Central Role of Fatty Liver in the Pathogenesis of Insulin Resistance in Obese Adolescents

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OBJECTIVE — We evaluated the role of fatty liver in the alteration of insulin sensitivity and β-cell function in two groups of obese adolescents, differing in hepatic fat content (hepatic fat fraction [HFF]) but with similar intrabdominal intramyocellular lipid content (IMCL) and overall degree of obesity.

RESEARCH DESIGN AND METHODS — We studied 23 obese adolescents with high HFF (HFF >5.5%) and 20 obese adolescents with low HFF (HFF <5.5%), matched for age, Tanner stage, BMI z score, and percentages of body fat, visceral fat, and IMCL. All subjects underwent an oral glucose tolerance test and a two-step hyperinsulinemic-euglycemic clamp, magnetic resonance imaging and 1H nuclear magnetic resonance to assess abdominal fat distribution, HFF, and IMCL, respectively.

RESULTS — The high HFF group showed significantly lower whole-body insulin sensitivity index (P = 0.001) and estimates of insulin secretion (P = 0.03). The baseline hepatic glucose production (EGP) rate was not different between the two groups. Suppression of EGP was significantly lower (P = 0.04) in the high HFF group during low-dose insulin; no differences were observed during the second step. Baseline fatty acids, glycerol concentrations, and clamp suppression of glycerol turnover did not differ between the groups. During the second step, the glucose disposal rate was significantly lower (P = 0.01) in the high HFF group.

CONCLUSIONS — Fatty liver, independent of visceral fat and IMCL, plays a central role in the insulin-resistant state in obese adolescents.

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Fatty liver and insulin resistance

Table 1—Clinical characteristics of the study cohort

|                      | High liver fat content | Low liver fat content | P value |
|----------------------|------------------------|-----------------------|---------|
| n                    | 23                     | 20                    |         |
| Race (C/AA/H)        | 10/3/10                | 6/8/6                 | 0.1     |
| Sex (male 19/female 24) | 11/12                | 8/12                  | 0.6     |
| Tanner stage (II/III/IV) | 4/10/9               | 2/11/7                | 0.7     |
| NGT/IGT             | 6/17                   | 8/12                  | 0.3     |
| Age (years)         | 13.8 ± 0.52            | 13.4 ± 0.54           | 0.5     |
| BMI (kg/m²)         | 35.5 ± 1.19            | 35.7 ± 1.87           | 0.9     |
| BMI z score         | 2.33 ± 0.06            | 2.25 ± 0.09           | 0.8     |
| Surface area (m²)   | 1.92 ± 0.05            | 1.96 ± 0.07           | 0.6     |
| Fat mass (kg)       | 35.6 ± 1.78            | 38.9 ± 3.16           | 0.4     |
| Lean body mass (kg) | 50.4 ± 2.26            | 53.9 ± 2.56           | 0.4     |
| Body fat (%)        | 40.5 ± 1.20            | 39.9 ± 1.19           | 0.6     |
| Body fat distribution|                        |                       |         |
| Abdominal           |                       |                       |         |
| Visceral fat (cm²)  | 65.3 ± 3.7             | 61.1 ± 5.81           | 0.4     |
| Subcutaneous fat (cm²)| 514.3 ± 30.8         | 545.4 ± 54.4         | 0.6     |
| Liver               |                        |                       |         |
| HFF (%)             | 17.1 ± 2.37            | 1.11 ± 0.34           | <0.0001 |
| Muscle              |                        |                       |         |
| EMCL (% water)      | 1.68 ± 0.50            | 1.86 ± 0.44           | 0.5     |
| IMCL (% water)      | 1.17 ± 0.11            | 0.93 ± 0.11           | 0.2     |

Data are n or means ± SEM. AA, African American; C, Caucasian; EMCL, extramyocellular lipid content; H, Hispanic; IGT, impaired glucose tolerance; NGT, normal glucose tolerance.

and 12 female; 10 Caucasians, 3 African Americans, and 10 Hispanics) in the high liver fat content group and 20 subjects (8 male and 12 female; 6 Caucasians, 8 African Americans, and 6 Hispanics) in the low liver fat content group. The two groups had different hepatic fat content but similar visceral fat and IMCL (Table 1). Before the formation of the two groups, we were unaware of potential differences in any of the outcomes measured. Thirty percent of the subjects were reported on previously (2).

The study protocol was approved by the institutional review board of Yale University School of Medicine. Written parental consent and child assent were obtained before the study.

Metabolic studies

OGTT and estimates of insulin sensitivity and β-cell function. Subjects were studied at the Yale Clinical Center Investigation at 8:00 A.M., after a 10-h overnight fast. All had an OGTT (7).

Estimates of insulin sensitivity were calculated using homeostasis model assessment of insulin resistance (HOMA-IR), defined by fasting insulin (micromunits per milliliter) × fasting glucose (milligrams per deciliter) (7) and whole-body insulin sensitivity index (WBISI), based on mean values of insulin (micromunits per milliliter) and glucose (milligrams per deciliter) obtained from the OGTT and the corresponding fasting values (7). The insulinogenic index (IGI), which represents early-phase insulin secretion, was calculated as IGI = Δ insulin (0–30 min)/Δ glucose (0–30 min) in micromunits per milliliter divided by the Δ glucose (0–30 min) in milligrams per deciliter (8). The disposition index (DI) was calculated as the product of the IGI and the WBISI, based on the curvilinear relation of these OGTT-derived variables, as described by our group in obese children and adolescents (8).

Areas under the curve (AUC) of insulin and glucose were calculated by the trapezium rule (9). Overall glucose-stimulated insulin secretion was calculated as the incremental AUC ratio (Δ AUCinsulin/Δ AUCglucose) (10).

Hyperinsulinemic-euglycemic clamp. After an overnight fast, at 7:00 A.M., two intravenous catheters, one for blood sampling and one for infusion of glucose, insulin, and tracers, were inserted in the antecubital vein of each arm after local lidocaine infiltration. The sampling arm was kept in a heated box for arterIALIZATION of blood. Whole-body insulin sensitivity was measured by two-step euglycemic clamp, by infusing insulin as a primed continuous infusion at 4 and 80 mU·m⁻²·min⁻¹. Each step lasted 2 h. A primed continuous infusion of 6,6-deuterium glucose and of ²H₅-glycerol were used to quantify the effects of insulin on glucose and glycerol turnover. To maintain the plasma enrichment of ²D-glucose constant at baseline value throughout the clamp, we used the Hot GINF method, as previously reported (5). Arterialized blood samples were collected every 10 min during the last 30 min of the baseline period and during each step of the clamp for measurement of glucose and glycerol enrichments, hormones, and substrates.

Imaging studies

Abdominal MRI and total body composition (dual-energy X-ray absorptiometry). Multislice abdominal MRI studies were performed on a Siemens Sonata 1.5 T system (11). Total body composition was measured by dual-energy X-ray absorptiometry with a Hologic scanner (Hologic, Boston, MA).

¹H-NMR spectroscopy: IMCL measurement. Localized ¹H NMR spectra of the soleus muscle were acquired on a 4T Biospec system (Bruker Instruments, Bil- lenica, MA) (12).

Fast-MRI: liver fat content. Liver fat content (hepatic fat fraction [% HFF]) was measured by MRI using the two-point Dixon method as modified by Fishbein et al. (13) and reported by Cali et al. (2).

Analytical procedures and calculations. Plasma glucose was determined with a YSI 2700 analyzer and plasma free fatty acids were determined by a colorimetric method. Plasma insulin, adiponectin, and leptin levels were measured with a radioimmunoassay (Linco, St. Charles, MO) and plasma C-peptide levels were measured using a kit (Diagnostic Products, Los Angeles, CA). Analysis of enrichments of ²D-glucose and ²H₅-glycerol in plasma was done as described elsewhere (5).

The glucose infusion rates were calculated during the last 30 min of each step of the clamp. Endogenous (mainly hepatic) glucose production (EGP) and glycerol turnover at baseline and during the two steps of the insulin clamp, along with the clamped glucose disposal rates, were calculated as reported previously (5). The percent suppression of EGP and glycerol turnover during low- and the high-dose insulin were used as indexes of the sensitivity of EGP and peripheral lipolysis to insulin. The percent suppression
of C-peptide levels during the two steps of the insulin clamp was used as index of the degree of endogenous insulin secretion.

Insulin clearance during the two steps of the clamp was calculated by dividing the rate of insulin infusion by the mean steady-state insulin level during the insulin infusion (14). In addition, to correct insulin contribution for the proportion of the residual endogenous insulin secretion, we also calculated the metabolic clearance rate of insulin, taking into account the concentrations of C-peptide before and at the end of each step of the clamp, as reported by Ferrannini et al. (15).

Statistical analyses
To test group differences in clinical and metabolic variables we used the Mann-Whitney U test. Differences in sex and race prevalence between groups were assessed by χ² test. Correlations were assessed by Spearman rank correlation.

Sample size calculations were based on the ability to detect differences in insulin sensitivity between subjects with high and low liver fat content. From an earlier study in obese adolescents, we observed a standardized difference of 1.0 (d = 1.00) in insulin resistance as measured by the crude HOMA-IR index (2). We expected similar differences in our current study for outcomes derived from the OGTT and the hyperinsulinemic-euglycemic clamp. With use of a two-sided Mann-Whitney U test, group sample sizes of 23 and 20 achieve 88% power at α = 0.05 to detect a standardized difference of 1.0 (d = 1.00) in insulin sensitivity.

Statistical analyses were performed with SPSS (16.0 for Windows; SPSS, Chicago, IL). All data are expressed as means ± SEM. P < 0.05 was considered statistically significant.

RESULTS
Anthropometric characteristics and body distribution
The sex, race, age distribution, BMI, BMI z score, surface area, fat mass, percent body fat, and lean body mass were similar between the groups. By design, the two groups were significantly discordant for percent HFF (P < 0.0001) but had similar visceral fat and subcutaneous fat amounts, as well as intramyocellular and extramyocellular muscle lipid contents (Table 1).

### Table 2—Metabolic characteristics of the study cohort

|                        | High liver fat content | Low liver fat content | P value |
|------------------------|------------------------|-----------------------|---------|
| n                      | 23                     | 20                    |         |
| Fasting glucose (mg/dl)| 96.7 ± 1.90            | 97.3 ± 2.09           | 0.8     |
| 2-h glucose (mg/dl)   | 140.6 ± 4.47           | 128.3 ± 6.95          | 0.2     |
| Fasting insulin (µU/ml)| 33.6 ± 2.54            | 26.6 ± 3.08           | 0.03    |
| 2-h insulin (µU/ml)   | 256.7 ± 32.9           | 174.5 ± 37.8          | 0.06    |
| Fasting C-peptide (pmol/ml)| 1,271.8 ± 81.3    | 954.6 ± 76.3          | 0.01    |
| HOMA-IR (dl · µU⁻¹)/(ml · mg⁻¹)| 10.9 ± 1.14     | 6.48 ± 0.73           | 0.002   |
| WBISI (dl · ml⁻¹)/(mg · µU⁻¹)| 1.0 ± 0.10     | 1.98 ± 0.27           | 0.001   |
| IG (dl · µU⁻¹)/(ml · mg⁻¹) | 3.5 ± 0.27      | 4.6 ± 0.65            | 0.1     |
| Disposition index [(dl · ml⁻¹)/(ml · mg⁻¹)]/[(dl · µU⁻¹)/(mg · µU⁻¹)] | 3.9 ± 0.38 | 6.2 ± 0.83 | 0.03 |
| Δ AUCinsulin/Δ AUCglucose (dl · µU⁻¹)/(ml · mg⁻¹) | 4.6 ± 0.50 | 4.8 ± 0.92 | 0.6 |
| Adiponectin (mg/l)    | 6.6 ± 0.61            | 12.5 ± 1.60           | 0.004   |
| Interleukin-6         | 2.47 ± 0.39           | 1.99 ± 0.25           | 0.8     |
| Leptin (µg/l)         | 33.7 ± 3.05           | 32.0 ± 3.74           | 0.6     |

Lipid profile
- Plasma fatty acids (µmol/l) 623.5 ± 42.0 553.5 ± 38.1 0.3
- Low-dose insulin infusion 436.1 ± 38.8 390.8 ± 35.6 0.4
- High-dose insulin infusion 109.6 ± 9.6 97.8 ± 11.6 0.3
- Plasma glycerol (µmol/l) 89.4 ± 5.29 82.3 ± 4.75 0.4
- Total cholesterol (mg/dl) 161.7 ± 7.09 152.5 ± 5.70 0.4
- LDL cholesterol (mg/dl) 91.8 ± 6.22 90.5 ± 4.84 0.9
- HDL cholesterol (mg/dl) 41.7 ± 1.80 46.3 ± 2.0 0.1
- Triglycerides (mg/dl) 116.4 ± 12.3 84.9 ± 8.16 0.05
- Liver enzymes (units/l)
  - Alanine transaminase 30.6 ± 4.41 15.4 ± 1.89 <0.0001
  - Aspartate aminotransferase 24.6 ± 1.34 19.7 ± 1.60 0.008
- Systolic blood pressure (mmHg) 119.6 ± 2.65 117.5 ± 2.81 0.4
- Diastolic blood pressure (mmHg) 70.9 ± 1.50 68.5 ± 2.44 0.4

Data are n or means ± SEM.

Estimates of insulin sensitivity and secretion
Fasting glucose concentrations were similar between the two groups; whereas the 2-h glucose levels tended to be higher in the high liver fat content group. The high liver fat content group showed significantly higher levels of fasting insulin (P = 0.03) and C-peptide levels and a trend for higher 2-h insulin concentrations (P = 0.06). The high liver fat content group had a significantly higher level of HOMA-IR (P = 0.002) and lower WBISI (P = 0.001) than the low liver fat content group (Table 2).

Although the high liver fat content group showed a trend for a lower first-phase insulin secretion (IGI) (P = 0.1), in agreement with previous reports (10,16) it was not statistically significant. No differences were observed in the incremental AUC ratio. However, when we calculated the disposition index, it was significantly lower in the high liver fat content group (P = 0.03).

### Metabolic characteristics
Fasting leptin concentrations were similar in the two groups, reflecting their equivalent amounts of adiposity. In contrast, plasma adiponectin concentrations were significantly lower in the high liver fat content group (P = 0.004) (Table 2). Interleukin-6 levels were not significantly different between the groups. Alanine transaminase and aspartate aminotransferase values and triglyceride levels were significantly higher in the high liver fat content group. Both groups had similar systolic and diastolic blood pressure values.

Measure of insulin sensitivity during the clamp
During the two steps of the clamp, plasma glucose concentrations were maintained at baseline values, and similar steady-state plasma insulin concentrations were achieved in both groups during the last 60 min of each step (first step 39.6 ± 3.0 vs. second step 39.6 ± 3.0).
plasma glycerol concentrations (Table 2) and basal glycerol turnover (14.9 ± 0.77 vs. 16.2 ± 0.81 mg·m⁻²·min⁻¹, in the high and low liver fat content groups, respectively) did not differ between the groups. In the high liver fat content group, we observed a trend for a lower percentage of systemic lipolysis suppression during the low-dose insulin infusion (P = 0.1), whereas during the high-dose insulin infusion suppression of lipolysis was similar in both groups (Fig. 1).

**Muscle**

During the high-dose insulin infusion, the peripheral glucose disposal rate was significantly lower (P = 0.01) in the high liver fat content group (Fig. 1); as expected no differences were seen between groups at the low insulin dose. In addition, we found a negative correlation between percent HFF and peripheral clamped glucose disposal rate during the high-dose insulin phase (r = -0.37, P = 0.02).

**CONCLUSIONS**— In the present study, we found that obese adolescents with high liver fat content, independent of visceral fat and IMCL, had 1) impaired insulin action in the liver and in muscle, 2) early defects in β-cell function, as shown by the low disposition index and 3) low adiponectin levels, and 4) a trend toward lower suppression of glycerol turnover during the low insulin dose.

These results suggest that the liver has a central role in the complex phenotype of the insulin resistance state in obese adolescents with fatty liver, as was also previously shown by Perseghin et al. (17). The current study, however, cannot prove causality. Unlike visceral fat and IMCL, the accumulation of fat in the liver may not be just a simple marker of insulin resistance. Previous studies from our group (3,5) showed that both visceral fat and IMCL are related to insulin sensitivity in obese adolescents. However, it should be noted that in those earlier studies we did not measure liver fat; therefore, we ignored an important ectopic fat. Furthermore, it is well known that visceral and intrahepatic fat are related to each other and that both are linked to the same metabolic abnormalities. In fact, in previous studies we found that alterations in glucose and lipid metabolism were seen with increases in both hepatic fat and visceral fat (2,3). Nevertheless, because of the simultaneous accumulation of fat in the liver, visceral depot, and skeletal muscle, we were unable to differentiate the individual effect of each one of these on putative defects in glucose and lipid metabolism. The results from this study do not prove that visceral fat and IMCL are not related to insulin resistance; rather they mainly suggest that by accounting for visceral fat and IMCL, hepatic fat is more than a simple marker of insulin resistance.

Results from our study are consistent with those of Fabbrini et al. (18), showing in adult obese subjects that intrahepatic fat, not visceral fat, is linked with metabolic complications of obesity. We expand on this theme by showing not only that the visceral fat may just be a marker of insulin resistance but also that IMCL may also be an innocent bystander. In addition, in our study it should be noted that the groups had relatively low visceral content as opposed to the study by Fabbrini et al. (18), in which the visceral volume was very high in absolute terms, thus making it difficult to ultimately exclude any influence of the visceral component on metabolic complications of obesity. Furthermore, given the young age of our subjects and the short duration of obesity, our results indicate that the in-
volvement of the liver in the pathogenesis of insulin resistance and its associated sequelae may be occurring very early.

What causes fat to accumulate excessively in the liver is not entirely clear. Inability of the subcutaneous adipose tissue to store triglycerides (3,19), such as in lipodystrophy, may be a possible cause (19). More recently, another mechanism has been proposed for ectopic fat accumulation, which involves alterations in the regulation of FFAs from plasma by CD36, a fatty acid transport protein (18).

The liver plays a central role in the regulation of glucose, fatty acid, and amino acid metabolism (20). It is the main source of hepatic glucose production and an important site of fatty acid disposal and insulin degradation (20). Most of these important functions have been found to be impaired in the presence of fatty liver in adults (18,21). In particular, the study of Gastaldelli et al. (22) clearly indicates that increased liver fat is associated with hepatic insulin resistance in obese adults with and without diabetes, whereas excess visceral fat affects primarily gluconeogenesis. Because EGP and lipolysis are more sensitive to suppression by insulin than stimulation of muscle glucose uptake, we used a low insulin infusion rate (4 mU • m⁻² • min⁻¹) to accurately quantify interindividual variations in hepatic insulin sensitivity and lipolysis. We found in obese adolescents with fatty liver that alterations in the sensitivity to insulin were clearly involving both liver and peripheral muscle insulin sensitivity. Although we were unable to show significant differences in suppression of lipolysis between the two groups, the high liver fat content group showed a lower trend of suppression of lipolysis and higher trend of FFA concentrations. However, FFA turnover may not necessarily be a reliable indicator of lipolysis because FFAs can be reesterified within adipose tissue; therefore, the possibility exists that the higher concentrations of FFAs are due to reduced reesterification in the high liver fat content group.

In this study, the presence of normal fasting and insulin-stimulated glycerol turnover and FFAs in the high liver fat content group argues against an FFA-induced insulin resistance and against a primary role of the adipose tissue. On the other hand, it should be noted that the high liver fat content group was less tolerant to glucose, suggesting that higher plasma glucose could push de novo lipo-
genesis and therefore contribute to the availability of nonsystemic fatty acids.

As expected, however, both the EGP and lipolysis were suppressed to the same extent during the high-dose insulin step. In contrast, this high dose was able to bring out the clear difference between the two groups in muscle insulin sensitivity.

Although the results of the present study are consistent with those reported by Klein and colleagues (23,24) in obese adolescents with fatty liver, it should be noted that the obese adolescents in their study had a liver fat content much greater (~26%) than those in our study (~15%). Thus, even at modest levels of hepatic fat content we found and established a global degree of insulin resistance involving the liver, muscle, and to some extent adipose tissue.

Although it was previously reported in adults that liver fat is associated with decreased insulin clearance (14,25), in the present study we were unable to find differences between the two groups. The reason for the lack of difference is not clear. Factors that may be implicated are the degree and duration of steatosis and sample size.

Our study also provides some evidence regarding β-cell function in the context of fatty liver. Using the disposition index, an estimate of β-cell function weighted by insulin sensitivity, we found that it was reduced by 30% in the group with fatty liver (Fig. 1), suggesting that the β-cells are unable to adequately compensate for the ambient level of insulin resistance and therefore are very vulnerable, thus increasing susceptibility to type 2 diabetes. Of note, the subjects with fatty liver had elevated 2-h glucose during the OGTT, which indicates an imminent prediabetic state.

A few limitations are worth noting here. First, given the cross-sectional nature of our study we were unable to prove causality, because it is possible that fatty liver is a primary factor in insulin resistance or that insulin resistance causes fatty liver. Second, we do not have a group of subjects who were matched for liver fat but differed for visceral fat and IMCL.

In summary, by accounting for visceral fat and IMCL, intrahepatic fat accumulation is more than a simple marker of insulin resistance in obese adolescents, being associated with impaired insulin sensitivity at the level of the liver, muscle, and adipose tissue.

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