The Effect of Age-Dependent Increase in Fat Mass on Peripheral Insulin Action is Saturable

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Insulin resistance and increased fat mass (FM) are common in human aging. We aimed to investigate the relationship between the age-dependent increase in FM and insulin resistance (by euglycemic hyperinsulinemic clamp technique), in a homogenous rodent model. The decline in insulin responsiveness was linear until late adulthood when body weight, FM, and epididymal fat reached a critical amount (r > .750, for all). Above this critical point, there was no further decline in insulin responsiveness with aging and with increased BW (p < .00001 for all spline curve analyses). This decline in insulin-mediated glucose uptake was accounted for by a decrease in whole body glycolytic rate with no change in the rate of glycogen synthesis. Thus, in this homogenous model, an early increase in FM is associated with impairment in insulin action until a critical FM is achieved, after which there is no additional insulin resistance with aging. We suggest that decreasing insulin responsiveness, in a heterogeneous group such as humans, will only occur within a specific accretion of visceral or total FM.

HUMAN and animal aging are characterized by a progressive increase in fasting and postprandial plasma insulin levels (1–3), suggesting an insulin-resistant state. Concomitantly with the increase in plasma insulin levels, rates of obesity are increased with aging, and approximately half of the U.S. population over age 50 is obese (4). An increase in fat mass (FM) typically occurs between age 30 and 70, and the ratio of FM over lean body mass (LBM) increases throughout human life (5–7). There is a common epidemiological association between insulin resistance and obesity; however, this relationship is confounded in humans by other coexisting conditions such as exercise capacity, dyslipidemia, hypertension, and genetic inheritance (8). Because of these confounding genetic and/or metabolic variables, animal models have been used to examine the relative impact of specific metabolic factors on glucose homeostasis.

Previous studies demonstrated that body mass index and FM were inversely correlated with glucose infusion rates during hyperinsulinemic clamp studies, independent of age (9,10). A recent multicenter study demonstrated similar peripheral insulin sensitivity in over 1,200 subjects of all ages and with a variety of FM. While increased FM was responsible for the majority of variability in insulin sensitivity at any age, insulin sensitivity was similar when data was analyzed separately in weight-matched young and old individuals (11). We had previously investigated whether aging is associated with peripheral insulin resistance in two rat models (2). Insulin-mediated glucose disposal appeared to decrease up to late adulthood (4 months of age), or up to a body weight (BW) of ~300 g. Thereafter, neither age nor body weight were associated with any further decrease in insulin-mediated glucose disposal. However, this observation was made in a relatively small group of animals.

The present studies were therefore designed to examine whether the effect of increased FM on peripheral insulin sensitivity is “saturable” and independent of age. We examined this question in a large number of aging rats providing adequate power for statistical modeling of the relationship between FM and insulin responsiveness with aging. We hypothesize that if the effect of FM on peripheral insulin sensitivity is saturable at a relatively young age, then aging per se is not further associated with defective insulin action.

MATERIAL AND METHODS

Animals.—Male Sprague–Dawley rats (purchased at 6 wk of age from Charles River Laboratories, Wilmington, MA) were ad libitum fed chow containing 64% carbohydrates, 30% proteins, and 6% fat with vitamin supplementation. Rats were sedentary and housed in individual cages subjected to a standard light (6 AM to 6 PM):dark (6 PM to 6 AM) cycle. They were studied at approximately 2 (n = 19), 4 (n = 20), 6 (n = 7), 12 (n = 7), 18 (n = 7) months of age. While this model is not a typical senescent animal model, it has the advantage of exhibiting changes in FM and visceral/abdominal fat with age that resembles these seen in human aging. One week before the in vivo study, rats were anesthetized with an i.p. injection of pentobarbital (50 mg/kg BW) and indwelling catheters were inserted in the right internal jugular vein and in the left carotid artery. The venous catheter was extended to the level of the right atrium and the arterial catheter was advanced to the level of the aortic arch. Recovery was continued until food intake was >20g/day for 3 days and BW was restored to the preoperative level. Studies were performed in awake, unstressed, chronically catheterized rats (2,12,13). After the conclusion of the study, fat pads from the epididymal, per-
inephric, and mesenteric areas were carefully dissected out and weighed in all rats studied.

**Body composition.**—Lean body mass (LBM) and FM were calculated from the whole body volume of distribution of water, estimated by tritiated water bolus injection in each experimental rat as described (2,3,14,15). 3H2O (20 μCi; New England Nuclear, Boston, MA) was injected intraarterially on the morning of the study. Steady state for 3H2O specific activity in rats is generally achieved within 30-45 min. Thus, five plasma samples were collected between 1- and 3-h post-injection. The distribution space of water (ml) was obtained by dividing the dpm injected by the steady-state specific activity of plasma water (dpm/ml) that was assumed to be 93% of the total plasma volume. LBM was calculated from the whole body water distribution space divided by .73. FM was calculated from the difference between total BW and LBM.

**Hyperinsulinemic euglycemic clamps.**—Insulin responsiveness (insulin-mediated glucose disposal at maximally stimulating insulin levels) was studied by using the euglycemic clamp technique as described (2,12,13,15). A primed-continuous infusion of insulin (18 μU/kg.min) was administered, and a variable infusion of a 25% glucose solution was started at time 0 and periodically adjusted to clamp the plasma glucose concentration at the basal level (=7 mM) for 2 h. All rats received a primed-continuous infusion of HPLC-purified [3-3H]-glucose (New England Nuclear, Boston, MA; 15-40 μCi bolus, 0.4 μCi/min), which was initiated at t = 0 and then maintained throughout the remainder of the study.

Plasma samples for determination of [3H]-glucose specific activity were obtained at 10 min. intervals throughout the insulin infusion. Plasma samples for determination of plasma insulin and free fatty acids (FFA) concentrations were obtained at 30 min intervals before (~30 and 0) and during the study. To prevent volume depletion and anemia, a solution (1:1 vol/vol) of 3.0 ml of fresh blood (obtained by heart puncture from a littermate of the test animal) and heparinized saline (10 U/ml) was infused for the duration of the study.

The study protocol was reviewed and approved by the Animal Care and Use Committee of the Albert Einstein College of Medicine.

**Whole body glycolysis and glycogen synthesis.**—The rate of glycolysis was determined by the rate of appearance of tritium from tracer glucose in plasma water. Specifically, because tritium on the C-3 position of glucose is lost to water during glycolysis, it can be assumed that plasma tritium is present either in tritiated water or [3-3H]-glucose (2,13,15). Thus, glycolysis can be analyzed from the slope of the line obtained from the wet counts over time. Plasma-tritiated water specific activity was determined by liquid scintillation counting of the protein-free supernatant (Somogyi filtrate) before and after evaporation to dryness. Muscle glycogen synthesis was quantitated by subtracting the glycolytic rate from the glucose uptake. We have obtained "wet" counts and presented data for glycolysis and glycogen synthesis in 28 of the rats studied.

**Analytical procedures.**—Plasma glucose was measured by the glucose oxidase method (Glucose Analyzer II, Beckman Instruments, Inc., Palo Alto, CA) and plasma insulin by radioimmunoassay using 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 after evaporation to dryness to eliminate tritiated water. Plasma nonesterified fatty acids concentrations were determined by an enzymatic method with an automated kit according to the manufacturer's specifications (Waco Pure Chemical Industries, Osaka, Japan).

**Calculations.**—Correlations were analyzed by simple linear regression. In addition, nonlinear regression was performed to fit an exponential decay model of glucose uptake versus parameters of body composition. Also, a piecewise regression model with an unknown knot point was used (16). This spline curve model consists of two regression lines joined at an unknown intersection (knot). This unknown knot can be statistically derived by nonlinear (NLLN) program in SAS windows, version 6.10. This model is expressed as: y = α + β1 x + β2 (x-k)h, y is glucose uptake, x represents parameters of body composition, k is the unknown knot to be estimated from the nonlinear regression procedure, and h is the indicator variable (which is equal to 1 if x is greater than the knot, and otherwise is 0). The model becomes y = α + β1 x if x is less than the estimated knot k and y = (α - β1 k) + (β1 + β2) x if x is greater than or equal to estimated knot k. The p values of the two slopes can be derived and will provide the information of how the outcome variable will change after the variable reached the estimated knot. The confidence interval of the unknown knot can also be derived from the nonlinear model. Other values are presented as the mean ± SEM.

**RESULTS**

**Body Composition**

Rat weights ranged from 272–595 g, and FM from 24–107 g. BW and FM were significantly linearly correlated (r = .943, p < .0001). Epididymal fat was the major contributor to the mass of the dissected fat pads, and correlated significantly with total BW (r = .925, p < .0001, Figure 1A). FM and epididymal fat weight were linearly correlated (r = .920, p < .0001, Figure 1B). Mesenteric and perinephric fat pads were also correlated to BW (r > .910, p < .0001 for all; data not shown). The weights of the epididymal, mesenteric, and perinephric fat pads were intercorrelated (r > .960, p < .0001 between all; data not shown).

**Insulin Clamp Study**

During the insulin clamp studies, the steady state plasma level of insulin, FFA, and glucose were (mean ± SEM) 2,212 ± 797 pM, 32 ± 0.8 mM, and 7.6 ± 0.9 mM, respectively. Residual hepatic glucose production during the clamp was 2.1 ± 0.9 mg/kg per min.

**Age, FM, and insulin-mediated glucose uptake (Table I).**—Between young adulthood (2 mo) and 6 mo FM nearly doubles, and significantly increases with age thereafter.
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Figure 1. (A) Relationship between BW and epididymal fat. (B) Relationship between FM and epididymal fat. n = 60, p < .0001 for both correlations.

Table 1. FM and Insulin-Mediated Glucose Uptake (Rd) According to Age. Rats Were Studied at 2 (n = 19), 4 (n = 20), 6 (n = 7), 12 (n = 7), and 18 (n = 7) Months of Age. FM and LBM Were Determined by Volume Distribution of 3H2O, and Rd was Determined by Hyperinsulinemic Euglycemic Clamp

<table>
<thead>
<tr>
<th>Age, months</th>
<th>FM, g</th>
<th>Rd, mg/kg LBM/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>32 ± 2</td>
<td>52 ± 2*</td>
</tr>
<tr>
<td>4</td>
<td>47 ± 4*</td>
<td>44 ± 3</td>
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<tr>
<td>6</td>
<td>59 ± 7*</td>
<td>42 ± 4</td>
</tr>
<tr>
<td>12</td>
<td>79 ± 9*</td>
<td>39 ± 5</td>
</tr>
<tr>
<td>18</td>
<td>101 ± 12*</td>
<td>39 ± 6</td>
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</table>

*p < .01 versus all others.

However, insulin-mediated glucose uptake expressed in terms of LBM decreases significantly between young (2 mo) and late adulthood (4 mo) and does not further decrease through aging.

Relationship between insulin-mediated glucose uptake and BW (Figure 2).—With simple regression analysis, BW was linearly and inversely correlated with the rate of glucose disappearance (Rd) when expressed in terms of kg BW (r = .814, p < .0001; data not shown). However, such an analysis would result in an underestimation of skeletal muscle glucose uptake in the older and more obese rats, because it does not take into account the low metabolic contribution of FM. When Rd was expressed in terms of kg LBM, it was still linearly and inversely correlated with BW, however, the correlation coefficient and significance were less robust (r = .678, p < .01; data not shown). The most significant and best fit model was achieved with a spline curve consisting of the two regression lines joined in a knot at 365 g with R^2 = .992 and p < .00001. R^2, which is defined as the regression of some of the squares over the total some of the squares (16), is an indicator of how well the spline curve is fitted by the data. For BW less than 365 g the slope was −.144X, but when BW was greater than 365 g the slope changed to −.003X. Simple regression line analysis reveals a significant inverse correlation between Rd and BW up to 365 g (r = −.814, p < .001). Thereafter, no significant change in Rd (r = −.095, p > .8; data not shown) with further increased BW.

Relationship between insulin-mediated glucose uptake and FM (Figure 3A).—Rd was inversely correlated with FM (r = −.706, p < .0001). However, a spline curve analysis best depicted the characteristic of this slope with the two lines intersecting at 53.8 g with R^2 = .992 and p < .00001 (with asymptotic 95% confidence interval of 46.3–61.3 g). Thus, an inverse relationship was observed between insulin-mediated glucose uptake and FM up to 53.8 g (r = −.715, p < .001) with no relationship at greater FM (r = −.089; p = .22).

Relationship between insulin-mediated glucose uptake and epididymal fat (Figure 3B).—Insulin-mediated glucose uptake was inversely correlated to FM (r = −.673, p < .001). With a spline curve analysis, a knot was determined at 5.80 g
Figure 3. (A) Relationship between FM and insulin-mediated glucose disposal. Spline knot analysis revealed a significant knot at 53.8 g \((p < .0001\) for the knot, \(n = 60\)). (B) Relationship between epididymal fat and insulin-mediated glucose disposal. Spline knot analysis revealed a significant knot at 5.80 g \((p < .0001, n = 60)\).

\[(R^2 = .994 \text{ and } p < .00001)\]. Thus, an inverse relationship was observed between insulin-mediated glucose uptake and epididymal fat up to 5.80 g \((r = -.767, p < .001)\) but with no relationship at greater FMs \((r = .242; p = .41)\).

When comparing rats weighing less than 365 g versus those weighing more, there were no differences in their plasma levels of insulin \(2,113 \pm 89\) and \(2,233 \pm 102\) pm), FFA \(.32 \pm .08\) and \(.33 \pm .09\) mM), glucose \((7.5 \pm 1.3\) and \(7.6 \pm 1.0\) mM), or in hepatic glucose production \((2.2 \pm .9\) and \(1.9 \pm .8\) mg/kg/min) during the insulin clamp. Glucose uptake averaged \(48.7 \pm 4.2\) mg/kg LBM/min in rats weighing <365 g and \(38.6 \pm 3.2\) mg/kg LBM/min in rats weighing >365 g \((p < .01)\).

Table 2. Basal Biochemical Characteristics of the Rats.

| Basal Plasma Levels of Glucose, Insulin, FFA, and Lactate in Rats With BW of <365 g \((n = 31)\) and >365 g \((n = 29)\). 
| Correlation \((r, p)\) Between BW and Plasma Levels of Glucose, Insulin, FFA, and Lactate, in the Rats Studied |
|---|---|---|---|---|
| Glucose, mM | Insulin, pM | FFA, mM | Lactate, mM |
| <365 g | 7.5 ± 1.3 | 282 ± 44 | .84 ± .12 | .47 ± .04 |
| >365 g | 7.6 ± 1.0 | 517 ± 62* | 1.06 ± .09* | .57 ± .06 |

\[\*p < .01 \text{ versus <365 g.}\]

Glycogen synthesis remained similar for the whole range of weights and ages.

Basal Biochemical Characteristics of Rats Weighing Less and More Than 365 g (Table 2)

Plasma insulin levels were \(\approx2\)-fold higher in rats with BW >365 g. Insulin levels were linearly correlated with BW, and did not plateau with increased weight (not shown). Plasma FFA levels were linearly correlated with BW and were higher by \(\approx25\%\) with BW >365 g. No change in plasma glucose or lactate levels were seen with increase in BW. There were no changes in these parameters when the rats >365 g were analyzed by age.

DISCUSSION

In this homogenous animal model, the correlation between insulin-mediated glucose uptake and body composition is bimodal. While insulin-mediated glucose uptake declines with increasing BW, FM, and epididymal fat, these correlations were saturable at a relatively young age (Figures 2, 3).

The hyperinsulinemic euglycemic clamp technique is the "gold standard" for the measurement of peripheral insulin action. While many studies demonstrated a decline in glucose utilization with age, results were often expressed in
In both animal and human models, FM may account for >30% of body weight but the efficiency of insulin-mediated glucose uptake in adipose tissue is much lower (=10%) than that of skeletal muscle (22). Thus, it is important to determine LBM in order to calculate the LBM-adjusted rates of insulin-mediated glucose uptake. Isotopically determined FM in rats has been previously shown to correlate with various determinants of body composition in rats (2,3,15). Such a correlation was present between epididymal fat and BW in our study (Figure 1). These correlations were also highly significant between BW and FM, % FM, and intraabdominal FM, and are in agreement with determination of carcass fat (23). To determine accurately the relationships between insulin responsiveness and body composition, we employed linear correlations and fitted exponential decays; however, the relationship with the best fit suggested two regression lines coming together at a point (intersection). Indeed, statistical analysis revealed that such a knot existed between insulin-mediated glucose uptake and BW, FM, and epididymal fat, suggesting a role for fat tissues in determining insulin-mediated glucose uptake. Epididymal fat is the largest i.p. FM, and its size correlated well with other visceral fat, particularly with the mesenteric fat (r = .996, p < .001), which is harder to accurately dissect and is also composed of blood vessels and nerves. Nevertheless, the relationship between the mesenteric fat and insulin-mediated glucose uptake generates a similar curve with a knot at 3.5 g (p < .001). Thus epididymal fat may be a marker not only for total FM but also for visceral fat, which is increased with aging (24) and has been suggested as a more metabolically important contributor to insulin resistance, than is subcutaneous obesity (25,26), in particular in aging (27). Because the rats that were studied were all post pubertal (by age, testicular descent, and by the appearance of balano posthitis groove), the differences observed in insulin action are related to changes that occur during aging rather than with maturation. In addition, caloric restriction is known to decrease FM and improve insulin action to the levels observed in young rats (28), further supporting the notion that FM determines insulin action.

This saturable relationship between obesity and insulin action is in agreement with a nonlinear correlation between the degree of obesity and insulin action (expressed per fat-free mass) made in Pima Indians (29). While the steep decline in insulin action with increasing FM occurred until it reached 28% of BW, a further increase in body fat was not accompanied by a further decline in insulin action. While the most important confounding factor in that study was the variation in exercise capacity, this was not a confounding factor in sedentary caged rats in this study. Efforts to confirm this observation in a Caucasian population may have failed because of the small sample size (29). The relationship between insulin-mediated glucose uptake and body mass index was inversely correlated (r = -.748); however, glucose uptake was expressed as the glucose infusion rate in terms of kg BW (30). This does not correct for large amounts of relatively inactive adipose tissue nor does it reflect the contribution of impaired suppression of hepatic glucose production in obesity to the total insulin-mediated glucose uptake. Yet the authors presented two distinct slopes relating to insulin action with increased BW, suggesting that their results may best be fitted by spline curve analysis.

It has been previously hypothesized that the decrease in glucose uptake may be determined by the chronically increased availability of such substrates as FFA and glycerol, which are often increased with obesity. Randle et al. (31) were the first to suggest that increased FFA availability will direct metabolism toward fat oxidation and away from glucose oxidation. Kim et al. (32) have recently demonstrated that glucose uptake decreased after 2 h of in vivo infusion of fatty acids. Furthermore, this decrease was accounted for by a decrease in glycolysis and not in glycogen synthesis, as is the case in our study. However a major decline in insulin-mediated glycogen synthesis was demonstrated in the insulin resistance of normoglycemic obese humans (33) and rats (2), and is considered impaired in type II diabetes mellitus. Furthermore such a defect is clearly developed with the initial accretion of fat after puberty (34). Thus, while decreased glycogen synthesis was not significantly decreased in our animals, it may still be impaired with increased FM. While significant differences were detected between plasma FFA levels under basal conditions, it should be pointed out that FFA levels were similar during insulin clamp studies. Other mechanisms that have been recently correlated between obesity and insulin-mediated glucose uptake are the secretion by fat cells of the bioactive peptide tumor necrosis factor-α (35), and the induction of insulin resistance by lipids through the glycosamine pathway (36).

Saturating effects of substrate and hormones in metabolism are well established. For example, increasing insulin levels over 2,000 pM in rats or 3,000 pM in humans will not trigger any further increase in glucose uptake, when the rate limiting step is at the postreceptor level (37). This example reflects pharmacological rather than physiological levels of effectors. While during most of evolution, humans presumably had a lower FM, the prevalence of obesity increases at a rate of 8% per decade in the last few decades, approaching >50% in adults over the age of 50 in the United States (4). It is possible that as a consequence of the current high levels of body fat, there is a saturating effect on skeletal muscle insulin-mediated glucose uptake. However, because basal plasma insulin levels increase with BW and do not plateau, this suggests that the insulin resistance further deteriorates with increased BW. This may be explained by our previous observation that the aging liver is resistant to the action of insulin proportionally to the increase in body weight (3), even when peripheral insulin action does not further decrease with aging (2).

These data in a rodent model demonstrated two major findings. (i) The effect of visceral and total FM on peripheral insulin action is saturable by mechanisms yet to be determined. (ii) Because this saturating effect occurred at a relatively young age, it demonstrates that aging per se is not associated with a further decrease in peripheral insulin sensitivity. However, the increases in visceral and total FM are typical of aging, subjecting the elderly population at risk for developing insulin resistance.
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