Differential Responses of Visceral and Subcutaneous Fat Depots to Nutrients

Francine H. Einstein,1 Gil Atzmon,2 Xiao-man Yang,2 Xiao-Hui Ma,2 Marielisa Rincon,3 Eric Rudin,2 Radhika Muzumdar,2,3 and Nir Barzilai2

Increased visceral adiposity is a pivotal component of the metabolic syndrome. Differential gene expression patterns of fat-derived peptides (FDPs) in visceral fat and subcutaneous fat have been characterized in the fasting state. Here we examined whether delivery of nutrients differentially affects the expression of FDPs in visceral fat versus subcutaneous fat (in the fed state). We increased the rate of glucose flux into adipose tissue of normal rats (n = 16) by hyperglycemia or hyperinsulinemia using the clamp technique. Glucose uptake was associated with increased expression of FDPs, including resistin (~5-fold), adiponectin (~2-fold), leptin (~15-fold), plasminogen activating inhibitor-1 (~10-fold), and angiotensinogen (~4-fold) in visceral fat, but markedly less in subcutaneous fat. Cytokine expression derived mainly from vascular/stromal/macrophage components of adipose tissue was less dramatically increased. Infusion of glucosamine amplified the results obtained by increasing glucose uptake into adipose tissue, suggesting that flux through the hexosamine biosynthetic pathway may serve as a mechanism for “nutrient sensing.” Nutrient-dependent expression of FDPs in visceral fat was also associated with increased plasma levels of several FDPs. Because a biologic sensing pathway can dynamically couple daily food intake to abnormal plasma levels of important FDPs, we challenge the practice of obtaining plasma samples from different fat depots relative to the other (13).

In this experiment, we evaluated gene expression and plasma levels of FDPs in response to nutrients. Although changes in glucoregulatory hormones have been documented after glucose tolerance tests and food boluses, nutrients may have a more complex and prolonged action affecting gene transcription, secretion, and ultimately plasma levels. For example, plasma levels of the FDP leptin are increased throughout the day, and its levels are maximal at ~2 a.m. (14), an effect that is linked to food intake and not a diurnal variation (15). This line of investigation is important because plasma levels of these surrogate markers of risk are proportional to fat depot size. In the central obesity typical of the metabolic syndrome, visceral fat accounts for up to 27% of total fat volume in obese humans (16), making an understanding of its regulation significant.

The hexosamine biosynthetic pathway (HBP) has a major role in directing hexose phosphates and glutamine to glycosylation and synthesis glycoproteins. Because the HBP accounts for only a small percentage (1–3%) of the glucose metabolized in myocytes, adipocytes, and other cells, it is hypothesized to be an ideal candidate for a cellular nutrient sensor, responding to energy availability and mediating different metabolic effects (17). Hyperglycemia or hyperinsulinemia, by increasing the flux through the HBP, can lead to insulin resistance and glycosylation of transcription factors such as Sp1 (17–19). Sp1 is an important transcription factor that can modulate expression of FDPs, particularly those involved in insulin action and the metabolic syndrome (20). Leptin gene expression in adipose and muscle tissues is increased during en-
hanced in vivo flux through HBP (21). Hyperglycemia and infusions of glucosamine increased levels of both plasminogen activating inhibitor-1 (PAI-1) (22) and angiotensinogen (23) in subcutaneous fat after 3 h, supporting the link between HBP and the induction of FDP.

To demonstrate nutritional regulation of FDPs, we aimed to increase glucose uptake in adipose tissue by either hyperglycemia or hyperinsulinemia for several hours and examined how the expression patterns of FDPs were altered. Glucosamine was also infused to determine whether changes in FDPs could be induced by a specific nutrient-sensing pathway. Finally, differences in gene expression between subcutaneous fat and visceral fat depots were compared to demonstrate a possible deleterious effect of the visceral fat depot.

RESEARCH DESIGN AND METHODS

Young adult F344 × BN rats (Harlan Worldwide, Somerville, NJ) were housed in individual cages and subjected to standard light (6:00 A.M. to 6:00 P.M.) and dark (6:00 P.M. to 6:00 A.M.) cycles. They were fed ad libitum using regular rat chow that consisted of 64% carbohydrate, 30% protein, and 6% fat with a physiological fuel value of 3.3 kcal/g. Rats were studied during young adulthood (3 months of age and ~300 g body wt). One week before the in vivo study, rats were anesthetized by inhalation of methoxyflurane, and indwelling catheters were inserted in the right internal jugular vein and in the left carotid artery (10,24–27). Recovery was continued until body weight was within 3% of the preoperative weight (~5–6 days). Chronically catheterized rats were studied ~16–24 h after their last feeding, while awake and unrestricted. In vivo studies. To mimic components of the in vivo physiologic postmeal conditions, we designed the following four protocols (n = 4 in each group):

1. Control study: saline was infused for 5 h.
2. Hyperglycemic clamp study: briefly, 25% dextrose was infused intravenously to raise plasma glucose concentration acutely to ~18 mmol/l. Somatostatin (1.5 μg · kg⁻¹ · min⁻¹) was infused to prevent endogenous insulin secretion. This plasma glucose concentration was maintained throughout the study using a variable infusion of insulin, periodically adjusted according to plasma glucose levels (22–24).
3. Hyperinsulinemic-euglycemic clamp study: this protocol was intended to increase and approximately match rates of glucose uptake in adipose depots to those obtained in the hyperglycemic clamp study. A primed continuous insulin infusion (3 μU · kg⁻¹ · min⁻¹) and a variable infusion of 25% dextrose was periodically adjusted to clamp the plasma glucose concentration at basal level (~7 mmol/l) for 5 h (26,27). Somatostatin (1.5 μg · kg⁻¹ · min⁻¹) was infused to prevent endogenous insulin secretion.
4. Glucose clamp study: we also included a glucose clamp study by directly increasing glucose levels in the carotid artery, directly by being phosphorylated to glucosamine-6-phosphate, its intravenous infusion (30 μmol · kg⁻¹ · min⁻¹) for 5 h (25) was used as a tool to directly increase glucosamine flux through the pathway without changing glucose flux. Somatostatin (1.5 μg · kg⁻¹ · min⁻¹) was infused to prevent exogenous insulin secretion. Insulin infusion (3 μU · kg⁻¹ · min⁻¹) was also used because glucosamine uptake is mediated by insulin-responsive glucose transporters. Infusion of 25% dextrose was titrated to maintain plasma blood glucose at a basal level (~7 mmol/l) for 5 h.

Thus, the hyperglycemia studies increased plasma glucose levels to allow for glucose flux largely through non-insulin-mediated glucose uptake. Although glucose levels were not physiologic, the glucose uptake into fat was. The hyperinsulinemia study attempted to approximately match glucose uptake to the hyperglycemic study. The glucosamine study attempted to increase glucosamine flux by increasing glucose concentration. The glucose clamp study was used to ensure that changes in the plasma levels of FDPs could be detected after initiation of expression.

All rats received a primed continuous (15–40 μCi/min) infusion of high-performance liquid chromatography–purified [3-H]glucose (New England Nuclear, Boston, MA) throughout the study to determine glucose fluxes. For determining glucose uptake in adipose tissue, a bolus of 2-[U-14C]deoxyglucose (20 μCi) was administered every 3 h throughout the entire course of a 15-h experimental period. At the end of the clamp study, rats were killed using 60 mg/kg body wt pentobarbital sodium intravenously through catheters that were previously implanted. The abdomen was quickly opened, and visceral and subcutaneous fat tissues were freeze-clamped in situ with aluminum tongs precooled in liquid nitrogen (28). The study protocol was reviewed and approved by the Animal Care and Use Committee of the Albert Einstein College of Medicine.

In vitro analysis

RNA extraction and labeling. Total RNA was isolated from both visceral and subcutaneous fat depots obtained from individual rats by direct homogenization in 1.0 ml of Trizol reagent (Life Technologies, Grand Island, NY). Total RNA from each depot from each rat in the group was obtained. The RNA was analyzed by 1% agarose gel that contained 2.2 mol/l formaldehyde before use. Further steps in the isolation process were performed according to the manufacturer’s recommended protocol. Subsequent purification and size selection of total RNA using RNAeasy columns (Qiagen, Valencia, CA) were also done as suggested by the manufacturer.

Quantification of expression by qRT-PCR. Real-time quantitative RT-PCR (qRT-PCR) was performed for transcript confirmation using a hot-start technique (Superscript II RT + Taq polymerase; Life Technologies, Gaithersburg, MD), with 2 μg of total RNA from individual rats as starting material and an annealing temperature of 58–60°C for 40–45 cycles, depending on the individual transcript being amplified. Resistor, tumour necrosis factor-α (TNF-α), leptin, adiponectin, interleukin (IL)-6, IL-10, PAI-1, angiotensinogen, glyceroldehyde-3-phosphate dehydrogenase (GAPDH) reference gene. Infusion of glucosamine increased levels of both plasminogen activator-1 (PAI-1) (22) and angiotensinogen (23) in subcutaneous fat after 3 h, supporting the link between HBP and the induction of FDP.

sites of FDP production. In addition to vascular and stromal sources of FDP, it has recently become apparent that adipose tissue macrophages may be numerous (20,30). Adipose tissue was obtained from above and immediately washed at least three times with saline to remove contaminating blood. The cells were then digested with collagenase type 1, 1 g/30 ml of Hanks’ balanced salt solution with 4% BSA (Worthington Biochemical, Lakewood, NJ) for 60 min at 37°C with intermittent shaking. The adipocytes were separated from the stromal-vascular fraction by centrifugation at 3,000 rpm for 10 min. CD14+ were separated from the stromal-vascular fraction by Dyna-beads (Dynal Biotech, Lake Success, NY) as per the manufacturer’s recommended method. The separated adipocytes and macrophages were washed by PBS and stored in Trizol for RNA extraction and analyzed by RT-PCR.

Analytical procedures. Plasma glucose was measured by the glucose oxidase method (Glucose Analyzer II; Beckman Instruments, Palo Alto, CA). Plasma insulin was measured by radioimmunoassay, using rat and human insulin as standards. Insulin-like growth factor (IGF)-I was measured in duplicate in the supernatants of Ba(OH)₂ and SnSO₄ precipitates of plasma samples (20 μl) after evaporation to dryness to eliminate tritiated water. For measuring plasma [U-14C]2-deoxyglucose, samples were deproteinized, and an aliquot of the supernatant was counted in a double-
channel β-counter after addition of 500 μl of water and 5 ml of liquid scintillation mixture. For measuring subcutaneous fat [U-14C]-2-deoxyglucose, frozen tissue samples were weighed and dissolved in 0.5 ml of 1 mol/l of HCl, two aliquots were taken. One was deproteinized with Ba(OH)2 and ZnSO4 and the other with 0% HClO4. The HClO4 supernatant contains both phosphorylated and unphosphorylated 2-deoxyglucose, whereas the Ba(OH)2 and ZnSO4 supernatants contain only the unphosphorylated form. The difference in disintegrations per minute between the two supernatants measures the fat content of 2-deoxyglucose phosphate, thus determining the rate of glucose uptake. Plasma levels of IL-6 (Rat IL-6 ELISA kit; Pierce Endogene) and adiponectin (Mouse Adiponectin ELISA kit; Linco Research) were measured by ELISA. Leptin was measured by radioimmunoassay (RAI; Linco Research), and PAI-1 activity was measured by chromogenic assay kit (Spectrolyse/P, PAI, Biopool International). Free fatty acids were measured using Waco NEFA C Kit (Waco Chemicals).

**Statistical analysis.** The significance of the results of qRT-PCR (fold expression of any gene by visceral fat/GAPDH over subcutaneous fat/GAPDH) was evaluated by the two-sample Student’s t test. Pearson correlation coefficients were calculated to estimate the linear relationship between variables. All values are presented as means ± SD. P < 0.05 was considered significant.

**RESULTS**

**Basal metabolic characteristics and glucose fluxes during saline, glucose, insulin, and glucosamine infusions.** Animals were matched in body weight, weight of perinephric fat pads, and other visceral fat pads (data not shown). During hyperglycemic clamps, glucose levels were maintained at target levels (~18 mmol/l), inducing a significant increase in total glucose uptake, subcutaneous fat glucose uptake, and visceral fat glucose uptake. In these studies, total glucose uptake was greater in subcutaneous fat compared with that in visceral fat, but a greater increase in glucose uptake was noted in visceral fat (~4-fold) compared with subcutaneous fat (~2-fold) over saline controls. Physiologic hyperinsulinemia (~60 μU/ml) induced an ~3-fold increase in total glucose uptake, and as seen with hyperglycemia, glucose uptake increased more dramatically in visceral fat (~3-fold) than in subcutaneous fat (~1.5-fold). Although glucosamine was infused during hyperinsulinemic conditions, total glucose uptake was significantly lower, possibly because glucosamine infusion induces insulin resistance beginning 2–3 h after the initiation of its infusion (25,31). We previously reported that glucose uptake was similar to that observed with hyperglycemia or hyperinsulinemia at 2–3 h (22,23). Similarly, glucose uptake into adipose tissue was not increased at the conclusion of the clamp, suggesting some resistance to insulin action in adipose tissue at the end of 5 h (Table 1).

**Selected basal FDP gene expression and the cellular compartment source.** Expression of resistin was ~10-fold greater in visceral fat than in subcutaneous fat. Adiponectin and IL-10 expression were also significantly greater in visceral fat, making these genes good markers of visceral fat. Leptin expression was significantly lower in visceral fat, whereas no marked differences were found in PAI-1, TNF-α, IL-6, or angiotensinogen expression in subcutaneous fat compared with visceral fat during saline infusion (Table 2).

Because expression of these peptides originates from different cellular components of adipose tissue (adipocytes, vascular/stromal, and macrophages), we assessed the relative contribution of each of these components to the expression of specific peptide. As indicated in Table 2, leptin, adiponectin, resistin, and angiotensinogen are expressed mainly in adipocytes. The cellular source of PAI-1 is mixed, whereas other peptides, including cytokines, are expressed more in nonadipocyte cells. When assessed per total mRNA obtained from per gram of adipose tissue, macrophages may contribute a small percentage even in visceral fat.

**Expression of FDPs during hyperglycemia and hyperinsulinemia.** Increased glucose uptake, whether by glucose or insulin, seemed to increase expression of FDPs significantly and more dramatically in visceral fat than
in subcutaneous fat. For example, resistin expression was modulated in both visceral fat and subcutaneous fat by both insulin and glucose, but both absolute and relative expression was greater in visceral fat. Absolute leptin expression was ~10-fold greater in visceral fat. Angiotensinogen, adiponectin, and PAI-1 expression were also more dramatic in visceral fat than in subcutaneous fat. IL-6 and TNF-α expression were not significantly modulated with insulin or glucose. IL-10 expression increased more in subcutaneous fat than in visceral fat (Fig. 1).

Expression of FDPs during glucosamine administration. The expression of resistin, leptin, angiotensinogen, adiponectin, PAI-1, IL-6, and IL-10 increased with glucosamine in both fat depots, but the change was more dramatic in visceral fat. TNF-α expression is also modulated by glucosamine but to a lesser extent than the other FDPs presented (Fig. 2).

Plasma levels of FDPs. Plasma leptin levels increased by twofold with hyperglycemia (0.87 ± 0.16 and 2.67 ± 0.53 ng/ml at 0 and 5 h, respectively) and with hyperinsulinemia (0.95 ± 0.09 and 1.81 ± 0.29 ng/ml at 0 and 5 h,
respectively) and by threefold with glucosamine infusion (0.89 ± 0.13 and 3.71 ± 0.22 ng/ml at 0 and 5 h, respectively). Levels during saline infusion were unchanged (0.90 ± 0.09 and 0.85 ± 0.11 ng/ml at 0 and 5 h, respectively). PAI-1 activity in plasma increased dramatically with hyperglycemia (7.9 ± 0.7 and 24.9 ± 1.5 units/ml at 0 and 5 h, respectively) and hyperinsulinemia (7.2 ± 0.5 and 35.6 ± 4.2 units/ml at 0 and 5 h, respectively) and was similar to saline control (7.2 ± 0.8 and 7.8 ± 0.6 units/ml at 0 and 5 h, respectively). IL-6 levels were doubled with hyperinsulinemia (38.3 ± 4.7 and 75.0 ± 10.5 pg/ml at 0 and 5 h, respectively) and tripled with glucosamine (43.2 ± 113.2 ± 14.9 pg/ml at 0 and 5 h, respectively), whereas levels were unchanged during saline infusion (37.3 ± 7.7 and 35.6 ± 6.2 pg/ml at 0 and 5 h, respectively). It is interesting that within this time course, plasma levels of adiponectin did not increase with hyperglycemia (0.58 ± 0.09 and 0.54 ± 0.12 µg/ml at 0 and 5 h, respectively), hyperinsulinemia (0.52 ± 0.11 and 0.54 ± 0.11 µg/ml at 0 and 5 h, respectively), or glucosamine (0.55 ± 0.08 and 0.55 ± 0.12 µg/ml at 0 and 5 h, respectively) and was similar to saline control (0.52 ± 0.09 µg/ml at 0 and 5 h, respectively).
DISCUSSION
We have demonstrated that acute increases in glucose uptake into adipose tissue by either hyperglycemia or hyperinsulinemia increased the expression of several FDPs in visceral fat to a greater degree than in subcutaneous fat. Some of these FDPs may be involved in modulating insulin action or are components of the metabolic syndrome. The basal ("static") expression of FDPs in visceral fat and subcutaneous fat were not predictive of the changes in FDP expression seen when exposed to delivery of a nutrient ("dynamic"). In addition, we have shown that increasing flux through the HBP increased the expression of FDPs, mimicking many of the effects exerted by hyperinsulinemia and hyperglycemia and providing evidence for its role as a "nutrient sensor." Finally, we have demonstrated significant increases in plasma levels of FDPs within the time course of the experiment.

Although the expression of many genes is commonly modulated by hyperglycemia, hyperinsulinemia, and glucosamine, other factors need to be considered to appreciate its physiologic significance. Although the absolute changes in leptin were more impressive than the fold increases, we present the remainder of the results as fold changes to emphasize the magnitude of change seen with nutrient delivery. Each gene has its own time course and magnitude of expression. In addition, each peptide may be translated, "packaged," and secreted by the components of adipose tissue, and each peptide has its own half-life in the circulation. Leptin exemplifies this concept. Leptin expression has been coupled to nutrient availability (21,32); it is secreted in a rapid time course and reaches maximal levels after midnight (15). We have measured significantly higher leptin and PAI-1 activity after 5 h. In contrast, although adiponectin expression increased significantly, plasma levels were not changed by any infusion. This may indicate that the time course has not been sufficient for significant increases to be seen, adiponectin clearance is increased in parallel, or the regulation of adiponectin secretion is uncoupled to its expression. Ours is not the first study to show a discrepancy between adiponectin expression and levels (33), suggesting complex regulation between adiponectin expression and secretion. Alternatively, although plasma levels of IL-6 were increased by insulin and glucosamine, IL-6 gene expression was not increased markedly. This may indicate that small changes in gene expression (as seen with glucosamine) can induce significant secretion of the protein, that nutrients can release stored IL-6, that regulation of IL-6 levels by nutrients is dependent on alterations in clearance, or that the source of IL-6 derives from another tissue. Because many FDPs do not have similar time courses, their plasma levels should be measured in humans in relationship to nutrient intake to establish the exact magnitude of this effect and the associated risk.

Perinephric fat was chosen to represent visceral fat because, whereas humans have a significant amount of omental fat and rodents have significant amount of gonadal fat, perinephric fat is common to both species. Although it has been suggested that visceral fat induces hepatic insulin resistance via a portal effect, there is significant evidence that other factors such as secretion of FDPs contribute independent of direct portal blood flow. In fact, removal of perinephric and epididymal fat pads in our rodent models, neither of which drain directly into the portal circulation, has been shown to improve hepatic and peripheral insulin resistance and modify FDP gene expression (7). This improvement in insulin sensitivity was seen despite the presence of mesenteric/omental fat. In addition, portal levels of free fatty acids did not differ between groups.

We have demonstrated that leptin, resistin, adiponectin, and angiotensinogen are derived mostly from adipocytes, whereas other FDPs, such as PAI-1 and TNF-α, originate in the vascular and/or stromal cell and macrophage compartments. Although macrophages express several cytokines, their number in adipose tissue is proportionally small compared with other cell types and may not indicate a major overall contribution. These different components of fat tissue may have specific regulation by factors other than those that modulate expression in adipocytes. For example, although the HBP exists in all of the cellular compartments of adipose tissue, their response to insulin may be limited. Moreover, some peptides, such as PAI-1 or angiotensinogen, are expressed in liver, which may be a major source contributing to their plasma levels. We previously suggested that angiotensinogen contribution from fat tissue in obesity exceeds the contribution from liver (23). Importantly, it is the expression contributed per adipose tissue mass that is the most physiologically relevant, independent of the actual cellular source.

Because glucosamine also activates specific genes that are activated by glucose or insulin, we suggest that HBP may be a major candidate for nutrient sensing for many FDPs. Similarly, increasing availability of free fatty acids may inhibit glycolytic enzymes and increase fructose-6-phosphate levels and the flux through the HBP, as we have demonstrated in muscle (34).

We have shown that the expression of FDPs after stimulation by nutrients markedly increased in visceral fat compared with subcutaneous fat. Several possible explanations for these differences exist. Because changes in gene expression during hyperglycemia and glucosamine studies were similar, we propose that the HBP may be at least partially responsible for this enhanced expression of FDPs in visceral fat. Nutrient-stimulated glucose uptake increased more in visceral fat than in subcutaneous fat and may activate HBP more significantly. It is also possible that the HBP is intrinsically more active in visceral fat possibly because increased free fatty acid flux in this tissue may shunt more fructose-6-phosphate through the HBP pathway. Differences in the number of vascular cells or macrophages relative to adipocytes may also contribute to the unique expression of visceral fat. We suggest that the "riskiest" visceral fat in humans may be determined by assessing FDP expression, potentially after nutrient stimulation. Furthermore, although the rodents studied are genetically homogeneous, individual sensitivity to nutrients may explain why some humans with abdominal obesity are at more risk than others.

In conclusion, we have shown that adipose tissue, in particular visceral fat, is not biologically static and
probably regulated by nutrients. We focused on several FDPs that are relevant for the metabolic syndrome and associated their increased expression with a novel biologic sensor. Unlike previous in vivo and epidemiologic studies, which are standardized for fasting, our study demonstrates that the cumulative effects of nutrient-coupled increases in gene expression may be significantly modulated throughout the day.

ACKNOWLEDGMENTS
This work was supported by grants from the National Institutes of Health (P01 AG021654 and RO1-AG 18381 to N.B) and the American Diabetes Association and by the Core Laboratories of the Albert Einstein Diabetes Research and Training Center (DK 20541).

We thank Dr. H. Shamoon, Dr. P. Scherer, and Dr. M. Hawkins for reviewing this manuscript and Rebecca Fabro and Adam Matos for assistance.

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