Accretion of visceral fat and hepatic insulin resistance in pregnant rats

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Submitted 4 September 2007; accepted in final form 5 December 2007


Insulin resistance (IR) is a hallmark of pregnancy. Because increased visceral fat (VF) is associated with IR in nonpregnant states, we reasoned that fat accretion might be important in the development of IR during pregnancy. To determine whether VF depots increase in pregnancy and whether VF contributes to IR, we studied three groups of 6-mo-old female Sprague-Dawley rats: 1) nonpregnant sham-operated rats (Nonpreg; n = 6), 2) pregnant sham-operated rats (Preg; n = 6), and 3) pregnant rats in which VF was surgically removed 1 mo before mating (PVF; n = 6). VF doubled by day 19 of pregnancy (Nonpreg 5.1 ± 0.3 g, Preg 10.0 ± 1.0 g, P < 0.01), and PVF− had similar amounts of VF compared with Nonpreg (PVF− 4.6 ± 0.8 g). Insulin sensitivity was measured by hyperinsulinemic-euglycemic clamp in late gestation in chronically catheterized unstressed rats. Glucose IR (mg·kg−1·min−1) was highest in Nonpreg (19.4 ± 2.0), lowest in Preg (11.1 ± 1.4), and intermediate in PVF− (14.7 ± 0.6; P < 0.001 between all groups). During the clamp, Nonpreg had greater hepatic insulin sensitivity than Preg [hepatic glucose production (HGP); Nonpreg 4.5 ± 1.3, Preg 9.3 ± 0.5 mg·kg−1·min−1, P < 0.001]. With decreased VF, hepatic insulin sensitivity was similar to nonpregnant levels in PVF− (HGP 4.9 ± 0.8 mg·kg−1·min−1). Both pregnant groups had lower peripheral glucose uptake compared with Nonpreg. In parallel with hepatic insulin sensitivity, hepatic triglyceride content was increased in pregnancy (Nonpreg 1.9 ± 0.4 vs. Preg 3.2 ± 0.3 mg/g; P < 0.05). Accretion of visceral fat is an important component in the development of hepatic IR in pregnancy, and accumulation of hepatic triglycerides is a mechanism by which visceral fat may modulate insulin action in pregnancy.

PREGNANCY IS A PERIOD OF SIGNIFICANT insulin resistance. Historically, placental hormones including placental growth hormone (HPGH), human placental lactogen (hPL), progesterone, and cortisol have been implicated, and an association between placental hormones and insulin resistance has been demonstrated (3, 4, 12, 20, 27, 31, 32). However, more recent evidence is conflicting (reviewed in Ref. 11) and suggests that, while all of these hormones play important roles in the physiological adaptation to pregnancy, only HPGH has emerged as a major factor in the development of insulin resistance. Kirwan et al. (22) introduced a paradigm shift from classical placental hormones as the primary mediators of insulin resistance when they found that plasma concentration of TNF-α, a cytokine, was most predictive of insulin sensitivity compared with placental hormones in women with normal glucose tolerance and those with gestational diabetes. Although it is also expressed in the placenta, TNF-α is one of many biologically active substances derived from adipose tissue that have been implicated as mediators of insulin resistance in nonpregnant states.

Adipose tissue is no longer considered an inert depot of energy storage but rather a metabolically active tissue with endocrine, paracrine, and autocrine functions. Central obesity is considered to be an essential component of the metabolic syndrome (1), and increased visceral fat is associated with decreases in peripheral and hepatic insulin sensitivity (5, 9, 14). Surgical extraction of visceral fat has been shown to reverse insulin resistance in young obese and old male rodents (13) and to delay the onset of diabetes in Zucker rats, a model of progressive obesity and diabetes (13). During pregnancy, maternal body fat increases on average >3 kg (19), with indirect evidence to suggest that visceral fat increases in particular (21). Furthermore, a significant negative correlation exists between fat mass and insulin sensitivity in late pregnancy (25).

The precise role of the placenta and visceral fat in the many metabolic changes that occur during pregnancy is unclear, but increasing consideration has been given to the role of adipose tissue lipolysis and adipokines in the development of insulin resistance (2). We sought to determine whether a significant amount of visceral fat accumulates during pregnancy in a rodent model. To determine whether decreased visceral fat accrual would alter insulin sensitivity, we performed euglycemic-hyperinsulinemic clamps in chronically catheterized rats in which visceral fat had been surgically removed before pregnancy. Furthermore, we explored the potential mechanisms through which visceral fat might modulate insulin action.

MATERIALS AND METHODS

These study protocols received review and approval and were carried out in accordance with the guidelines and regulations of the Albert Einstein College of Medicine Animal Care and Use Committee. To determine the extent of accretion of visceral fat (VF) and its impact during pregnancy, three groups of age-matched adult female Sprague-Dawley (SD) rats were studied: 1) nonpregnant virgin sham-operated rats (n = 6), 2) pregnant sham-operated rats (n = 6), and 3) pregnant females, in which VF was surgically removed 1 mo before mating (n = 6). Rats were housed in individual cages and subjected to standard light (6 AM to 6 PM) and dark (6 PM to 6 AM) cycles.

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They were fed regular rat chow ad libitum consisting of 64% carbohydrate, 30% protein, and 6% fat.

**VF removal and sham operations.** Rats in all groups were anesthetized (pentobarbital sodium, 50 mg/kg body wt), and surgeries were performed at -16 wk of age. At 16 wk of age, SD rats have reached adult body weight, and age-related increases in VF are minimal. In the PVF- group, all visible VF (parametrial and perinephric) was surgically excised through a vertical midline incision. The mesentery fat was left intact because of the high morbidity associated with its removal. The amount of VF removed was ~10–20% of total fat mass (13). For sham operations, the abdominal cavity was incised, and the VF was mobilized from its surrounding tissues but not removed. Clinical features of recovery were monitored after surgery with particular attention to food intake and body weight changes.

**Mating.** In the pregnant groups, females were mated approximately 4 wk after surgery. The male and female were housed together overnight, and the female was examined each morning for evidence of copulation. The day that the ejaculatory plug was visualized on the female’s vulva was considered day 1 of a 22-day gestation.

**Catheter placement.** Four to six days before the in vivo clamp study (days 13–15 in the pregnant groups), rats were anesthetized by inhalation of methoxyflurane, and indwelling catheters were inserted in the right internal jugular vein and in the left carotid artery as previously described (17). Recovery was continued until body weight was within 3% of the preoperative weight in nonpregnant females and until body weight exceeded the preoperative weight in pregnant females (~4–6 days). Chronically catheterized rats were studied on day 19 in the pregnant groups ~6 h after their last feeding while awake, unrestrained, and unstressed.

**Euglycemic-hyperinsulinemic clamp protocol.** Each group received a primed-continuous infusion (0.5 μCi/min bolus, 0.05 μCi/min maintenance) of high-performance liquid chromatography-purified [3-3H]glucose throughout the study to determine glucose fluxes. A primed-continuous infusion of insulin (3 mU·min−1·kg−1) was given to acutely increase and then maintain high, but physiological, plasma insulin levels. Somatostatin (1.5 μg·kg−1·min−1) was infused to prevent endogenous insulin secretion. A variable infusion of 25% dextrose was adjusted periodically to clamp the plasma glucose concentration in all groups at basal level (~5.7 mmol/L) for nonpregnant females. Blood was sampled through the venous catheter at times 0, 30, 60, 70, 80, 90, 95, 100, 105, 110, 115, and 120 during the 120 min of the study. At the end of all clamp studies, rats were killed using pentobarbital sodium (100 mg/kg body wt) intravenously. The abdomen was quickly opened, and organ and tissue samples were freeze clamped in situ with aluminum tongs precooled in liquid nitrogen, weighed, and stored in -80°C for later in vitro analysis. The uterus was removed, and pups were counted and weighed.

Under steady-state conditions for plasma glucose, the rate of glucose disappearance (Ra) is equal to the rate of glucose appearance (Rg). Ra was determined by the infusion rate/specific activity for plasma [3H]glucose during the last 30 min of the clamp during steady-state conditions. Plasma [3H]glucose specific activity was measured in duplicate in the supernatants of Ba(OH)2 and ZnSO4 precipitates (Somogyi procedure) of plasma samples (20 μl) after they were evaporated to dryness to eliminate tritiated water. The hepatic glucose production (HGP) during the clamp was calculated as the difference between the tracer-derived Ra and the glucose infusion rate.

Plasma glucose was measured by the glucose oxidase method (Glucose Analyzer II; Beckman Instruments, Palo Alto, CA), and plasma insulin was measured by radioimmunoassay using rat insulin standards. Plasma nonesterified fatty acid (free fatty acids; FFA) concentrations were determined by an enzymatic method with an automated kit according to the manufacturer’s specification (Waco Pure Chemical, Osaka, Japan). TNF-α, interleukin-6 (IL-6), adiponectin, leptin, and plasminogen activating inhibitor-1 (PAI-1) activities were measured using standardized kits as previously described (10).

Triglyceride content was measured by triglyceride kit (GPO-Trinder; Sigma Diagnostics, St. Louis, MO). Frozen liver tissue (200 mg) was homogenized in 4 ml of 2:1 chloroform-methanol. The solution was vortexed and filtered through Sharkskin. After addition of 0.2 ml of 0.58% NaCl solution, the filtrate was centrifuged at 1,000 rpm for 5 min. The upper phase was aspirated. The chloroform phase was further analyzed. The sample was evaporated in chloroform to a volume of <1 ml. A final volume of 1 ml was made with the addition of chloroform. Ten to twenty microliters of chloroform-containing samples, water, or standard solution of glycerol were added to the cuvettes. One milliliter of reagent was added. After incubation at 30°C for 10 min, the samples were read in a spectrophotometer at 540 nm.

In *vitro* analysis. Total RNA was isolated from placenta and visceral adipose tissue obtained from individual rats in the pregnant control group as described previously (10). Quantification of expression of fat-derived peptides by real-time PCR was performed for transcript confirmation using equal amounts of total RNA from individual rats as starting material. Annealing temperature and number of cycles depended on the individual transcript being amplified. Adipokine expression (i.e., TNF-α, PAI-1, IL-6, resistin, adiponectin, and leptin) was measured as previously described (10). GAPDH, a “housekeeping” gene, was measured by real-time PCR and used to account for any tissue degradation, which could alter peptide expression.

**Statistical analysis.** All values are presented as means ± SE. The significance of group differences was evaluated using repeated measures ANOVA for multiple comparisons. The significance of the results of quantitative real-time PCR (fold expression of any gene over GAPDH) was evaluated by the Kruskal-Wallis test.

**RESULTS**

Three groups of 6-mo-old female SD rats were studied: 1) nonpregnant sham-operated rats (Nonpreg; n = 6), 2) pregnant sham-operated rats (Preg; n = 6), and 3) pregnant rats in which VF was surgically removed 1 mo before mating (PVF-; n = 6). Nonpreg, Preg, and PVF- were studied using hyperinsulinemic-euglycemic clamps. The mean age at the time of examination was similar for all of the groups (Table 1). There was no statistically significant difference in weight between the pregnant groups (Preg 362.1 ± 15.6 g, PVF- 360.7 ± 19.4 g). VF was increased by twofold in the pregnant controls (10.0 ± 1.0 g) compared with the nonpregnant controls (5.1 ± 0.3 g). Although the PVF- group had all visible perinephric and parametrial fat resected, its remnants grew to a similar volume as that found in the nonpregnant group (PVF- 4.6 ± 0.8 g).

Pregnancy characteristics were similar in the pregnant groups; there was no significant difference in maternal weight gain, number of pups per litter, or the mean pup weight at day 19 of gestation.

<table>
<thead>
<tr>
<th>Table 1. Body composition and pregnancy characteristics</th>
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<tbody>
<tr>
<td>Nonpregnant, mean (±SE)</td>
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<tr>
<td>Age, wk</td>
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<tr>
<td>Body weight, g</td>
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<tr>
<td>VF, g</td>
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<tr>
<td>Maternal weight gain, g</td>
</tr>
<tr>
<td>No. of pups/litter</td>
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<td>Apgar score, g</td>
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Nonpregnant, nonpregnant sham-operated rats; Pregnant, pregnant sham-operated rats; Pregnant VF-, pregnant rats in which visceral fat (VF) was surgically removed 1 mo before mating. *P < 0.05 compared with Nonpregnant. †P < 0.05 compared with Pregnant.
Plasma insulin levels were similar in the three study groups at 120 min of the hyperinsulinemic clamp study (Nonpreg 66.1 ± 7.2, Preg 62.8 ± 6.3, PVF− 59.9 ± 3.5 μU/ml; P = not significant (NS)). Glucose infusion rate (GIR) was highest in Nonpreg (19.4 ± 2.0 mg·kg\(^{-1}\)·min\(^{-1}\)), lowest in Preg (11.1 ± 1.4 mg·kg\(^{-1}\)·min\(^{-1}\)), and intermediate in the PVF− group (14.7 ± 0.6 mg·kg\(^{-1}\)·min\(^{-1}\); P < 0.001 between all groups) (Fig. 1). Decreased accretion of VF in pregnancy improved overall insulin sensitivity, although not quite to the level seen in the nonpregnant group. HGP in Nonpreg was 5.3 ± 1.4 mg·kg\(^{-1}\)·min\(^{-1}\), while Preg had significantly higher HGP during the clamp (9.3 ± 0.5 mg·kg\(^{-1}\)·min\(^{-1}\); P < 0.01) with similar insulin levels. With a decreased amount of VF, the groups had significantly lower peripheral glucose uptake (Preg 14.7 ± 1.4 meq/ml; P < 0.01 compared with Nonpreg; Table 2). After 120 min of hyperinsulinemia, FFA remained significantly increased in the pregnant group (Nonpreg 0.5 ± 0.2 vs. Preg 1.5 ± 0.1 meq/ml; P < 0.05). Hepatic triglyceride content was also increased in pregnancy (Nonpreg 1.9 ± 0.4, Preg 3.2 ± 0.3 mg/g; P = 0.06), although it did not quite reach statistical significance. In PVF−, FFA was similar to that in Nonpreg under both basal and hyperinsulinemic conditions (PVF− 2.0 ± 0.2 basal and 1.0 ± 0.2 meq/ml at 120 min; P = NS). However, the percentage of insulin-induced suppression of FFA was not different between the pregnant groups. PVF− rats had significantly lower hepatic triglyceride compared with Preg rats. (PVF− 1.3 ± 0.4 mg/g; P < 0.05 compared with Preg). These data demonstrate that, in parallel with increased hepatic insulin resistance, there is an increase in the accumulation of hepatic triglyceride in pregnancy, which may be mediated through the accretion of VF.

Next, we sought to examine plasma and tissue expression of several adipokines associated with insulin resistance. Plasma levels of TNF-α, PAI-1, and leptin were significantly increased in both pregnant groups compared with nonpregnant controls (Table 3). No change was seen in plasma levels of IL-6 or adiponectin during pregnancy or with the removal of VF. To aid in determining the source of circulating peptides, we compared visceral adipose tissue and placental expression of the adipokines in the pregnant control rats. Tissue from the PVF− group was not used because the surgical manipulation may have altered gene expression. Placental tissue expressed significantly more PAI-1 and resistin compared with visceral adipose tissue. IL-6 expression was significantly greater in visceral adipose compared with placenta. No statistical difference was seen in expression of TNF-α, leptin, or adiponectin.

**DISCUSSION**

We present evidence that insulin resistance in pregnant rodents is associated with changes that occur in maternal body fat distribution, specifically the increase in visceral adiposity. Our data show that pregnancy is marked by a significant increase in accretion of visceral fat. Furthermore, we used a model of surgical removal of visceral fat in the preconception period to effectively decrease visceral fat in late gestation and revealed a complete restoration of hepatic insulin sensitivity. Although the decrease in visceral fat did not completely reverse pregnancy-related changes in insulin action (as determined by GIR), this novel finding demonstrating the importance of visceral fat in the hepatic insulin resistance of pregnancy has not been previously appreciated. We demonstrate the association between progressive maternal visceral adiposity and hepatic insulin resistance during pregnancy.

A reduction in insulin-induced suppression of HGP in pregnancy has been found in some animal models (16, 29) and human studies (7, 35) but not in others (8, 24). Many of the...
differences in the findings stem from the varying methodologies used, including the degree of hyperinsulinemia and the plasma concentration at which the glucose was clamped. In this study, we used somatostatin to suppress endogenous insulin production and achieved similar levels of physiological hyperinsulinemia in all groups. In pregnancy, the ability of insulin to suppress HGP is dependent to some extent on plasma glucose levels (29), and fasting glucose concentration is lower in pregnant animals compared with nonpregnant animals (24). Therefore, we chose to clamp the glucose at similar levels in all groups and used fasting concentration for nonpregnant females (∼5.7 mmol/l) as the target level to avoid the counterregulatory effects of relative hypoglycemia in the nonpregnant rats. This glucose level in pregnant animals is within a physiological range (not hyperglycemia) and most likely led to an increase in glucose uptake by the fetal unit with minimal, if any, effect on range (not hyperglycemia) and most likely led to an increase in glucose metabolism, FFA, and hepatic TG content.

**Table 2. Metabolic characteristics, FFA, and hepatic TG content**

<table>
<thead>
<tr>
<th></th>
<th>Nonpregnant, mean (±SE)</th>
<th>Pregnant, mean (±SE)</th>
<th>Pregnant VF−, mean (±SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose, mmol/l</td>
<td>5.7 (±0.3)</td>
<td>5.9 (±0.2)</td>
<td>5.7 (±0.2)</td>
</tr>
<tr>
<td>Insulin, μU/ml</td>
<td>66.1 (±7.2)</td>
<td>62.8 (±6.3)</td>
<td>59.9 (±3.5)</td>
</tr>
<tr>
<td>Basal FFA, meq/ml</td>
<td>1.7 (±0.04)</td>
<td>3.2 (±0.3)</td>
<td>2.0 (±0.2)</td>
</tr>
<tr>
<td>FFA at 120 min, meq/ml</td>
<td>0.5 (±0.2)</td>
<td>1.5 (±0.1)</td>
<td>1.0 (±0.2)</td>
</tr>
<tr>
<td>Hepatic TG content, mg/g</td>
<td>1.9 (±0.4)</td>
<td>3.2 (±0.3)</td>
<td>1.3 (±0.4)</td>
</tr>
</tbody>
</table>

FFA, free fatty acids; TG, triglyceride. *P < 0.05 compared with Nonpregnant. †P < 0.05 compared with Pregnant. ‡P = 0.06 compared with Nonpregnant.

rodent placenta and perhaps a species-specific difference in the amount that reaches the maternal compartment. We found a small increase in the amount of plasma adiponectin in the PFV− rats compared with pregnant controls, but this did not reach statistical significance. Although adipose tissue and placenta express many peptides, the amount from each tissue that reaches maternal circulation is unclear.

The metabolic role of each peptide is somewhat more complicated than simply the source of circulating levels. Circulating TNF-α may exert a direct effect on insulin-stimulated glucose uptake in skeletal muscle by promoting serine phosphorylation of the insulin receptor substrate-1 (IRS-1) (18, 30), and in pregnancy, serine phosphorylation is increased in skeletal muscle late in gestation (34). Additionally, TNF-α is a chronic regulator of lipolytic tone in adipose tissue (23). Adipose tissue TNF-α is not secreted into circulation, and much of its effects are thought to be a result of autocrine and paracrine actions (28). Placenta-derived circulating TNF-α may have contributed to pregnancy-induced peripheral insulin resistance, while the increase in plasma free fatty acids may have been mediated in part by TNF-α derived from adipose tissue.

The association between hepatic insulin resistance and hepatic triglyceride accumulation is well established in the nonpregnant state (34). Triglyceride accumulation and nonalcoholic fatty liver disease represent the hepatic component of the metabolic syndrome. We demonstrate that plasma free fatty acids and hepatic triglyceride content are increased in pregnancy. When visceral fat was decreased, plasma free fatty acids and hepatic triglyceride content were comparable to levels seen in nonpregnant females and paralleled the improved hepatic insulin sensitivity. Obese women with fat accumulation in the liver before pregnancy may be at particular risk for glucose intolerance with the added burden in pregnancy. Furthermore, some animal models (33) and human studies (26) suggest that the accumulation of triglycerides in the liver may precede hepatic insulin resistance. After pregnancy, hepatic triglyceride accumulation may have important ramifications for the long-term health of the mother, especially in cases of postpartum weight retention and if pregnancy results in permanent changes in body fat distribution (15).

Many physiological adaptations occur in pregnancy, including accretion of visceral fat, accumulation of triglycerides in the liver, and a reduction in peripheral and hepatic insulin

![Fig. 2. Relative expression of fat-derived peptides (FDP) in VF and placental tissue during pregnancy. Quantitative real-time PCR is shown as fold expression of any gene over GAPDH. All values are presented as means ± SE. PAI-1, plasminogen activating inhibitor-1. *P < 0.05.](http://ajpendo.physiology.org/)

**Table 3. Fat-derived peptide plasma levels**

<table>
<thead>
<tr>
<th></th>
<th>Nonpregnant, mean (±SE)</th>
<th>Pregnant, mean (±SE)</th>
<th>Pregnant VF−, mean (±SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma active PAI-1, ng/ml</td>
<td>332 (±106)</td>
<td>2,941 (±169)</td>
<td>2,460 (±497)</td>
</tr>
<tr>
<td>Plasma TNF-α, ng/ml</td>
<td>135.4 (±19)</td>
<td>267.7 (±162)</td>
<td>216.2 (±137)</td>
</tr>
<tr>
<td>Plasma leptin, ng/ml</td>
<td>1.6 (±0.5)</td>
<td>3.6 (±0.1)</td>
<td>3.1 (±0.3)</td>
</tr>
<tr>
<td>Plasma adiponectin, ng/ml</td>
<td>3.2 (±0.2)</td>
<td>3.3 (±0.5)</td>
<td>3.7 (±0.2)</td>
</tr>
<tr>
<td>Plasma IL-6, ng/ml</td>
<td>38.5 (±9.7)</td>
<td>54.6 (±14.3)</td>
<td>41.2 (±15.3)</td>
</tr>
</tbody>
</table>

PAI-1, plasminogen activating inhibitor-1; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6. *P < 0.05 compared with Nonpregnant.
sensitivity. The cause of changes in insulin action seems to be multifactorial. We demonstrate the importance of visceral fat in the hepatic insulin resistance of pregnancy, which may be mediated by the accumulation of triglycerides in the liver. Excessive increases in visceral fat, whether preceding the pregnancy or due to excessive weight gain, may be a modifier of risk for gestational diabetes and type 2 diabetes later in life. The dramatic increase in obesity in reproductive-age women over the past 20 years makes the delineation of changes in maternal body fat distribution and the role of visceral fat in the insulin resistance of human pregnancy a matter of escalating relevance.

ACKNOWLEDGMENTS

We thank Hong-Qiang Liang for technical help.

GRANTS

This work is supported by a Research Training Scholarship Award from the American Association of Obstetricians and Gynecologists Foundation with the American Board of Obstetrics and Gynecology and the Society for Maternal-Fetal Medicine (to F. H. Einstein), National Institute on Aging Grants AG-21654 and AG-18381 (to N. Barzilai), and the core laboratories of the Albert Einstein Diabetes and Research Training Center.

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