympathetic fibers.[5] Insulin has a direct effect on brown adipose tissue lipogenesis, stimulating fatty acid synthesis both in vivo[6] and in brown adipocytes in vitro,[7] and its concentration in plasma changes in parallel with the diet-induced changes in brown adipose tissue thermogenesis. Therefore, during the adjustments of thermogenesis in DIT situations, the combined effects of the 2 factors, sympathetic outflow to brown adipose tissue and plasma insulin levels, are probably of major importance for the corresponding adjustments of lipogenesis.

The experiments in the present work were designed to investigate the relative importance of sympathetic activity and of insulin in the adjustments of brown adipose tissue lipogenesis. Using a preparation, the rats adapted to a high-protein, carbohydrate-free diet, whose metabolic characteristics make it adequate for this purpose. We have shown[8] that adaptation of rats to a high-protein diet results in a marked reduction in brown adipose tissue thermogenic capacity. Confirming the parallelism between thermogenesis and lipogenesis, fatty acid synthesis, estimated in vivo with ^3H_2O, was also decreased in brown adipose tissue from these animals.[8,9] We have further shown that norepinephrine content and rates of norepinephrine turnover are reduced in brown adipose tissue from rats adapted to the high-protein diet,[10] suggesting that a decrease in the sympathetic outflow to the tissue was responsible, at least in part, for the reduction in both thermogenesis and lipogenesis. However, rats adapted to the high-protein diet also have low levels of plasma insulin,[11] and the hormonal deficit at tissue level probably contributed, to an unknown extent, to reduce brown adipose tissue lipogenesis in these animals. The present experiments were prompted by the verification that replacement of the high-protein diet by a control, balanced diet for 24 hours restores norepinephrine fractional turnover rate and turnover rate values to those of normally-fed animals[10] and by the results of experiments reported here showing that substitution of their diet for the same period was sufficient to normalize the reduced brown adipose tissue lipogenesis. To investigate the relative importance of the sympathetic nervous system and of insulin levels in the control of brown adipose tissue lipogenesis, we examine in the present work the effects of denervation and of

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acute insulin deficiency on the reactivation of brown adipose tissue lipogenesis of rats adapted to the high-protein diet after reversion of their diet to a balanced diet. In addition to in vivo rates of fatty acid synthesis, the activities of 4 enzymes involved in lipogenesis were determined at several time intervals after diet reversion. Changes in plasma levels of glucose, insulin, and glucagon induced by replacement of the diet are also reported.

MATERIALS AND METHODS

Male Wistar rats weighing initially 110 to 120 g were housed in suspended, wire-bottom cages, with water ad libitum, in a room kept at 25° ± 2°C with a 12-hour light/dark cycle. The animals were adapted for 21 days to a 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 2 diets, which were approximately isocaloric and contained equal amounts of vitamins and minerals, have been described in detail.[8] 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 2 groups of rats.[11] At the end of 21-day adaptation period, when they were used for the experiments, the animals weighed 200 to 220 g. In all 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 the different metabolic parameters were measured within the next 24 hours.

Unilateral Denervation of Brown Adipose Tissue

Under ether anesthesia, a small incision was made between the scapula, and the interscapular brown adipose tissue was carefully dissected from the surrounding muscle and white adipose tissue. Five branches of the right intercostal nerve bundles that contain sympathetic fibers entering the right side of interscapular brown adipose tissue were isolated and cut off at a length of approximately 5 mm. Surgical denervation was performed 6 days before using the animals for the experiments. Preliminary studies showed that 6 days after surgery, the norepinephrine content of the denervated side of the tissue, measured as described,[12] was reduced to less than 2% of values in the control, innervated, side. They also showed that after 6 days, rates of fatty acid synthesis and the activities of lipogenic enzymes (measured as indicated below) in the innervated side did not differ significantly from those in brown adipose tissue from nonoperated rats.

Anti-insulin serum was obtained from guinea-pigs following the recommendations of Makulu and Wright.[13] The samples of serum obtained in the different phases of the immunization procedure were pooled together and stored at -20°C. Preliminary trials showed that a single intraperitoneal injection of 3 mL of serum induced in rats an increase in blood glucose (to 300 to 400 mg/dL), which persisted for about 6 hours. In the diet reversion experiments, the rats received two 3 mL injections of anti-insulin serum, the first 12 hours after diet replacement and the second 6 hours later.

In Vivo Lipogenesis Measurements

In vivo rates of fatty acid synthesis were evaluated by the rate of incorporation of tritiated water into brown adipose tissue fatty acids. ^3H_2O (3 mCi in 0.5 mL saline) was injected intraperitoneally into fed, nonanesthetized animals. In the experiments of diet reversion, the label was injected before and 3, 6, 12, and 24 hours after the replacement of the diet of rats adapted to the high-protein diet. One hour after ^3H_2O injection, the animals were killed by decapitation and blood samples were collected for determination of plasma water specific radioactivity. The entire interscapular brown adipose tissue or the right and left pads of unilaterally denervated tissue were rapidly removed, carefully cleaned of adhering fat and muscle, and weighed. Tissue total lipids were extracted with 2:1 chloroform-methanol, and ^3H_2O was removed from the lower phase (predominantly chloroform) by washing 3 times with an upper phase mixture.[14] After each shaking, the tubes were briefly centrifuged to sharpen the phase boundary, and the upper phase was aspirated and discarded. The chloroform phase was evaporated to dryness under N_2, and the triacylglycerols were hydrolyzed with ethanolic potassium hydroxide for 1 hour at 70°C. After extraction of nonsaponifiable lipids and acidification with sulfuric acid, the ^3H-labeled fatty acids were extracted with petroleum ether, and the extract was evaporated to dryness in a scintillation vial and dissolved in toluene-2,5-diphenyloxazole (PPO) for counting in a Beckman (Fullerton, CA) LS 7600 spectrometer. Body water specific activity was determined directly on aliquots of diluted (20 times) plasma dissolved in toluene-triton-PPO. Rates of tissue fatty acid synthesis were calculated following the assumptions of Windmueller and Spaeth.[15]

Measurements of Enzyme Activities

The activities of glucose-6-phosphate dehydrogenase (G6PDH), malic enzyme (ME), and ATP-citrate lyase (CLY) were determined in 100,000 g supernatants of tissue homogenates prepared as previously described.[9] G6PDH was assayed as described by Lee,[16] and ME was assayed by the method of Ochoa,[17] with the modifications proposed by Hsu and Lardy.[18] Both assays were performed by measuring the formation of reduced nicotinamide adenine dinucleotide phosphate (NADPH). CLY was assayed as described by Srere,[19] measuring the rate of oxidation of NADH. The assay of acetyl-CoA carboxylase (ACCX) activity was performed as described by Halestrap and Denton[20] 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 composition of the assay mixture for the 4 enzymes was identical to that of a previous study.[9] Protein concentration of tissue homogenates was determined by the method of Lowry et al.[21]

Other Methods of Chemical Analysis

The plasma concentration of insulin and glucagon was determined by radioimmunoassay using commercial kits from Amersham (Little Chalfont, UK) and Linco (St Charles, MO), respectively. The concentration of plasma glucose was determined with glucose oxidase in a Beckman glucose analyzer. Tissue total lipids were determined gravimetrically after extraction by the procedure of Folch et al.[14]

Statistical Methods

Differences between groups were analyzed by 1- or 2-way analysis of variance (ANOVA), as appropriate, with $P < .05$ as the level of significance.

RESULTS

The data in Table 1 show that the weight of interscapular brown adipose tissue fat pads was lower in rats adapted to the

Table 1. Weight and Lipid Content of Innervated and Denervated Brown Adipose Tissue Fat Pads in Controls and in Rats Adapted to the High-Protein Diet

		Innervated	Denervated
Tissue weight (mg)	N	166 ± 10 (25)	149 ± 7 (25)
	HP	128 ± 7* (25)	116 ± 6 (25)
Lipid content (mg/g)	N	303 ± 16 (5)	360 ± 24 (5)
	HP	281 ± 13 (5)	326 ± 17 (5)

NOTE. Values are means ± SE with the number of rats in parentheses.

Abbreviations: N, controls; HP, high-protein diet.

*$P < .05$ *v* N.

high-protein diet than in animals fed the balanced diet, but did not differ significantly in denervated and innervated pads in both experimental groups. The data also show that there was a tendency for the lipid content to be higher in denervated pads in the 2 groups, although statistical significance was not attained ($P < .10$).

Plasma Levels of Glucose, Insulin, and Glucagon

The data in Fig 1 show that basal levels of plasma glucose (Fig 1A) and of insulin (Fig 1B) were lower in rats fed the high-protein diet than in controls and increased markedly after their diet was replaced by the balanced diet. From 6 hours after diet reversion until the end of the experimental period (24 hours), plasma levels of both glucose and insulin were significantly higher than those of rats fed the balanced diet. In contrast, basal levels of plasma glucagon were higher in rats adapted to the high-protein diet and decreased sharply to levels lower than those of control rats after diet reversion, returning to values similar to those of control rats only at the end of the experimental period (Fig 1C).

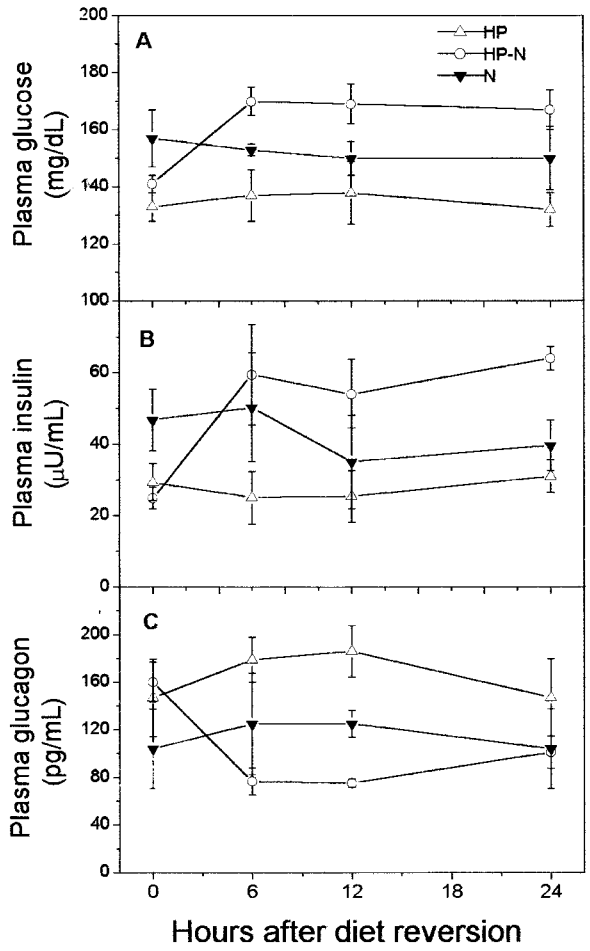

Fig 1. Plasma levels of glucose, insulin, and glucagon of rats adapted to the high-protein diet before (HP) and after (HP-N) replacement of their diet by the control (N) diet. Levels in control rats at coinciding periods of time are also shown. Values are mean ± SE of 4 to 6 animals.

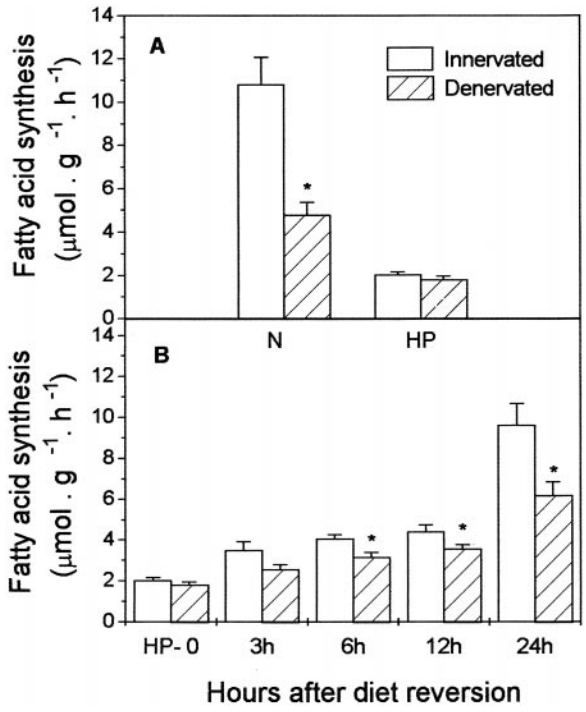

Fig 2. Effect of denervation (A) on the rate of brown adipose tissue fatty acid synthesis in rats adapted to the high-protein diet (HP) and in controls (N) and (B) on the reactivation of tissue fatty synthesis of HP diet-adapted rats after diet reversion. Values are mean ± SE from 10 rats in (A) and 5 to 7 rats in (B). *$P < .05$ *v* innervated.

Effect of Denervation on In Vivo Fatty Acid Synthesis

As shown in Fig 2A, in vivo rates of fatty acid synthesis in denervated pads from control rats fed the balanced diet were reduced to about 50% of rates in innervated pads. Rates of fatty acid synthesis in brown adipose tissue from rats adapted to the high-protein diet were already very low and were not significantly affected by denervation. The data in Fig 2B show that after replacement of the high-protein diet by the balanced diet lipogenesis in innervated pads increased progressively. The increase was more pronounced between 12 and 24 hours of diet reversion. At the end of the experimental period, rates of fatty acid synthesis did not differ significantly from those in controls fed the balanced diet. Figure 2B also shows that in the denervated side, the rate of increase in fatty acid synthesis after diet reversion was slower than in the intact pad both at the 0- to 12-hour and at the 12- to 24-hour intervals.

Effect of Denervation on Lipogenic Enzymes

In rats fed the balanced diet, denervation produced a relatively small (19%) reduction in the activities of ME and G6PDH, which was statistically significant only for ME (Fig 3A and B). The effect of denervation on CLY and ACCX was more marked, the activities of these enzymes decreasing about 29% and 47%, respectively, of values in intact pads (Fig 3C and D). The activities of the 4 enzymes were already markedly reduced in brown adipose tissue from rats adapted to the

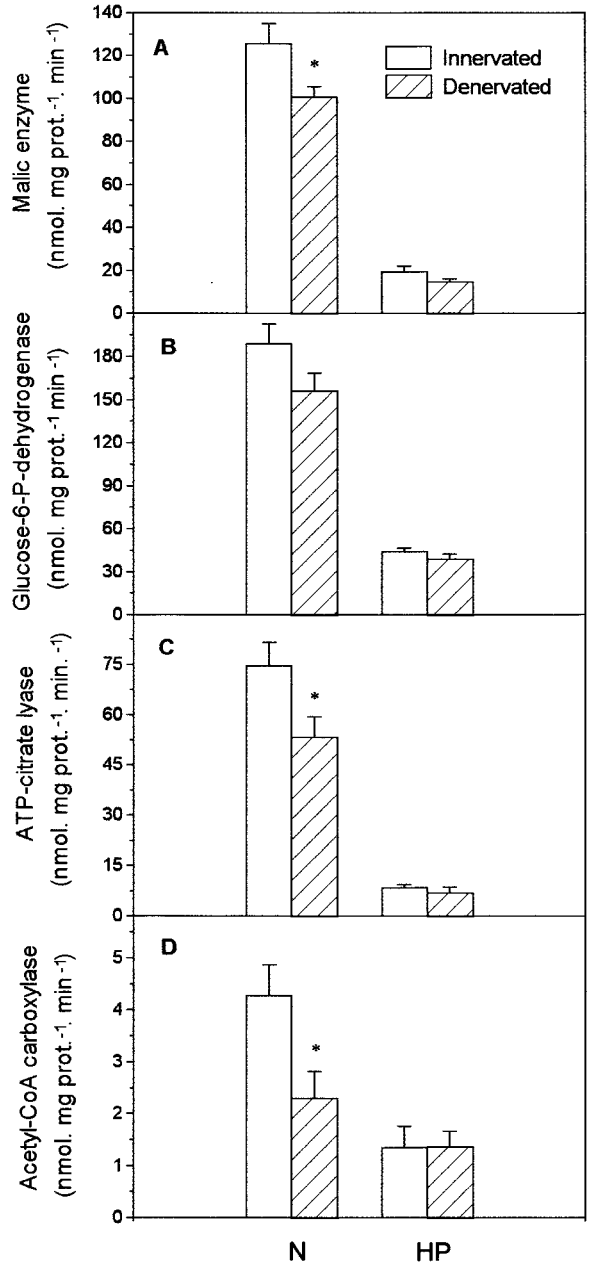

Fig 3. Effect of brown adipose tissue denervation on the activity of malic enzyme (A), glucose-6-phosphate dehydrogenase (B), ATP-citrate lyase (C), and acetyl-CoA carboxylase (D) in rats adapted to the high-protein diet (HP) and controls (N). Values are mean ± SE of 5 to 9 animals. *$P < .05$ *v* innervated.

high-protein diet, and no significant change was observed after denervation (Fig 3A to D). Figure 4A to D show that except for an increase in the activity of ME between 6 and 12 hours, no significant change in the activity of brown adipose tissue enzymes in the innervated side was observed during the first 12 hours after replacement of the high-protein diet by the control diet. During the 12- to 24-hour period, the activity of ME remained unchanged, but the activities of G6PDH, ACCX, and CLY increased by 39%, 71%, and 122%, respectively (Fig 4A to D). However, in contrast to the rates of fatty acid synthesis, at the end of the experimental period, the activities of the 4 enzymes remained well below the levels in intact pads of rats fed the balanced diet. In the denervated side, the activity of the enzymes was even lower than that in innervated pads, both at the initial 12-hour period and at the end of the experimental period (Fig 4A to D).

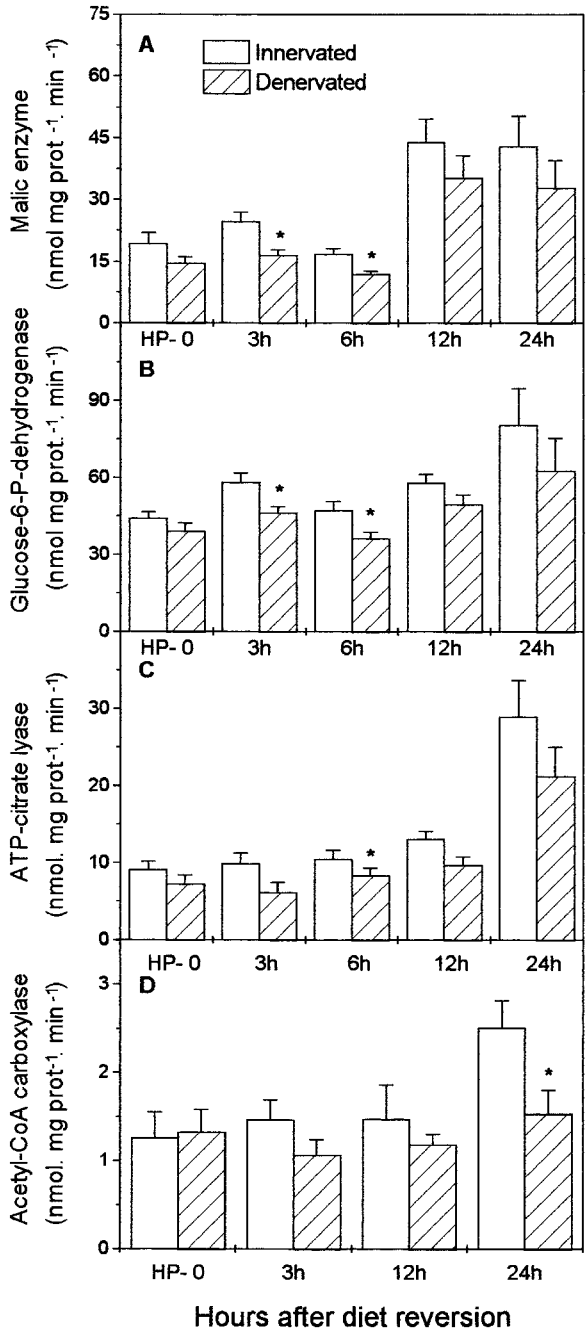

Fig 4. Effect of brown adipose tissue denervation on the activity of malic enzyme (A), glucose-6-phosphate dehydrogenase (B), ATP-citrate lyase (C), and acetyl-CoA carboxylase (D) after diet reversion in rats adapted to the high-protein diet (HP). Values are mean ± SE from 4 to 6 animals. *$P < .05$.

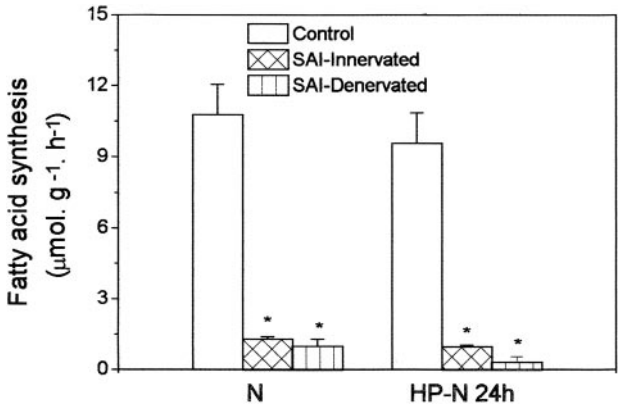

Fig 5. Effect of acute insulin deficiency induced by anti-insulin serum (SAI) on the rate of fatty acid synthesis in denervated and intact brown adipose tissue fat pads from control (N) rats and from rats adapted to the high-protein diet 24 hours after diet reversion (HP-N 24h) (see Materials and Methods for details). Values are mean ± SE from 4 to 6 rats. *$P < .01$.

Effect of Acute Insulin Deficiency on In Vivo Fatty Acid Synthesis

To induce an insulin insufficiency during the period of greater increase in fatty acid synthesis after diet reversion (12 to 24 hours), rats adapted to the high-protein diet were injected with anti-insulin serum 12 and 18 hours after replacement of their diet. Coincident injections were administered to rats fed the balanced diet. Measurements were made after the rats fed the high-protein diet completed 24 hours of diet reversion. The results in Fig 5 show that treatment with anti-insulin serum resulted in a drastic reduction in fatty acid synthesis in brown adipose tissue from control rats and almost completely blocked the increase in fatty acid synthesis induced by diet reversion in brown adipse tissue from rats adapted to the high-protein diet, both in denervated and intact pads.

DISCUSSION

The data of the present study clearly show that the integrity of brown adipose tissue innervation is essential not only for the maintenance of normal rates of fatty synthesis, but also for reactivation of tissue lipogenesis in situations in which this process is reduced, as is the case of rats fed a high-protein, carbohydrate-free diet. In the present study, denervation of normally-fed animals resulted in a 50% reduction of brown adipose tissue fatty acid synthesis rate, an effect that is consistent with the observation that electrical stimulation of the ventromedial hypothalamic nucleus[3,4] or of brown adipose tissue sympathetic fibers[5] rapidly stimulates fatty acid synthesis, suggesting that brown adipose tissue lipid synthesis is under direct control of the sympathetic nervous system.[22] Confirming previous results from this laboratory,[9] adaptation to the high-protein diet resulted in a 80% decrease in brown adipose tissue lipogenesis, a reduction more pronounced than that induced by tissue denervation in control rats. The extremely low rates of in vivo fatty acid synthesis in rats fed the high-protein diet are probably due to the additive effects of a reduced sympathetic activity[10] and a local insufficiency of insulin,[11] a hormone that has been shown to stimulate fatty acid synthesis both in vivo[6] and in vitro.[7] Because the high-protein and the control diet are approximately isocaloric, the reduction in norepinephrine turnover rate in brown adipose tissue from rats adapted to the high-protein diet should be attributed to the composition of their diet. As discussed previously,[8] both the high-protein diet and the lack of carbohydrates may be responsible for the reduction in brown adipose tissue sympathetic activity, with the lower levels of blood glucose in rats in the absorptive period probably contributing to inhibition of the hypothalamic sympathetic outflow. Indeed, microinjections of glucose into the ventromedial hypothalamic nucleus increases the firing rate of brown adipose tissue sympathetic fibers.[23,24] It has been found that insulin decreases the firing rate of sympathetic fibers when injected into the ventromedial hypothalamus,[25,26] but may increase this process when injected into the nucleus suprachiasmaticus, depending on the time of day.[27] Thus, more information is needed to clarify the role of the low levels of insulin in the reduction of brown adipose tissue sympathetic activity in rats adapted to the high-protein diet.

The experiments with diet reversion demonstrate the importance of the integrity of brown adipose tissue innervation for an efficient reactivation of lipogenesis. In innervated fat pads, replacement of the high-protein diet by the balanced diet for 24 hours restored in vivo fatty acid synthesis to normal levels. In contrast, despite the marked increases in plasma glucose and insulin levels induced by diet reversion, fatty acid synthesis in denervated pads increased more slowly, remaining 40% lower than in intact pads after 24 hours. On the other hand, the results of the experiments with hemidenervation also show that even in the complete absence of sympathetic activity, an adequate hormonal/metabolic environment of the tissue can maintain an active brown adipose tissue lipogenesis, albeit at a reduced rate.

In the experiments in which the high-protein diet was replaced by the balanced diet, administration of anti-insulin serum during the period of maximal increase in fatty acid synthesis (12 to 24 hours after diet reversion) induced a drastic reduction in tissue fatty acid synthesis, which attained rates even somewhat lower than those observed before diet replacement. This effect of anti-insulin serum was independent of tissue innervation and was also obtained in control rats fed the balanced diet. Although the magnitude and rapidity of brown adipose tissue lipogenesis inhibition in these experiments suggest that lack of insulin at the tissue level was the predominant factor, the possibility exists that a decreased sympathetic outflow to the tissue, induced by the hormonal/metabolic conditions prevailing after anti-insulin serum injection, such as high levels of blood glucose, contributed to the effect observed. Whatever the contribution of a decrease in sympathetic activity, the experiments with anti-insulin serum indicate that an active brown adipose tissue lipogenesis cannot be maintained in the absence of insulin. The stimulatory effect of insulin on lipogenesis is enhanced by the sympathetic nervous system and, as shown by the results of denervation experiments in this study, insulin alone, even at high concentrations, cannot maintain efficient rates of brown adipose tissue fatty acid synthesis. It has been found that norepinephrine administration or electric stimulation of sympathetic nerves produces a marked increase in brown adipose tissue glucose utilization, estimated with

2-deoxyglucose.[28,29] It has also been shown in cultured brown adipocytes that norepinephrine stimulates glucose transport directly, independently of insulin, by enhancing the functional activity of GLUT 1 though a cyclic adenosine monophosphate (cAMP)-dependent mechanism.[30,31] However, further experiments are needed to clarify the biochemical mechanisms underlying the synergism between the sympathetic nervous system and insulin.

The present results show that brown adipose tissue denervation in normally-fed rats induced, in parallel with the reduction in lipogenesis, a decrease in the activities of lipogenic enzymes, which was more marked for ACCX and CLY. In rats adapted to the high-protein diet, the markedly reduced lipogenesis was accompanied by low levels of the 4 enzymes examined, which were not further decreased by denervation. The response of enzyme activities to the change of diet in animals adapted to the high-protein diet was much slower than that of fatty acid synthesis, the 4 enzymes remaining well below the levels in brown adipose tissue from normally-fed animals after 24 hours of diet reversion. A comparison of the changes in the activity of the enzymes during the period of 12 to 24 hours after diet substitution show that the greatest increases occurred in the activities of CLY and ACCX. Thus, the changes in the activities of these 2 enzymes, which catalyze reactions of the fatty acid synthesis pathway, show a better correlation with brown adipose tissue lipogenesis than the generators of H^+, ME, and G6PDH. The NADPH produced by the latter enzymes is also used in other metabolic pathways, and their activities have not always been found to correlate with rates of fatty acid synthesis in lipogenic tissues.[32]

In summary, the present data show that denervation reduces by 50% fatty acid synthesis in brown adipose tissue from normally-fed rats and impairs the restoration of tissue lipogenesis induced by diet reversion in rats adapted to a high-protein diet. Administration of anti-insulin serum drastically reduces brown adipose tissue lipogenesis in control rats and blocks the reactivation of lipogenesis after diet reversion in rats fed a high-protein diet. The data suggest that efficient and rapid adjustments of brown adipose tissue lipogenesis require sympathetic activation, and that the tissue can maintain significant, albeit reduced, rates of lipogenesis in the absence of sympathetic stimulation, but not in the absence of insulin.

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