Preferential Channeling of Energy Fuels Toward Fat Rather Than Muscle During High Free Fatty Acid Availability in Rats

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The preferential channeling of different fuels to fat and changes in the transcription profile of adipose tissue and skeletal muscle are poorly understood processes involved in the pathogenesis of obesity and insulin resistance. Carbohydrate and lipid metabolism may play relevant roles in this context. Freely moving lean Zucker rats received 3- and 24-h infusions of Intralipid (Pharmacia and Upjohn, Milan, Italy) plus heparin, or saline plus heparin, to evaluate how an increase in free fatty acids (nonesterified fatty acid [NEFA]) modulates fat tissue and skeletal muscle gene expression and thus influences fuel partitioning. Glucose uptake was determined in various tissues at the end of the infusion period by means of the 2-deoxy-[1-3H]-D-glucose technique after a euglycemic-hyperinsulinemic clamp: high NEFA levels markedly decreased insulin-mediated glucose uptake in red fiber–type muscles but enhanced glucose utilization in visceral fat. Using reverse transcriptase–polymerase chain reaction and Northern blotting analyses, the mRNA expression of fatty acid translocase (FAT)/CD36, GLUT4, tumor necrosis factor (TNF)-α, peroxisome proliferator–activated receptor (PPAR)-γ, leptin, uncoupling protein (UCP)-2, and UCP-3 was investigated in different fat depots and skeletal muscles before and after the study infusions. GLUT4 mRNA levels significantly decreased (by ~25%) in red fiber–type muscle (soleus) and increased (by ~45%) in visceral adipose tissue. Furthermore, there were marked increases in FAT/CD36, TNF-α, PPAR-γ, leptin, UCP2, and UCP3 mRNA levels in the visceral fat and muscle of the treated animals in comparison with those measured in the saline-treated animals. These data suggest that the in vivo gene expression of FAT/CD36, GLUT4, TNF-α, PPAR-γ, leptin, UCP2, and UCP3 in visceral fat and red fiber–type muscle are differentially regulated by circulating lipids and that selective insulin resistance seems to favor, at least in part, a prevention of fat accumulation in tissues not primarily destined for fat storage, thus contributing to increased adiposity and the development of a prediabetic syndrome. *Diabetes* 50:601–608, 2001

In obesity, excessive energy storage as fat is mainly due to an imbalance between energy intake and expenditure, and the preferential channeling of excess calories as fat rather than protein or glycogen may play an important role in the development and maintenance of the disease. Carbohydrates and lipids share common metabolic pathways, which may be important in the regulation of energy metabolism and fuel partitioning. Insulin stimulates glucose oxidation in skeletal muscle and adipose tissue, but increased blood levels of other macronutrients may interfere with glucose oxidation: for example, although basal glucose utilization remains unaffected by lipid infusion, insulin-mediated glucose utilization decreases, as measured by means of the euglycemic-hyperinsulinemic clamp technique (1). Furthermore, positron emission tomography scanning using fluorodeoxyglucose as a tracer shows that this inhibition mainly takes place in skeletal muscle (2). Free fatty acid (nonesterified fatty acid [NEFA])-induced insulin resistance saves scarce glucose for central nervous system requirements, but this becomes counterproductive in obesity because it inhibits glucose utilization when there is no need to save it. Glucose and NEFA might thus be channeled toward tissues (such as adipose tissue in which insulin sensitivity is maintained or even improved) and contribute to triglyceride synthesis, although the cellular and molecular mechanisms responsible for these processes (particularly for defective muscle glucose uptake) are still under debate. It has been suggested that tumor necrosis factor (TNF)-α may play a relevant role because it is overexpressed in the adipose tissue of obese insulin-resistant rodents and humans, and TNF-α antibodies counteract insulin resistance in the fat and muscle of genetically obese Zucker rats (3).

In addition to substrate competition, which is a very complex metabolic phenomenon involving much more molecular and cellular events than those firstly hypothesized by Randle et al. (4) and others (5–7), other processes and factors are known to regulate fuel partitioning and fat
deposition, including the hormonal milieu (i.e., insulin levels) and the expression of genes whose products play active roles in tissue energy homeostasis, such as leptin and uncoupling protein (UCP)-2 and UCP3 (8,9). Recent evidence suggests that once inside the cells, the fatty acids derived from the uptake of the NEFA released by the hydrolysis of circulating triglyceride-rich lipoproteins or from circulating fatty acids bound to serum albumin (10) act as signaling molecules. They bind to and activate the peroxisome proliferator–activated receptor (PPAR) protein family, a new class of nuclear receptors that can link fatty acids by means of a program of gene expression (11). PPAR-γ modulates the expression of genes involved in adipogenesis, lipid storage, and metabolism, such as fatty acid translocase (FAT)/CD36 (12). Furthermore, it has been hypothesized that cross-talk may exist between insulin action and PPAR-γ functions in mature adipocytes because the thiazolidinediones, a new class of antidiabetic drugs that improve in vivo insulin sensitivity in humans (13), have been found to bind and activate PPAR-γ (14). PPAR-γ is also expressed at lower levels in skeletal muscle (15), but its role in this tissue is not yet fully understood.

We have recently suggested that circulating NEFAs in healthy human subjects directly favor the accumulation and metabolism of fatty acids in adipose depots by modulating the expression of some of these specific adipose genes (16). However, that study did not provide any information concerning the effect(s) of circulating NEFAs on muscle metabolism and gene expression. The aim of the present study was therefore to investigate fuel partitioning during exposure to high plasma NEFA levels similar to those induced by high-fat diets. In particular, we compared the expression of different genes involved in the peripheral control mechanisms of energy balance in white fat and skeletal muscle and their relationships with overall and tissue glucose metabolism.

RESEARCH DESIGN AND METHODS

Surgical procedure and infusion protocol. Adult male lean (Fa/?) Zucker rats (Charles River, Lecco, Italy) of 200 g body weight were housed at 24°C with a 0700–1900 light cycle and had free access to water and food pellets. All of the animals were studied in the morning after 6 h of fasting. The study protocol was approved by the institutional review board of the University of Padova.

Surgical procedure was as previously described (17). At the end of anesthesia, the animals were placed in individual cages with a single slot for the catheters, which were kept under continuous tension. The patency of the arterial catheter was maintained by means of a slow infusion of saline solution.

In a first series of experiments, four groups of six animals each were studied. Twelve hours after surgery, an infusion of 20% triglyceride emulsion (Intralipid; Pharmacia, Uppsala, Sweden) plus heparin (20 U/ml) was started and continued at a rate of 0.7 ml/h for either 3 or 24 h in the two experimental groups, whereas the two control groups received saline solution. Twelve hours after surgery, an infusion of 20% triglyceride emulsion (Intralipid; Pharmacia and Upjohn, Milan, Italy) was started and continued at a rate of 0.7 ml/h for either 3 or 24 h in the two experimental groups, whereas the two control groups received saline solution.

RESULTS

Metabolic parameters. Intralipid plus heparin infusion significantly increased plasma NEFA and insulin levels in comparison with the saline plus heparin–treated rats, but no significant differences in blood glucose were observed (Table 1). Intralipid treatment was also followed by a significant increase in serum leptin levels, which was evident after only 3 h of infusion (Table 1). No significant differences in food intake and body weight were noted between the two groups of animals (data not shown).
Glucose infusion rate and tissue glucose utilization index during a euglycemic-hyperinsulinemic clamp.

The glucose infusion rate was significantly lower in the Intralipid-infused rats than in the saline-treated rats after both 3 and 24 h of treatment (38.14 ± 1.74 vs. 18.38 ± 1.26 mg · kg⁻¹ · min⁻¹; P < 0.0002) and 24 h of treatment (38.14 ± 1.74 vs. 17.89 ± 1.44 mg · kg⁻¹ · min⁻¹; P < 0.0002). Figure 1 shows that the glucose utilization index was significantly reduced in the same animals in all of the oxidative muscles considered (the red portion of the quadriceps and gastrocnemius, and the soleus) after both 3 (Table 2) and 24 h (Fig. 1) of treatment. Increased NEFA availability was followed by an enhanced glucose uptake in visceral adipose tissue after 3 (Table 2) and 24 h (Fig. 1), but no difference was observed in white fiber–type muscles (white portion of quadriceps and gastrocnemius) or in the subcutaneous fat depot at either time point (Table 2 and Fig. 1). On the basis of these results, the following experiments were performed only in visceral fat and red fiber–type muscle (soleus).

Effects of Intralipid plus heparin infusion on gene expression in visceral fat and red fiber–type skeletal muscle. Postinfusion RT-PCR analysis using specific primers of the RNA isolated from visceral fat, and red fiber–type muscle showed a marked increase in FAT/CD36 mRNA in comparison with the levels in the saline-treated animals after both 3 and 24 h (Fig. 1) of treatment. Increased NEFA availability was followed by an enhanced glucose uptake in visceral adipose tissue after 3 (Table 2) and 24 h (Fig. 1), but no difference was observed in white fiber–type muscles (white portion of quadriceps and gastrocnemius) or in the subcutaneous fat depot at either time point (Table 2 and Fig. 1). On the basis of these results, the following experiments were performed only in visceral fat and red fiber–type muscle (soleus).

Because it has been widely reported that TNF-α can affect adipose tissue and muscle metabolism at various levels and it has been demonstrated that TNF-α modulates GLUT4 expression (22), we measured TNF-α’s mRNA levels by RT-PCR after Intralipid plus heparin infusion. Figures 3A and 4A show that the levels of TNF-α mRNA were markedly higher in fat and soleus after both 3 and 24 h in comparison with those measured in the saline plus heparin–treated animals.

In addition, because the expression of PPAR-γ protein appears to be modulated by calorie intake of fat (23), we investigated whether increased levels of plasma fatty acids could affect PPAR-γ mRNA levels and found that these were high after both 3 and 24 h in red fiber–type muscle (Fig. 4C), but only after 3 h in visceral fat (Fig. 3C). Moreover, the Intralipid infusion markedly increased

**TABLE 1**

| Metabolic parameters measured during Intralipid plus heparin infusion or saline plus heparin infusion in normal-weight male Zucker rats |
|-----------------|-----------------|-----------------|
|                 | Baseline        | 3 h             | 24 h            |
| Free fatty acid (μmol/l) |                 |                 |                 |
| Saline          | 833.9 ± 69.5    | 694.2 ± 180.5   | 852.0 ± 54.0    |
| Intralipid      | 811.2 ± 56.9    | 1817.4 ± 241.0† | 1861.7 ± 183.1† |
| Glucose (mmol/l) |                 |                 |                 |
| Saline          | 5.7 ± 0.7       | 5.3 ± 0.6       | 5.7 ± 0.3       |
| Intralipid      | 5.3 ± 0.2       | 6.0 ± 0.4       | 5.7 ± 0.2       |
| Insulin (mU/l)  |                 |                 |                 |
| Saline          | 30.1 ± 3.2      | 33.8 ± 9.5      | 23.5 ± 3.8      |
| Intralipid      | 35.1 ± 5.2      | 74.4 ± 13.5†    | 65.9 ± 15.0†    |
| Leptin (ng/ml)  |                 |                 |                 |
| Saline          | 1.66 ± 0.16     | 1.47 ± 0.16*    | 0.84 ± 0.14*    |
| Intralipid      | 1.92 ± 0.17     | 2.41 ± 0.30*†   | 2.37 ± 0.28*†   |

Data are means ± SE. *P < 0.05 vs. baseline; †P < 0.05 vs. saline plus heparin infusion.
NEFA AND GENE EXPRESSION IN FAT AND MUSCLE

The glucose utilization index was derived from the amount of [3H]-2DG measured in the various tissues as previously described (19), thus using the accumulation of [3H]-2DG as an index of the glucose metabolic rate. *P < 0.05 vs. saline plus heparin infusion; †P < 0.01 vs. saline plus heparin infusion.

Because adipose UCP2 has been found to be upregulated by a high-fat diet (24), we investigated whether increased plasma fatty acid levels affect the expression of UCP2 and UCP3 in the visceral adipose tissue and skeletal muscle of normal-weight rats. Figure 5C shows that the mRNA levels of UCP2 were increased 24 h after Intralipid plus heparin infusion in both fat and muscle with respect to those of saline-treated rats. Figure 5D shows that the mRNA levels of UCP3 were increased 24 h after Intralipid plus heparin infusion in muscle, but not in visceral fat, with respect to those of saline-treated rats. Both UCP2 and UCP3 mRNA levels were unchanged after 3-h Intralipid plus heparin infusion (data not shown).

DISCUSSION
The experimental approach used in this study mimics in a short time the increased circulating NEFA levels observed with a high-fat diet. As shown by the reduced glucose infusion rate during a euglycemic-hyperinsulinemic clamp (which was probably due to increased lipid use by red fiber–type muscles), insulin resistance develops as a result of the greater NEFA availability driven by Intralipid plus heparin infusion. Early enhancement of the gene expression of FAT/CD36 (which is suggested to be involved in NEFA uptake [25]) was evident in rat muscle after 3 h of infusion, and red fiber–type muscle insulin-dependent glucose uptake measured by [3H]-2DG technique decreased in accordance with the competitive action of NEFA on glucose metabolism (4–7).

A number of molecular mechanisms may be involved. First, GLUT4 mRNA levels were reduced in the red fiber–type muscles of the rats infused with Intralipid plus heparin after both 3 and 24 h, and TNF-α gene expression (which is extremely low even if measured by RT-PCR under baseline conditions) was markedly enhanced. This finding is relevant because, although high TNF-α expression has been demonstrated in the adipose tissue of insulin-resistant obese rodents (26) and humans (27), the fact that circulating TNF-α levels in insulin-resistant obese subjects were not detected in all studies (28) raised doubts as to whether adipose-released TNF-α really affects muscle metabolism. Our finding that high levels of circulating NEFAs can directly increase TNF-α mRNA levels in red fiber–type muscle suggests that the putatively increased TNF-α protein may act in a paracrine way in muscle tissues. Because an inverse linear relationship between the maximum glucose disposal rate and muscle TNF-α has been reported (29), this may constitute a critical point in the development of insulin resistance. It has been shown that TNF-α impairs insulin receptor signaling (30,31) and that TNF-α knockout mice have more GLUT4 protein expressed in muscle tissue (32). Our findings suggest that greater NEFA availability may shift red fiber–type muscle metabolism to preferential use of lipids rather than carbohydrates as fuel substrates. This may be at least partially due to the induction of TNF-α and the inhibition of GLUT4 gene expression.

There is a simultaneous increase in the insulin sensitivity of visceral but not subcutaneous fat. The Intralipid infusion significantly increased GLUT4 gene expression in visceral adipose tissue, thus suggesting that energy fuels may be preferentially partitioned to fat rather than muscle. It is interesting to note that both GLUT4 and TNF-α increased in visceral adipose tissue but not in red fiber–type muscles. GLUT4 favors glucose utilization, whereas TNF-α counteracts excessive fat storage by means of various mechanisms (e.g., by stimulating lipolysis [33] and decreasing the activity of adipose tissue lipoprotein lipase [34]). A similar pattern of events has been observed in the

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**TABLE 2**

<table>
<thead>
<tr>
<th>Subcutaneous adipose tissue</th>
<th>Visceral adipose tissue</th>
<th>White quadriceps</th>
<th>Red quadriceps</th>
<th>White gastrocnemius</th>
<th>Red gastrocnemius</th>
<th>Soleus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.13 ± 0.23</td>
<td>0.32 ± 0.07</td>
<td>4.86 ± 0.65</td>
<td>13.46 ± 1.53</td>
<td>3.64 ± 1.16</td>
<td>10.36 ± 1.17</td>
</tr>
<tr>
<td>Intralipid</td>
<td>0.95 ± 0.07</td>
<td>0.90 ± 0.17*</td>
<td>4.23 ± 0.50</td>
<td>8.60 ± 1.55*</td>
<td>3.83 ± 0.59</td>
<td>9.10 ± 1.33</td>
</tr>
</tbody>
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*P < 0.05 vs. saline plus heparin infusion; †P < 0.01 vs. saline plus heparin infusion.

FIG. 2. FAT/CD36 expression after 3 or 24 h of treatment with saline plus heparin (□) or Intralipid plus heparin (■) infusion in the red fiber–type muscle (muscle) and visceral fat (v.a.t.) of normal-weight rats. Top panels: representative agarose gels showing RT-PCR analysis of FAT/CD36 and β-actin mRNA content in fat and muscle samples of one animal. Bottom panels: densitometric analysis of the ratios of FAT/CD36/β-actin mRNA abundance normalized to arbitrary units. Bars represent the mean ± SE of six animals (*P < 0.05, **P < 0.01 vs. saline plus heparin infusion). The FAT/CD36 mRNA levels did not change after saline infusion.
dynamic phase of several animal models of obesity in which fat accumulation is accompanied by increased glucose uptake and GLUT4 gene expression in adipose tissue, and insulin resistance develops at the muscle level (17).

The increased expression of PPAR-γ and leptin in both muscle and adipose tissue after Intralipid infusion may be a means of counterbalancing the development of insulin resistance. After Intralipid infusion, all of the studied tissues showed a higher expression of the PPAR-γ gene, a major adipogenic transcription factor that is poorly expressed in preadipocytes but turned on during adipogenesis (35) and that regulates the expression of most adipogenic genes.

**FIG. 3.** GLUT4, TNF-α, and PPAR-γ mRNA levels after 3 or 24 h of treatment with saline plus heparin (□) or Intralipid plus heparin (■) infusion in the visceral fat (v.a.t.) of normal weight rats. Top panels: representative autoradiograms showing Northern blotting analysis of GLUT4 and β-actin mRNA (A) and representative agarose gels showing RT-PCR analysis of TNF-α (B), PPAR-γ (C), and β-actin (B and C) mRNA content in fat samples of one animal. Bottom panels: densitometric analysis of the ratios of GLUT4/β-actin, TNF-α/β-actin, and PPAR-γ/β-actin mRNA abundance normalized to arbitrary units. Bars represent the mean ± SE of six animals (*P < 0.05 and **P < 0.01 vs. saline plus heparin infusion). The GLUT4, TNF-α, and PPAR-γ mRNA levels did not change after saline plus heparin infusion.

**FIG. 4.** GLUT4, TNF-α, and PPAR-γ mRNA levels after 3 or 24 h of treatment with saline plus heparin (□) or Intralipid plus heparin (■) infusion in the red fiber-type muscle (soleus) of normal-weight rats. Top panels: representative autoradiograms showing Northern blot analysis of GLUT4 and β-actin mRNA (A) and representative agarose gels showing RT-PCR analysis of TNF-α (B), PPAR-γ (C), and β-actin (B and C) mRNA content in muscle samples of one animal. Bottom panels: densitometric analysis of the ratios of GLUT4/β-actin, TNF-α/β-actin, and PPAR-γ/β-actin mRNA abundance normalized to arbitrary units. Bars represent the mean ± SE of six animals (*P < 0.05, **P < 0.001, ***P < 0.0001 vs. saline plus heparin infusion). The GLUT4, TNF-α, and PPAR-γ mRNA levels did not change after saline plus heparin infusion.
pocyte genes (35). Three different groups have recently demonstrated that PPAR-γ is required for adipogenesis in vivo (36–38), and Barroso et al. (39) have recently reported that two loss-of-function PPAR-γ mutations are associated with severe insulin resistance and type 2 diabetes in humans. Although such mutations are rare, the implication that PPAR-γ is required for normal insulin sensitivity at least in some individuals is an important advance.

It can also be speculated that the high level of PPAR-γ expression observed by us may confer some protection against the development of the skeletal muscle insulin resistance associated with increased circulating NEFA levels. This is consistent with the fact that thiazolidinediones (TZDs) may improve insulin action in skeletal muscle by upregulating the expression of PPAR-γ (40). Furthermore, Kubota et al. (37) demonstrated that, although apparently normal on a regular diet, mice that are heterozygous for PPAR-γ deficiency are partially resistant to high-fat diet–induced obesity and markedly resistant to the insulin resistance that normally accompanies such obesity. Both PPAR-γ overactivity due to TZD stimulation and PPAR-γ underactivity due to haploinsufficiency may protect against NEFA-induced insulin resistance. Lowell (41) suggested that PPAR-γ can be seen as a “thrifty gene” that promotes fat storage to survive starvation when food is scarce and produces excessive fat storage leading to disease when food is plentiful. The mechanism by means of which the TZD-mediated stimulation of PPAR-γ improves insulin resistance has not been definitively established (42), but it is probably related to the transcription factor’s ability to increase the number of small adipocytes by stimulating adipogenesis and to decrease the number of large adipocytes, which are known to produce excess amounts of TNF-α and free fatty acids. Although not affecting adipogenesis, PPAR-γ haploinsufficiency limits further adipocyte hypertrophy, possibly by increasing leptin expression. Kubota et al. (37) have reported that leptin mRNA and circulating protein levels are elevated in high-fat–fed PPAR-γ1/2 mice, possibly because of a decrease in the PPAR-γ–mediated inhibition of leptin gene expression.

Under our experimental conditions, high levels of circulating NEFAs induced leptin expression in both fat and muscle and increased plasma leptin levels. We did not measure body composition (in particular body fat content), but it seems unlikely that the rapid induction of leptin gene expression observed after Intralipid infusion was a consequence of an increase in fat mass. Although the effects of leptin on satiety are mainly due to its activation of hypothalamic receptors (43), there is also substantial evidence that leptin has important peripheral effects (44,45), including the stimulation of lipolysis with lipid oxidation in white fat pads ex vivo (46) and decreasing in vivo tissue triglyceride stores (47,48). It can therefore be hypothesized that, under our experimental conditions, leptin has an autocrine/paracrine function, i.e., it limits excess fat storage in adipose tissue. Furthermore, leptin mRNA, which is generally undetectable in the skeletal muscle of nonobese rodents (5,49), increased 24 h after an increase in plasma NEFA levels. In skeletal muscle, leptin may be important in the regulation of intermediate metab-
olism (particularly lipid oxidation and storage) (1,45,48), energy expenditure (50), and insulin signaling (51,52). Interestingly, it has been reported that, regardless of its effect on food intake, leptin leads to a marked improvement in insulin sensitivity and glucose disposal in a mouse model of lipodystrophy (53). Leptin expression in the fat and muscle of subjects with high circulating NEFA levels could therefore be involved in counterbalancing excessive fat accumulation and the development of insulin resistance.

Finally, our data demonstrate that Intralipid infusion increased UCP2 and UCP3 mRNA in both adipose tissue and muscle. These findings are in line with the enhanced UCP2 gene expression in both tissues observed in obese subjects (54,55), whose circulating NEFA levels are commonly high. It has been reported that PPAR-γ agonists induce UCP2 gene expression in adipocytes (56), and TNF-α administration increases UCP2 and UCP3 mRNA levels in rat skeletal muscle (57). This molecular event may be ultimately aimed at dissipating energy excess, but UCP2 and UCP3 may also be involved in fatty acid metabolism in the case of a large supply of nutrients (55,58).

In conclusion, our findings suggest that NEFAs are involved in the partitioning of calories to fat by modulating the expression of various genes: FAT/CD36, which probably plays a crucial role in the development of insulin resistance in skeletal muscle; PPAR-γ, which promotes cell differentiation and fat storage and also modulates peripheral insulin sensitivity; and leptin and UCPs, which control energy intake and dissipation. Although it is difficult with our experimental approach to attribute the effects seen on the mRNA levels only to elevated plasma NEFAs because also insulin, leptin, and triglyceride levels are increased, nevertheless, it seems possible to speculate that the above mechanisms during a high-fat diet may unbalance energy homeostasis and channel the fuels toward adipose tissue, thus leading to obesity.

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