Fat accretion and the regulation of insulin-mediated glycolysis synthesis after puberty in rats

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Banerjee, Swati, Paul Saenger, Meizhu Hu, Wei Chen, and Nir Barzilai. Fat accretion and the regulation of insulin-mediated glycolysis synthesis after puberty in rats. Am. J. Physiol. 273 (Regulatory Integrative Comp. Physiol. 42): R1534–R1539, 1997.—Peripheral insulin sensitivity decreases after puberty in both humans and rodents and can be explained mostly by a reduction in insulin-mediated glycolysis synthesis. We tested the hypothesis that the increase in postpubertal fat mass (FM), reflecting an alternative energy store, regulates a decrease in the capacity to store muscle glycogen. We studied Sprague-Dawley rats (n = 21) before puberty (Pre) or after puberty (at 4 mo of age) in groups that were either ad libitum fed (Post) or moderately caloric restricted (CR). FM (by $^{3}$H$_{2}$O isotope dilution technique) was decreased by >40% in CR compared with Post. Glucose uptake (R$_{b}$ by 18 mU·kg$^{-1}·$min$^{-1}$ hyperinsulinemic clamp) was 63 ± 8 mg·kg$^{-1}·$min$^{-1}$ in Pre and decreased to 39 ± 2 mg·kg$^{-1}·$min$^{-1}$ in Post (P < 0.001). However, it increased in CR to 53 ± 2 mg·kg$^{-1}·$min$^{-1}$ (P < 0.001 vs. Post). This increase in R$_{b}$ was mainly accounted for by an increase in glycogen synthesis (R$_{g}$ glycoseysis determined by the rate of conversion of $^{3}$H-labeled glucose to $^{3}$H$_{2}$O) from 23 ± 2 in Post to 33 ± 2 mg·kg$^{-1}·$min$^{-1}$ in CR (P < 0.001; 38 ± 7 mg·kg$^{-1}·$min$^{-1}$ in Pre). Correction of glycogen synthesis in CR to near-prepubertal levels was further supported by directly assayed muscle glycogen content after insulin stimulation that was 45% higher and by a 35% enhanced accumulation of $^{3}$H glucose into glycogen. No changes in the enzyme kinetics of glycogen synthase or phosphorylase were observed. An additional group of 2-mo-old postpubertal ad libitum-fed rats was matched with CR for lean body mass but had more FM. This group demonstrated 25% lower rates of insulin-mediated glycogen synthesis compared with CR, further supporting the notion that a moderate reduction of FM prevents the decline in insulin responsiveness and glycolysis synthesis occurring after puberty. These data suggest a cause-effect relationship between the increased deposition of fat and the reduced ability to store glucose in skeletal muscle after puberty.

MATERIAL AND METHODS

Animals. Weanling (≈3 wk old) male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were used for this study. These rats are relatively obese and hyperphagic, and caloric restriction is used not as underfeeding but as normofeeding. Prepubertal rats (Pre) were studied within 1 wk (n = 5), and the remaining rats were divided into three groups. One group was fed ad libitum and studied postpu-
berty at 4 mo of age (Post, n = 5). The second group was kept on ~60% of the diet of the ad libitum-fed rats and was studied postpubertally at 4 mo of age (CR, n = 6). A third group was studied postpubertally at 2 mo of age with animals matched for LBM to CR (LBM-C, n = 5). All rats were fed a standard chow that contained 64% carbohydrates, 30% proteins, and 6% fats, with a physiological fuel value of 3.30 kcal/g chow, and vitamin supplements. The rats were sedentary and were housed in individual cages. They were subjected to a standard light (6 AM-6 PM)-dark (6 PM-6 AM) cycle. Pre animals were housed in individual cages. They were subjected to a standard and vitamin supplements. The rats were sedentary and were 6% fats, with a physiological fuel value of 3.30 kcal/g chow, chow that contained 64% carbohydrates, 30% proteins, and

On the morning of the study, 20 µCi of 3H2O (NEN, Boston, MA) was injected intra-arterially. Steady state for 3H2O specific activity in rats is generally achieved within 30–45 min, and 8 samples were collected between 1 and 1.5 h after injection.

The distribution space of water was obtained by dividing the total radioactivity injected by the steady-state specific activity of plasma water, which was assumed to be 93% of the total plasma volume. LBM was calculated from the whole body distribution space, divided by 0.73 (the percent water content of LBM). FM was calculated as the difference between total body weight and LBM. Because carcass protein was not measured in Pre-Post- and CR rats, we assumed similar distribution volume of water in all rats. Epididymal fat pads were dissected and weighed after the completion of the study as an additional index of adiposity.

Hyperinsulinemic-euglycemic clamp. Studies were performed in awake, unstressed, chronically catheterized rats (4, 22, 24). All rats received a primed continuous (15–40 µCi bolus, 0.4 µCi/min) infusion of high-performance liquid chromatography-purified [3-3H]glucose (NEN, Boston, MA) was injected intra-arterially. Steady state for 3H2O specific activity in rats is generally achieved within 30–45 min, and 8 samples were collected between 1 and 1.5 h after injection.

The distribution space of water was obtained by dividing the total radioactivity injected by the steady-state specific activity of plasma water, which was assumed to be 93% of the total plasma volume. LBM was calculated from the whole body distribution space, divided by 0.73 (the percent water content of LBM). FM was calculated as the difference between total body weight and LBM. Because carcass protein was not measured in Pre-Post- and CR rats, we assumed similar distribution volume of water in all rats. Epididymal fat pads were dissected and weighed after the completion of the study as an additional index of adiposity.
0.45% mercaptoethanol, 15 mM G-1-P (50 µCi/mmol), and 3.4 mg/ml glycogen.

Analytic procedures. Plasma glucose was measured by the glucose oxidase method (GlucoseAnalyzer II; Beckman Instruments, Palo Alto, CA), and plasma insulin was measured by radioimmunoassay with the use of rat and porcine insulin standards. Plasma (3H) glucose radioactivity was measured in duplicate on the supernatants of Ba(OH)2 and ZnSO4 precipitates of plasma samples before and after evaporation to dryness to eliminate tritiated water. We determined plasma nonesterified fatty acid concentrations using an enzymatic method with an automated kit according to the manufacturer’s specifications (Wako Pure Chemical Industries, Osaka, Japan). Glycerol was determined using the GPO-Trinder reagents for the quantitative enzymatic determination of glycerol in plasma at 540 nm (Sigma, St. Louis, MO).

Calculations. Rates of whole body glycolysis were estimated from the increment per unit time in tritiated water [disintegrations·min⁻¹ (dpm)·ml⁻¹]×body water (ml)/[3H]-glucose specific activity (dpm/mg). All values are presented as means ± SE. Comparisons between groups were made with the use of repeated-measures analysis of variance where appropriate. Where F ratios were significant, further comparisons were made with the use of Student’s t-tests.

RESULTS

Body composition and biochemical characteristics of experimental rats. Although CR animals attained ~75% of the weight of Post animals (Table 1), the total and epididymal FM of CR animals was reduced by approximately one-half, whereas their LBM was reduced by only ~20%. All groups had similar 6-h fasting glucose levels, whereas plasma insulin, FFA, and glycerol levels were very significantly lower in Pre. When CR is compared with Post, basal plasma insulin level was decreased by ~40% and plasma glycerol level was decreased by nearly one-third, whereas plasma FFA level was similar.

Rd, glycolysis, and glycogen synthesis. During the insulin clamp studies, insulin levels were increased to similar, maximally glucose-uptake stimulating levels (2,412 ± 250, 2,538 ± 128, and 2,921 ± 80 µM in Pre, Post, and CR, respectively; Fig. 1). Steady-state plasma glucose levels were also similar in all groups (7.1 ± 0.5 mM). Hepatic glucose production was invariably suppressed to <2 mg·kg⁻¹·min⁻¹ (controls 12–15 mg·kg⁻¹·min⁻¹), and plasma FFA level decreased by >60% in all groups. Rd (Fig. 1) was increased by 36% in CR to 53 ± 2 compared with 39 ± 2 mg·kg⁻¹·min⁻¹ in Post (P < 0.001; 63 ± 8 mg·kg⁻¹·min⁻¹ in Pre). Glycolysis in CR was mildly increased to 21 ± 1 from 16 ± 3 mg·kg⁻¹·min⁻¹ in Post (P < 0.01; 25 ± 6 mg·kg⁻¹·min⁻¹ in Pre). Glycogen synthesis (Rd-glycolysis) was increased to 33 ± 2 mg·kg⁻¹·min⁻¹ in CR from 23 ± 2 mg·kg⁻¹·min⁻¹ in Post (P < 0.001), similar to 38 ± 7 mg·kg⁻¹·min⁻¹ in Pre. Thus more than two-thirds of the improvement in insulin-mediated Rd in the CR rats resulted from improvement in glycogen synthesis, whereas the rest of the improvement in Rd could be attributed to the increase in glycolysis.

Table 1. Body composition and biochemical characteristics of Sprague-Dawley rats

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Pre</th>
<th>Post</th>
<th>CR</th>
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<tr>
<td><strong>Body composition</strong></td>
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<tr>
<td>Body weight, g</td>
<td>81 ± 8*</td>
<td>337 ± 25</td>
<td>258 ± 12†</td>
</tr>
<tr>
<td>FM, g</td>
<td>1.5 ± 1.0*</td>
<td>47.0 ± 5.6</td>
<td>27.3 ± 6.4†</td>
</tr>
<tr>
<td>LBM, g</td>
<td>80 ± 4*</td>
<td>299 ± 19</td>
<td>233 ± 11†</td>
</tr>
<tr>
<td>Epididymal fat, g</td>
<td>0.3 ± 0.1*</td>
<td>4.8 ± 0.5</td>
<td>2.2 ± 0.3†</td>
</tr>
<tr>
<td><strong>Biochemical characteristics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose, mM</td>
<td>7.2 ± 0.3</td>
<td>7.4 ± 0.8</td>
<td>7.4 ± 0.6</td>
</tr>
<tr>
<td>Insulin, pM</td>
<td>139 ± 24*</td>
<td>348 ± 34</td>
<td>208 ± 5†</td>
</tr>
<tr>
<td>FFA, mM</td>
<td>0.33 ± 0.04*</td>
<td>1.05 ± 0.08</td>
<td>0.94 ± 0.07</td>
</tr>
<tr>
<td>Glycerol, µM</td>
<td>30 ± 7*</td>
<td>182 ± 18</td>
<td>125 ± 32†</td>
</tr>
</tbody>
</table>

Values are means ± SE. Body weight, fat mass (FM), lean body mass (LBM), epididymal fat pads, plasma glucose, insulin, free fatty acids (FFA), and glycerol levels in prepubertal (Pre), postpubertal (Post), and calorie-restricted (CR) rats. *P < 0.001 vs. Post and CR; †P < 0.01 vs. Post.
mediated Rd was increased in CR by 18%, and although the insulin levels were comparable, body weight but n
(45% less FM compared with LBM-C.

Muscle glycogen synthesis was also determined by two other methods (Table 2). Muscle glycogen stores were ~45% higher in the CR compared with Post. Maintenance of prepubertal glycogen synthesis was further supported by enhanced insulin-stimulated accumulation of [3H]glucose into glycogen, with CR incorporating ~35% more than Post.

Muscle GSy and GP activities. No differences were noted in the Vmax for UDP-glucose of GSy (Table 2). Insulin-activated (decrease in Km for UDP-glucose) GSy activity in all groups was comparable. No differences were noted in the AMP-independent form of GP activity.

Comparison with LBM-matched control. Because CR resulted in a decrease in LBM compared with Post (Table 3), we compared a subgroup of CR rats (n = 4) with an additional group of ad libitum-fed 2-mo-old rats (n = 5) with identical LBM (LBM-C). CR had similar body weight but ~30% less FM compared with LBM-C. Although the glucose and FFA levels were comparable, the insulin levels were ~30% higher in LBM-C. Insulin-mediated Rd was increased in CR by 18%, and although glycolysis was unchanged, most of the improvement in Rd resulted from the increase in glycogen synthesis. The lower glycogen synthesis in the LBM-C was further confirmed by an ~40 decrease in muscle glycogen (74 ± 6 μmol/g wet wt) as well as by a milder decreased incorporation of [3H]glucose into glycogen (4.9 ± 1.4 × 10^7 dpm/g muscle).

Table 3. Major characteristics of 4-mo-old CR and 2-mo-old ad libitum-fed rats matched for LBM

<table>
<thead>
<tr>
<th></th>
<th>CR</th>
<th>LBM-C</th>
</tr>
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<tbody>
<tr>
<td>LBM, g</td>
<td>226 ± 5</td>
<td>223 ± 11</td>
</tr>
<tr>
<td>FM, g</td>
<td>25.2 ± 2.7</td>
<td>35.4 ± 3.3*</td>
</tr>
<tr>
<td>Insulin, pM</td>
<td>212 ± 6</td>
<td>273 ± 18*</td>
</tr>
<tr>
<td>R4, mg·kg⁻¹·min⁻¹</td>
<td>54 ± 2</td>
<td>46 ± 2*</td>
</tr>
<tr>
<td>Glycolysis, mg·kg⁻¹·min⁻¹</td>
<td>21 ± 1</td>
<td>20 ± 3</td>
</tr>
<tr>
<td>GSy, mg·kg⁻¹·min⁻¹</td>
<td>33 ± 1</td>
<td>26 ± 3*</td>
</tr>
</tbody>
</table>

Values are means ± SE. LBM-C, 2-mo-old ad libitum-fed rats matched with CR for LBM. * P < 0.01 vs. CR.

DISCUSSION

In this study we used caloric restriction as a specific probe to examine the effects of increase in postpubertal FM on peripheral insulin action. We have demonstrated that by partially preventing the fat accumulation that occurs during and after puberty, we were able to maintain insulin-mediated Rd and glycogen synthesis at near prepubertal levels. This improvement occurred when compared with both ad libitum-fed age-matched animals and ad libitum-fed younger but LBM-matched controls.

Fat stores were significantly reduced by careful caloric restriction without affecting puberty as determined by testicular descent and balano-preputial separation (17). Furthermore, steady weight gain was preserved, ensuring a steady increase in LBM in the animals studied. Reduction of fat stores was demonstrated by radioisotope techniques measuring LBM and by dissecting out the discrete epididymal, perinephric, and mesenteric fat pads, all of which decreased proportionately. However, although the Pre animals had negligible amounts of visceral and subcutaneous fat, fat deposition was only partially prevented to ~40% of age-matched Post.

Although basal glucose levels were similar in all animals, postpubertal plasma insulin levels were decreased by ~40% in the CR compared with the Post group. In addition, CR rats had a decreased plasma glycerol level, indicating a decrease in FM and lipolysis. Although FFA levels were similar in the CR group compared with the Post group, the rate of lipid oxidation throughout human puberty is dissociated from the rate of lipolysis (3), suggesting that plasma FFA level may be a poor indicator of FFA fluxes. Thus, although elevated concentrations of circulating FFA may contribute to insulin resistance, normalization of these levels does not appear to be required to restore insulin sensitivity. Indeed, on the basis of the lower FM and plasma insulin and glycerol levels in CR, we expected this model to partially restore the impaired insulin-mediated Rd to prepubertal levels. In fact, insulin-mediated Rd was decreased after puberty in the CR to near prepubertal levels compared with Post. Because it could be argued that CR rats had lower LBM when compared with age-matched controls, we further matched LBM of CR with that of younger Post rats and still maintained higher insulin-mediated Rd. In fact, the difference between these groups (Table 3) resulted from an increased FM in the 2-mo-old rats, further suggesting that FM plays a role in establishing insulin sensitivity postpubertally.

Several lines of evidence suggest that the improvement in Rd seen in the CR rats was largely a result of an improvement in glycogen synthesis. When calculated as the difference between Rd and glycolysis, glycogen synthesis rates were significantly improved in the CR compared with Post and LBM-C (Fig. 1 and Table 3). In addition, the [3H]glucose incorporation in muscle glycogen was increased in the CR group compared with the controls. Finally, the insulin-induced increase in muscle...
glycogen was also higher in the CR rats (Table 2). This occurred with no changes in the kinetics of muscle GSy or in the activity of GP. Thus the improvement in the ability to store muscle glycogen by CR supports the hypothesis that the decreased glycogen synthesis and insulin resistance in post pubertal rats is predominantly a function of the fat accumulation seen after puberty. Earlier studies in humans have characterized the insulin resistance of type II diabetes and suggested defective glycogen storage. These studies, with the use of indirect calorimetry or $^{13}$C nuclear magnetic resonance, have demonstrated a major decline in insulin-mediated glycogen synthesis (27). Moreover, such a decrease was demonstrated in the insulin resistance of normoglycemic obese subjects (11). Such a defect in insulin-mediated glycogen synthesis has also been demonstrated directly in diabetic (22) and obese (4) rats. Our data suggest that this defect in glycogen storage, which is typical in other insulin resistance states, is initiated early in life with the appearance of FM. A milder decrease in the rate of glycolysis was demonstrated in this study and has been previously demonstrated in humans during puberty by indirect calorimetry (3). A mild decrease in glycolysis was also demonstrated in diabetic and obese humans (11) and in obese rats (4). Thus, whereas glycogen synthesis accounts for much of the decrease in insulin-mediated $R_d$ after puberty, postpubertal fat accretion or maturation may also substantially affect the glycolytic and glucose oxidative pathways.

One mechanism that may signal the muscle to decrease its capacity to store glycogen is an increase in substrates such as FFA and glycerol, which are associated with increased FM. Acute increase in plasma FFA levels in humans is associated with a reduction in glucose oxidation (31), supporting the Randle hypothesis that glucose-FFA substrate competition is the initial event that leads to insulin resistance (20). However, intralipid infusion did not consistently cause a decrease in insulin-mediated $R_d$ or produce changes in the activities of important enzymes that should be impaired as a result of glucose-FFA substrate competition (26). Recent evidence suggests that, although an experimentally induced elevation in plasma FFA levels promptly decreases the rate of glycogen oxidation, it took $>4$ h to significantly impair insulin-mediated $R_d$ and glycogen synthesis (6, 7). Thus the downregulation of insulin action requires additional “downstream” intracellular steps to sense increased energy stores in fat.

In summary, the transition to puberty results in an increased deposition of fat and a decrease in insulin sensitivity. We demonstrated that this FM-mediated insulin resistance is characterized by a marked decrease in the ability to store muscle glycogen. This model demonstrates a cause-effect relationship between the increased deposition of fat and the reduced ability to store glucose in muscle in postpubertal rats.

**Perspectives**

If FM accretion is associated with a decrease in the muscle capacity to store glycogen in response to insulin, what are the mechanisms by which fat tissue “cross talks” with muscle? To deliver such a message to the muscle, candidates should ideally be secreted from fat, their plasma levels should reflect FM, and they should impair insulin action on glucose storage. The most commonly accepted candidate is the substrate FFA, and, although it was generally accepted that the acute increase of FFA causes an immediate “switch” from glucose to lipid oxidation (20), its effect on the decrease of insulin-mediated glycogen synthesis is markedly delayed. A recent hypothesis suggests that the hexosamine pathway acts as a sensor that reacts to an increase in intracellular nutrients such as FFA by decreasing insulin sensitivity (23). Indeed, prolonged FFA infusion to rats increased the accumulation of the end products of the glucosamine biosynthetic pathway, followed by the development of insulin resistance and a decrease in the rate of glycogen synthesis (14). In addition to substrates, fat is becoming increasingly recognized as a metabolically active endocrine tissue that secretes bioactive peptides and may contribute to the changes in the $R_d$ with enhanced fat stores. For example, impairment in tyrosine kinase-dependent insulin signaling and the development of peripheral and hepatic insulin resistance were shown to be induced by tumor necrosis factor-α (12, 16). Although leptin is secreted from fat in proportion to body weight and may play a role in the control of body composition and glucose tolerance, it has been suggested that some obese subjects are resistant to the action of leptin (18). This latter example suggests that a mechanism of failure to communicate between fat and muscle may result in obesity and insulin resistance. These mechanisms, which may be involved in the cross talk between FM and skeletal muscle and which are causing a decrease in insulin action on $R_d$ and glycogen storage, are targets for further investigation.

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