Glucose Uptake, Glucose Transporter GLUT4, and Glycolytic Enzymes in Brown Adipose Tissue From Rats Adapted to a High-Protein Diet

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In vivo rates of glucose uptake, insulin-responsive glucose transporter (GLUT4) content, and activities of glycolytic enzymes were determined in brown adipose tissue (BAT) from rats adapted to a high-protein, carbohydrate-free (HP) diet. Adaptation to the HP diet resulted in marked decreases in BAT glucose uptake and in GLUT4 content. Replacement of the HP diet by a balanced control diet for 24 hours restored BAT glucose uptake to levels above those in rats fed the control diet, with no changes in GLUT4 levels in 4 of 5 animals examined. BAT denervation of rats fed the control diet induced a 50% reduction in glucose uptake, but did not significantly affect the already markedly reduced BAT hexose uptake in HP diet-fed rats. It is suggested that the pronounced decrease in BAT glucose uptake in these animals is due to the combined effects of the HP diet-induced reductions in plasma insulin levels and in BAT sympathetic activity. Adaptation to the HP diet was accompanied by decreased activities of hexokinase, phosphofructo-1-kinase, and pyruvate kinase (PK). The activity of BAT PK in HP diet-fed rats was reduced to about 50% of controls, and approached normal levels 24 hours after diet reversion. BAT denervation induced a small (15%) decrease in BAT PK activity in control rats, but did not affect the activity of the enzyme in HP diet-adapted rats. Also, denervation did not interfere with the restoration of PK activity induced by diet substitution. Treatment with anti-insulin serum resulted in an almost 50% reduction in PK activity in both innervated and denervated BAT from rats fed the control diet, but caused a much smaller (≈20%) decrease in BAT from HP diet-fed rats. Furthermore, anti-insulin serum administration completely suppressed the restoration of BAT PK activity induced by diet reversion. These data suggest that, differently from glucose uptake, BAT PK activity is predominantly controlled by hormonal/metabolic factors.

RATS ADAPTED to a high-protein, carbohydrate-free (HP) diet have been used in our laboratory as a model system to investigate adaptive mechanisms in energy-linked metabolic processes. We have previously shown that in vivo lipogenesis, assessed by the rate of incorporation of tritium from ^3H_2O into tissue fatty acids, is markedly reduced in the carcass, liver, and white adipose tissue from rats adapted to the HP diet.[1,2] Despite the reduced lipogenic activity in both liver and adipose tissue, body fat stores of rats fed the HP diet were well preserved, with carcass FA amounting, after 30 days on the diet, to about 85% to 90% of values in controls fed a balanced, carbohydrate-rich diet.[1] In subsequent studies we obtained evidence indicating that this relatively small loss of body fat could be due, at least in part, to a HP diet-induced reduction of brown adipose tissue (BAT) thermogenic capacity,[3] with a consequent decrease in overall body energy expenditure and increased metabolic efficiency. The reduced thermogenesis in BAT from rats adapted to the HP diet was indicated by decreases in tissue weight, mitochondrial protein, cytochrome oxidase activity, and mitochondrial binding of guanosine diphosphate (GDP), as well as by a reduced response of the tissue temperature to norepinephrine infusion. These signs of reduced brown adipose tissue thermogenesis were accompanied by a reduction in lipogenic activity, estimated in vivo by the incorporation of ^3H_2O into tissue fatty acids.[3] Recently, we have demonstrated that in vivo rates of incorporation of ^{14}C-glucose into fatty acids are also reduced in BAT from rats adapted to the HP diet, and that the tissue of these animals have a higher capacity to synthesize glyceride-glycerol from noncarbohydrate sources (glyceroneogenesis), as shown by increases in the activity of phosphoenolpyruvate carboxykinase and in the rate of incorporation of ^{14}C-pyruvate into glyceride-glycerol in vitro.[4]

The experiments of the present work were designed to further clarify the adaptive changes induced in BAT glucose metabolism by the HP diet, and to obtain more information about the control of glucose utilization by the tissue. To this end, we investigated (1) the effect of the diet on the in vivo rate of glucose uptake, on the content of the insulin-responsive glucose transporter (GLUT4), and on the activity of glycolytic enzymes: hexokinase, phosphofructo-1-kinase, and pyruvate kinase (PK); (2) the effect of short-term replacement of the HP diet by a balanced diet on the uptake of glucose, on the amount of GLUT4, and on the activity of PK, which catalyzes the reaction that is circumvented during glyceroneogenesis; and (3) the effect of BAT denervation on glucose uptake and on the activity of PK. The effect of acute insulin deficiency on the activity of PK was also investigated.

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 min-

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erals, have been described in detail.[3] 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.[5] At the end of 21-day adaptation period, when they were used for the experiments, the animals weighed 180 to 200 g. In all experiments of diet reversion, the diet of the rats adapted to the HP diet was replaced by the control diet at 7:30 PM and the metabolic parameters were measured after 24 hours.

Glucose Uptake In Vivo

The technique used was based on the method of Sokoloff et al[6] as modified by Ferré et al.[7] 2-Deoxy[1-^{3}H]-glucose (30 μCi; 11Ci/mmol) in 0.2 mL of 0.9% NaCl was injected as a bolus in fed nonanesthetized rats through a silastic catheter (Dow Corning, Midland, MI) inserted into the right jugular vein 2 days before the experiment. With the rat free in its cage, blood samples of 0.15 mL were taken 1, 3, 5, 10, 20, 30, and 60 minutes after label injection for determination of 2-deoxy[1-^{3}H]-glucose concentration (in terms of radioactivity). After the last blood sample, the rats were killed by cervical dislocation and the interscapular BAT was rapidly removed, cleaned free of adhering fat and muscle, and immediately immersed in plastic tubes containing 1 mol/L NaOH. After total digestion of the tissue, the content of 2-deoxy-[1-^{3}H]-glucose-6-phosphate (2-DG-P) was determined as described by Ferré et al.[7] The plasma glucose concentration was determined with glucose oxidase in a Beckman (Fullerton, CA) glucose analyzer.

Calculations

Rates of glucose uptake were determined from the 2-deoxy-[1-^{3}H]glucose/glucose ratio versus time curves and tissue 2-DG-P using an equation derived from a 2-compartment (plasma and tissue) mathematical model.[6,7] Plasma levels of glucose did not change significantly during the experimental period, as required by the technique used.

Unilateral Denervation of BAT

Under ether anesthesia, a small incision was made between the scapulae and the interscapular BAT 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 BAT were isolated and cut off at a length of approximately 5 mm. Surgical denervation was performed 6 days before the utilization of 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 ,[8] was reduced to less than 2% of values in the control, innervated side.

Western Blotting for GLUT4 Protein Analysis

The procedure used was as previously described.[9] Briefly, a fat-free extract fraction (FFE) of BAT was prepared from BAT homogenates and equal amounts (40 μg) of membrane sample proteins were solubilized in Laemmli's sample buffer, subjected to sodium dodecyl sulfate polyacrylamide gel electropheresis (SDS-PAGE) (10%), electrophoretically transferred to nitrocellulose paper, and blocked with bovine serum albumin in phosphate-buffered saline (PBS). The sheets were incubated with an anti-GLUT4 antiserum during 3 hours at 37°C and the membranes were then washed and incubated with ^{125}I–protein-A during 2 hours at room temperature. After washing and exposing the membranes to x-ray film for 5 days, the autoradiographic blots were quantified by densitometry. The results are expressed as arbitrary units (AU) per microgram of protein loaded in the electrophoresis or as AU per milligram tissue, calculated from the total protein yield, the volume of FFE, and the tissue weight.

Anti-insulin Serum

Anti-insulin serum was obtained from guinea-pigs following the recommendations of Makulu and Wright.[10] 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.

Measurement of Enzyme Activity

Hexokinase and phosphofructo-1-kinase. Portions of BAT were homogenized in ice-cold 50 mmol/L Tris buffer, containing 5 mmol/L $MgCl_2$, 1 mmol/L EDTA, and 20 mmol/L mercaptoethanol, pH 8.2. After centrifugation at 10,000 $\times$ *g* for 15 minutes and removal of the floating fat layer, the supernatants were centrifuged for 60 minutes at 100,000 $\times$ *g*. Aliquots of new supernatant were used to determine enzyme activities.[11,12]

Pyruvate kinase. The tissue was homogenized in ice-cold 0.1 mol/L Tris-HCl buffer, pH 7.4, containing 50 mmol/L potassium fluoride, 7.5 mmol/L EGTA and 30% glycerol. The enzyme activity was determined[13] in 100,000 $\times$ *g* supernatants prepared as described above. The composition of the assay mixture for the 3 enzymes was identical to that of a previous study.[14] The concentration of protein in homogenates was determined by the method of Lowry et al ,[15] using bovine serum albumin as a standard. The concentration of plasma insulin was determined by radioimmunoassay using a commercial kit from Amersham (Little Chalfont, UK).

Statistical Methods

Results are expressed as means $\pm$ SEM and differences between means were analyzed by 1- or 2-way analysis of variance (ANOVA), as appropriate, with $P < .05$ as the criterion of significance.

RESULTS

The data in Fig 1 show that the rate of glucose uptake in vivo was markedly reduced in BAT of rats adapted to the HP diet, with rates in HP rats amounting to about 25% of control values. Replacement of the HP diet by the balanced, control diet restored the tissue capacity to utilize glucose, rates of hexose uptake attaining levels even higher than those in normally fed control rats 24 hours after diet reversion (Fig 1). Figure 1 also shows that BAT denervation of rats fed the control diet caused a decrease in the tissue uptake of glucose, with in vivo rates in denervated pads about 50% lower than rates in intact pads. The already very low levels of glucose uptake in BAT from HP diet-fed rats were not affected by denervation (Fig 1).

As shown in Fig 2, adaptation to the HP diet resulted in a decrease of GLUT4 content of BAT, which attained about 50% of levels in control rats, when expressed per milligram tissue. The effect of replacement of the HP diet by the normal diet on GLUT4 content was examined in 5 HP-fed rats (Fig 2). In contrast to the marked effect of diet reversion on the rate of glucose uptake, the amount of BAT GLUT4 was not significantly affected in 4 HP-adapted rats after 24 hours of diet substitution. In one animal, however, GLUT4 content was within the range of control values after the same period of diet replacement.

The results of measurements of the glycolytic enzymes are shown in Fig 3. The reduction in the rate of glucose utilization

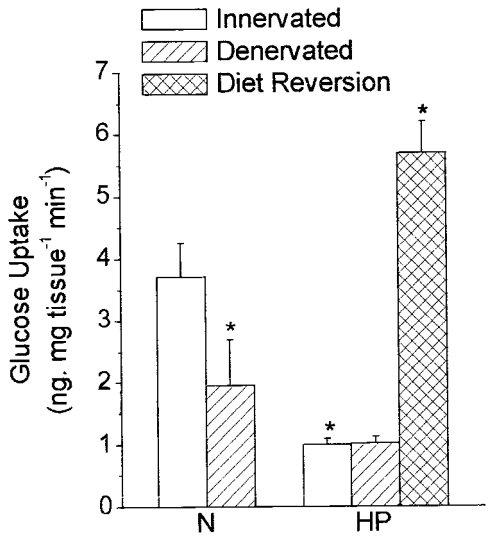

Fig 1. Effect of denervation on in vivo rates of glucose uptake by BAT from rats adapted to a HP diet or to a balanced (N) diet; and effect of replacement for 24 hours of the diet of HP diet-adapted rats by the N diet. Data are means ± SE from 6 to 8 animals. *$P < .05$ *v* innervated, N diet.

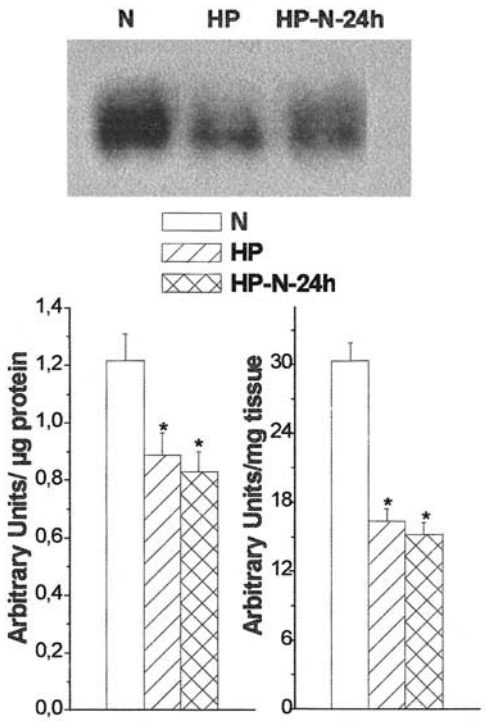

Fig 2. Effect of adaptation of rats to the HP diet, and of replacement by the balanced (N) diet for 24 hours (HP-N-24h), on BAT GLUT4 protein content. Data are expressed as AU per mg protein or per mg tissue (see Methods). Values are means ± SE from 6 rats (HP or N diet) and from 4 of the 5 HP-N-24h rats examined. In the 5th animal, GLUT4 content (27 AU/mg tissue) was within the normal (N diet) range. *$P < .05$ *v* N diet. (Top) Representative autoradiographies from N diet, HP diet, and HP-N-24h animals.

by BAT was accompanied by decreased activities of tissue hexokinase, phosphofructo-1-kinase, and PK (Fig 3). The decrease was greater for hexokinase (to ≈15% of control values) than for phosphofructo-1-kinase and PK (to 50% to 60% of controls).

Because PK catalyzes a reaction that is circumvented by glyceroneogenesis, a processes which we have shown to be activated in BAT from rats adapted to the HP diet,[4] we also investigated the effects of diet reversion, denervation, and acute insulin deficiency on the activity of this enzyme. The results of the experiments with denervation of BAT are shown in Fig 4. The data in Fig 4 show that denervation induced a relatively small (15%) reduction in the activity of PK in tissues from rats fed the balanced diet, and did not affect significantly the activity of the enzyme in HP diet-adapted rats, in which the activity was already 50% lower than in controls. Figure 4 also shows that replacement of the HP diet by the balanced diet induced a marked increase in the activity of BAT PK, the levels of the enzyme approaching those in controls after 24 hours of diet reversion. Furthermore, the stimulation of PK activity induced by diet substitution was not affected by previous denervation of brown adipose tissue from HP-diet adapted or control rats (Fig 4). The results of the experiments with acute insulin deficiency are shown in Fig 5. In these experiments, rats adapted to the HP diet were injected with anti-insulin serum 12 and 18 hours after replacement of the diet. Coincident injections were administered to rats fed the balanced diet. Measurements were made after the rats fed the HP diet had completed 24 hours of diet reversion. The data in Fig 5 show that treatment with anti-insulin serum resulted in a marked (almost 50%) reduction in the activity of PK in BAT from rats fed the balanced diet, the decrease being only slightly greater in denervated pads. The anti-insulin serum-induced reduction in the enzyme activity

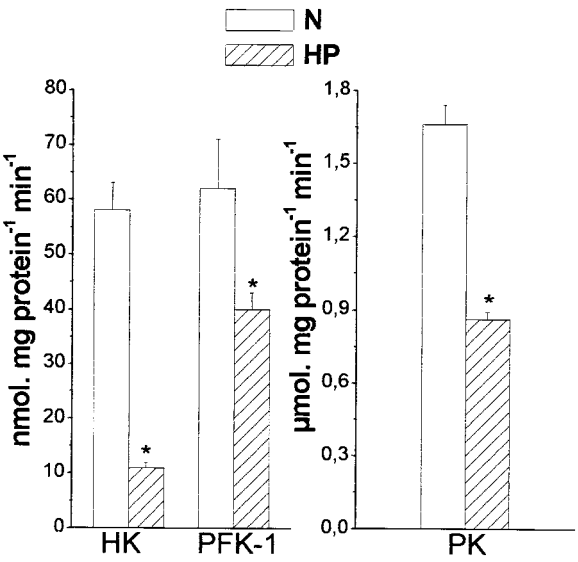

Fig 3. Effect of adaptation of rats to the HP diet on the activities of BAT hexokinase (HK), phosphofructo-1-kinase (PFK-1), and PK. Values are means ± SE from 6 animals. *$P < .05$ *v* balanced (N) diet.

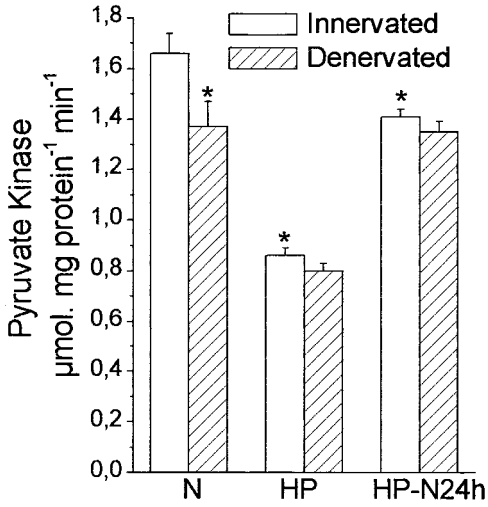

Fig 4. Effect of denervation on the activity of BAT PK in rats adapted to the HP diet or to a balanced (N) diet, and in HP diet-adapted rats 24 hours after replacement of their diet by the control diet (HP-N 24h). Values are means ± SE from 6 animals. *$P < .05$ *v* innervated, N diet.

was much smaller (≈20%) in intact or denervated pads from rats adapted to the high protein diet. Figure 5 also shows that anti-insulin serum administration completely blocked the increase in PK activity induced by diet reversion in both intact and denervated pads from HP-diet adapted rats.

DISCUSSION

The data of the present work clearly show that adaptation to a HP diet results in a marked reduction in the utilization of glucose by BAT, evidenced not only by a marked decrease in the rate of uptake of glucose by the tissue in vivo, but also by the reduced activity of key glycolytic enzymes: hexokinase, phosphofructo-1-kinase, and PK. The data also show that BAT hemidenervation, a procedure that does not affect plasma insulin levels, causes a 50% reduction in the in vivo rate of glucose uptake by denervated pads from normally fed rats (Fig 1). This finding is consistent with the observations that electrical stimulation of BAT sympathetic nerves,[16] as well as norepinephrine administration,[17] produces a significant increase in glucose utilization, estimated by 2-deoxy-glucose. Also, in cultured brown adipocytes, norepinephrine has been shown to stimulate glucose transport directly, independently of insulin.[18,19] We have previously shown that the turnover of norepinephrine, which is mainly dependent on the sympathetic flux to the tissue, is markedly reduced in BAT from HP-adapted rats.[20] Since the levels of plasma insulin are also reduced in these animals[5,21] (19 ± 4 µU/mL in the HP diet-adapted rats of the present study *v* 41 ± 6 µU/mL in controls), the pronounced reduction in glucose uptake in these animals (more marked than that produced by BAT denervation in normally fed rats, Fig 1) was probably due to the combined effects of a reduced sympathetic flow and a local deficiency of insulin, a hormone that has been shown in numerous studies[22] to stimulate glucose utilization both in vivo and directly in vitro. This conclusion is supported by the finding that the tissue content of the insulin-responsive glucose transporter (GLUT4) is reduced in rats adapted to the HP diet (Fig 2). The already reduced sympathetic activity may have been the main reason for the lack of significant effects of BAT denervation in HP-adapted rats (Fig 1).

The finding that, in contrast to the marked increase (to values above controls) in the rates of glucose-uptake 24 hours after diet reversion (Fig 1), the concentration of GLUT4 protein did not increase in 4 of the 5 HP-adapted animals examined (Fig 2), indicates that diet replacement induced a rapid activation of glucose utilization by a mechanism independent of the content of this transporter. A similar pattern has been observed after refeeding of starved rats, which have low BAT levels of mRNA and GLUT4 protein. Despite dramatic increases in glucose utilization 2 and 4 hours after refeeding, no significant changes in the concentration of GLUT4 mRNA was detected.[23] Also, it has been found that acute (4-hour) cold exposure of fasted rats produced a significant increase in glucose uptake, but had no effect on BAT mRNA and GLUT4 levels, which increased with more prolonged exposition to cold.[24] Since BAT sympathetic activity increases after diet reversion of HP-adapted rats,[20] as well as in the above situations, it is possible that the initial increase in glucose utilization was due to a GLUT1-mediated stimulation of glucose transport by norepinephrine. In brown adipocytes, norepinephrine has been shown to stimulate glucose transport, independently of insulin, by increasing the affinity or activation of GLUT1 isoform, with no apparent increase in GLUT1 protein in the plasma membrane.[18,19]

Previous studies from this laboratory have demonstrated that glyceroneogenesis, the synthesis of glycerol-3-P from noncarbohydrate sources, is not only present but is very active in rat BAT, and increases significantly in rats adapted to the HP diet.[4] PK catalyzes the P-enol-pyruvate→pyruvate reaction in the glycolytic pathway that is reverted during glyceroneogenesis,

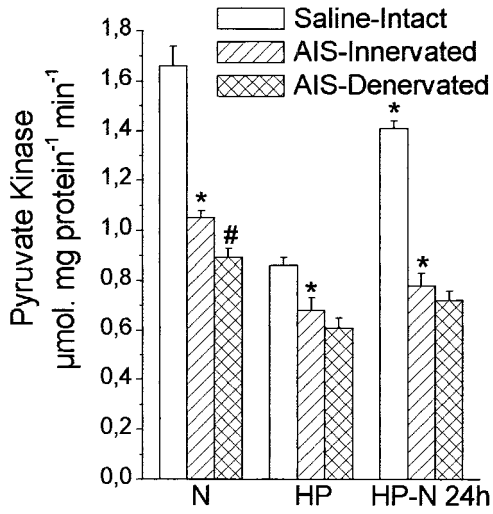

Fig 5. Effect of anti-insulin serum (AIS) administration on the activity of BAT PK in rats adapted to the HP diet or to a balanced (N) diet, and in HP-diet adapted rats 24 hours after replacement of their diet by the control diet (HP-N 24h). Values are means ± SE from 5 or 6 rats. *$P < .05$ *v* control, #$P < .05$ *v* AIS-treated, innervated.

and the reduction in the activity of this enzyme in BAT from HP-diet adapted rats (Fig 4) minimizes the recycling of substrates, increasing the efficiency of the glyceroneogenic process. The data of the present work strongly suggest that the control of BAT PK is exerted by hormonal/metabolic (insulin), rather than neural (sympathetic) factors. Thus, tissue denervation induced only a relatively small (≈15%) reduction of PK activity in normally fed rats and did not affect significantly the already reduced activity of the enzyme in HP-adapted rats (Fig 4). Furthermore, denervation did not interfere with the marked increase (to almost normal levels) in the activity of PK induced by replacement of diet of rats fed the protein diet (Fig 4). On the other hand, administration of anti-insulin serum produced a 40% to 50% reduction in the activity of the enzyme in both intact and denervated pads from control rats, and a smaller (≈20%) reduction in tissues from HP-fed rats (Fig 5). The importance of insulin is further evidenced by the complete block by anti-insulin serum of the PK activation induced by diet reversion not only in denervated, but also in intact pads (Fig 5), in spite of the fact that tissue sympathetic activity is restored after diet reversion.[20] We have recently investigated,[21] using the same experimental design, the control of enzymes of the lipogenic pathway in BAT. Although insulin was also found to be essential to maintain the activity of adenosine triphosphate (ATP)-citrate lyase and acetyl-coenzyme A (CoA) carboxylase, a participation the sympathetic nervous system was clearly observed. Thus, in contrast to the present results with PK, denervation caused in normally fed rats a marked reduction in the activity of both citrate lyase and acetyl-CoA carboxylase and significantly impaired the diet reversion-induced increase in the activity of the 2 enzymes in HP diet-adapted rats.[21] Further studies are needed to clarify the reasons for these differences in the effects of sympathetic system on BAT metabolism. Because of its metabolic characteristics, the rat adapted to a HP diet seems to be a experimental model useful for the elucidation of the biochemical mechanisms involved in the complex interplay of hormonal/metabolic and neural factors that control BAT activity.

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