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Hormonal regulation of intracellular lipolysis in C57BL/6J mice: effect of diet-induced adiposity and data normalization

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Abstract

The breakdown of intracellular triglycerides in adipose tissue provides fatty acids and glycerol as substrates for oxidation. However, the exposure of target organs to excess free fatty acids is associated with the development of insulin resistance and impaired regulation of carbohydrate metabolism, suggesting that the control of triglyceride breakdown is an important factor in balancing health and disease. We have studied the temporal influence of diet-induced changes in adiposity on the response of intracellular lipolysis to epinephrine ± insulin using freshly isolated adipocytes from C57BL/6J mice fed a low-fat (10% kcal) or high-fat (HF, 45% kcal) diet for 1, 4, or 12 weeks. In this model, we also tested how data normalization affects the interpretation. The contribution of the epididymal fat to total body mass increased by ~15%, 45%, and 100% after 1, 4, and 12 weeks of HF diet consumption, respectively. In addition, HF feeding led to an increase in fasting insulin, that is, ~2-fold greater in HF- vs low-fat–fed mice at 4 and 12 weeks. We found that diet-induced changes in adiposity did not alter the lipolytic response to epinephrine when data were normalized per DNA (ie, per cell); however, the lipolytic potential of the organ (ie, the lipolytic rate per cell multiplied by the total number of cells) was increased in isolated adipocytes after 4 and 12 weeks of HF feeding. We also observed a marked impairment in insulin-mediated inhibition of epinephrine-stimulated lipolysis after 4 and 12 weeks of HF feeding, demonstrating that diet-induced adiposity leads to insulin resistance in adipocytes. In conclusion, HF feeding in mice leads to greater rates of lipolysis via (1) an increase in the number of fat cells and (2) a defect in insulin signaling in adipocytes. The combination of these 2 alterations on the control of intracellular lipolysis suggests a mechanism(s) that (partly) explains how target organs could be exposed to excess lipid-derived energy substrates, for example, free fatty acids and glycerol.

1. Introduction

Increased utilization of fatty acids by target organs (eg, liver, skeletal muscle, and pancreatic β-cells) impairs the regulation of whole-body carbohydrate metabolism. In fact, the metabolism and/or the accumulation of lipid in liver, muscle, and β-cells appears to play a greater role than frank obesity in the development of insulin resistance. For example, impaired insulin-stimulated glucose disposal can be induced in virtually any randomly selected nondiabetic, healthy subject in a few hours via lipid infusion [1,2]. Those observations suggest that nutrients have a direct and strong effect on the pathogenesis of insulin resistance [3,4].

Although it is generally accepted that increased lipid utilization by target organs readily affects the regulation of carbohydrate homeostasis, there is uncertainty as to whether free fatty acids (FFAs), fatty acyl–coenzyme As, diacylglycerols, and/or other products (eg, changes in the concentration of citric acid, shifts in the intracellular redox state, etc) are responsible for impairing insulin action [5,6]. Furthermore, it is unclear as to whether the exposure of one tissue vs another to excess lipids is more important in the development of insulin resistance. Although numerous studies have examined possible mechanisms of lipid-induced toxicity in target organs, our interpretation regarding the relationship(s) between lipids and insulin resistance leads us to ask, "Why are excess lipids available for use by liver, muscle, β-cell, etc?" We formulated the

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general hypothesis that if lipids are properly stored in adipose tissue, until needed, insulin resistance will not develop. Indeed, Bluher et al [7] have demonstrated that increased FFAs appear to be a critical first step in the pathogenesis of impaired glucose tolerance, as elevated fatty acids were observed before changes in tumor necrosis factor-α, insulin-like growth factor–I, growth hormone, and angiotensin II.

Recent work by our laboratory suggests a novel role for white adipose tissue in maintaining daily lipid flux [8]. For example, although the apparent $t_{1/2}$ of stored triglycerides is seemingly long (~20 days) in normal mice, we found that there is a relatively large flux of lipid(s) in/out of epididymal fat pads; the absolute rate of triglyceride turnover is proportional to the daily lipid intake. These observations agree with the seminal work of Schoenheimer and Rittenberg [9] and the more recent hypothesis proposed by Frayn [10] regarding the importance of adipose tissue in managing daily lipid flux. In addition, we have found comparable and extensive rates of triglyceride turnover regardless of whether mice were fed a low-fat (LF) diet or a carbohydrate-free diet [11], implying that glucose availability is not immediately a limiting factor in triglyceride synthesis in vivo.

We initiated a study to determine the influence of diet-induced changes in fat mass (or altered adiposity) on the regulation of intracellular lipolysis via epinephrine and insulin in isolated adipocytes. To examine the impact of temporal changes in weight gain on the regulation of lipid flux, C57BL/6J mice were fed an LF or a high-fat (HF) diet for up to 12 weeks; and the responses to hormonal stimuli were determined. Although many investigators agree that the control of lipolysis is impaired in obese subjects and that increased lipolysis is implicated in the development of insulin resistance, this notion becomes less clear in terms of biological meaning when data are normalized against different parameters. For example, Bougneres et al [12] reported that basal rates of lipolysis were greater in obese children; however, when the data were expressed per unit of fat mass, obese children had ~32% less lipolysis than lean controls. Therefore, the use of our ex vivo model allowed us to normalize the data against different end points and to determine the physiologic response of individual cells vs an entire organ.

2. Materials and methods

2.1. Chemicals and supplies

Unless specified, all chemicals and reagents were purchased from Sigma-Aldrich (St Louis, MO). Diets were purchased from Research Diets (New Brunswick, NJ). Epinephrine hydrochloride (±) was used in all incubations. Insulin (human Novolin) was purchased from Henry Schein (Melville, NY). Round-bottom polystyrene culture test tubes were purchased from Fisher Scientific (Rockville, MD).

2.2. Biological experiments

The C57BL/6J mice (male, 5 weeks old) were purchased from Jackson Laboratories (Bar Harbor, ME). On arrival, all mice were placed on the high-carbohydrate, LF diet (#D12450B; kilocalorie distribution equals to 10% fat, 70% carbohydrate, and 20% protein) for 5 days to acclimate. Next, mice were split either to continue on the LF diet or to switch to a low-carbohydrate, HF diet (#D12451; kilocalorie distribution equals to 45% fat, 35% carbohydrate, and 20% protein) for 1, 4, or 12 weeks. The night before each experiment, mice were individually housed and fed ~50% of their normal daily intake (ie, ~1 g of food). Note that we used a "semifasted" state because a complete overnight fast would have substantially decreased the mass of the fat pad and thus made it difficult to obtain a sufficient amount of material for a given experiment, especially in the LF-fed groups.

On the day of the study, mice were weighed and killed by cervical dislocation. Blood was collected via cardiac puncture; plasma was separated and frozen (It should be noted that cervical dislocation affects blood clotting; ie, the heart can stop beating or go into fibrillation. Consequently, in some mice, we could only obtain ~150 μL of sample.). Both epididymal fat pads were dissected, weighed, and rinsed with ice-cold Krebs bicarbonate buffer (KRB) containing 10 mmol/L HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and 10 mmol/L glucose (pH 7.4). Fat pads were pooled from a given mouse; the tissue was blotted dry, minced, and digested for 30 minutes in KRB containing 2% bovine serum albumin (fatty acid free, BSA) and collagenase type II (1 mg/mL) in a shaking water bath (80 rpm, 37°C) [13]. After digestion, the plastic flask was gently swirled several times; and the adipocytes were filtered from the undigested tissue through 240-μm nylon mesh (Sefar America, Depew, NY). Adipocytes were let to stand to float, and the collagenase buffer was aspirated. Adipocytes were then washed 3 times with fresh collagenase-free KRB-BSA buffer before being resuspended in a known volume of KRB-BSA buffer with 10 mmol/L glucose; because we aimed to incubate ~250 000 cells per tube, the adipocyte suspension was counted using a hemocytometer immediately before splitting into separate plastic culture tubes (Fisherbrand, Fisher Scientific) for studies (see below). Using this preparation, it was possible to study each mouse as an "n of 1"; that is, enough adipocytes were isolated to allow either the epinephrine-stimulation studies or the insulin-inhibition studies (see below). It should be noted that adipose tissue contains various cell types, for example, adipocytes, progenitor cells, endothelial cells, and macrophages. Although digestion with collagenase and subsequent washing are sufficient to liberate adipocytes and remove

virtually all contaminating cells (ie, nonadipocytes), our method was not able to separate large and small fat cells.

2.2.1. Stimulation of intracellular lipolysis by epinephrine

Aliquots containing 200 μL of a well-mixed adipocyte suspension (~250 000 cells per incubation) were added to 200 μL of KRB-BSA buffer + 10 mmol/L glucose and incubated with increasing concentrations of epinephrine (0 to 10 μmol/L final concentration) for 1 hour in a gently shaking water bath (80 rpm, 37°C). Note that the "0-mmol/L" sample serves as a control to account for basal lipolysis. The incubation was stopped by the addition of 500 μL of chloroform. The sample was vortexed and centrifuged to separate lipolytic products, that is, the aqueous phase containing glycerol and genomic DNA and the chloroform phase containing released fatty acids and intracellular triglycerides. Samples were frozen until further analyses.

2.2.2. Inhibition of epinephrine-stimulated lipolysis by insulin

Aliquots containing 200 μL of a well-mixed adipocyte suspension (~250 000 cells per incubation) were added to 200 μL of KRB-BSA buffer + 10 mmol/L glucose. Cells were incubated in the presence of epinephrine at the EC_{50} for a given group (determined from the experiments outlined above). To inhibit epinephrine-stimulated lipolysis, increasing concentrations of insulin (up to 10 nmol/L) were added to the incubations and let run for 1 hour in a shaking water bath (80 rpm, 37°C). The incubation was stopped by the addition of 500 μL of chloroform. The sample was vortexed and centrifuged to separate lipolytic products, that is, the aqueous phase containing glycerol and genomic DNA and the chloroform phase containing released fatty acids and intracellular triglycerides. Samples were frozen until further analyses.

2.3. Analytical procedures

Rates of lipolysis were calculated from the release of lipolytic products, that is, glycerol and FFAs. The concentrations of glycerol and FFAs were determined spectrophotometrically using free glycerol reagent (Sigma) and nonesterified fatty acid C (Wako Chemicals, Richmond, VA) kits, respectively. Glycerol was determined by reacting 20 μL of the aqueous sample phase, and FFAs were determined by evaporating 20 μL of the chloroform phase to dryness and redissolving using the nonesterified fatty acid kit. The concentration of genomic DNA was determined using a fluorometric assay by reacting 15 μL of aqueous sample phase with PicoGreen (Molecular Probes, Carlsbad, CA). Plasma glucose and insulin were determined using commercially available kits from Sigma and Linco (St. Charles, MO), respectively. In all cases, the concentrations were determined by comparing against assays of known standards.

2.4. Calculations

We have used various end points to normalize the rates of hydrolysis of intracellular triglycerides. Lipolysis data were calculated using 3 equations:

$$\text{Intracellular lipolytic rate} = \text{nanomoles glycerol/micrograms DNA} \times \text{time} \quad (1)$$

$$\text{Fractional lipolytic rate} = \text{nanomoles glycerol/grams triglyceride} \times \text{time} \quad (2)$$

$$\text{Lipolytic potential}* = \text{intracellular lipolytic rate} \times \text{total micrograms DNA} \quad (3)$$

*We calculated the total micrograms of adipocyte DNA as follows. First, the ratio of triglyceride to DNA was determined in isolated adipocytes. Second, we determined the quantity of triglyceride per gram of adipose tissue. As demonstrated by Rodbell [13], virtually all of the triglyceride in adipose tissue is present in adipocytes; therefore, the total adipocyte DNA can be estimated using the equation:

$$\text{Fat pad (grams)} \times (\text{grams triglyceride/grams fat pad}) \times (\text{micrograms DNA isolated cells/grams triglyceride isolated cells}) \quad (4)$$

Although this exact equation is not used in the literature, Di Girolamo and coworkers [14,15] have used a conceptually similar approach. Namely, the assumption with this logic is that adipocytes within a tissue should be relatively homogeneous in size, that is, a relatively constant ratio of triglyceride to DNA. Data in the literature suggest that that assumption is reasonable under conditions similar to ours; for example, intraorgan adipocyte cell size (which reflects the ratio of triglyceride to DNA) is generally stable in animals once they reach maturity, provided that they are not subjected to starvation [14-17]. In cases of overnutrition (eg, HF feeding), one expects that new (smaller) fat cells will be rapidly filled, which leads to a relatively homogeneous population. However, we suspect that the validity of Eq. (4) may be questionable in conditions where markedly heterogeneous samples of adipocytes are expected, for example, during treatment with peroxisome proliferator–activated receptor–γ agonists.

The lipolytic rate data were best fit to sigmoidal curves using Origin (OriginLab, Northampton, MA); initial (basal) rate, maximal rate, and the EC_{50} values for the respective studies were determined.

The percentage of suppression of epinephrine-stimulated lipolysis by insulin was calculated using the equation:

$$\left([\text{LP}]\text{EPI}_{\text{x}\mu\text{mol/L}+\text{INS}z\text{nmol/L}} - [\text{LP}]\text{EPI}_{0\mu\text{mol/L}}\right) / \left([\text{LP}]\text{EPI}_{\text{x}\mu\text{mol/L}} - [\text{LP}]\text{EPI}_{0\mu\text{mol/L}}\right)] \times 100, \quad (5)$$

where [LP] is the concentration of the lipolytic product (eg, glycerol) at a given dose of epinephrine ($EPI_{x\ \mu mol/L}$) or

Table 1 Characteristics of C57BL/6J mice

Dietary intervention	Weight (g)	Glucose (mmol/L)	Insulin (pmol/L)	Epididymal fat (% body weight)	Epididymal adipocytes		
					Lipid (g)	DNA (μg)	Lipid to DNA ratio
1 wk							
LF	23 ± 1	7.92 ± 0.16	59 ± 5	1.8 ± 0.2	0.32 ± 0.08	24 ± 5	0.013 ± 0.002
HF	24 ± 2	8.14 ± 0.24	65 ± 8	2.1 ± 0.3	0.41 ± 0.06	28 ± 4	0.015 ± 0.001
4 wk							
LF	24 ± 2	7.88 ± 0.22	66 ± 9	2.1 ± 0.3	0.39 ± 0.07	30 ± 5	0.013 ± 0.002
HF	27 ± 1*	8.67 ± 0.33	113 ± 13*	3.0 ± 0.3*	0.72 ± 0.12*	42 ± 5*	0.019 ± 0.003*
12 wk							
LF	27 ± 2	8.21 ± 0.36	75 ± 10	2.4 ± 0.4	0.49 ± 0.11	34 ± 4	0.014 ± 0.002
HF	34 ± 3*	8.39 ± 0.18	139 ± 18*	4.8 ± 0.6*	1.50 ± 0.18*	53 ± 8*	0.029 ± 0.005*

Animals were fed a high-carbohydrate, LF diet vs a low-carbohydrate, HF diet for 1, 4, or 12 weeks. In all cases, data are shown as mean ± SEM; n = 5 to 8.
* $P < .01$ LF vs HF at same time.

epinephrine + insulin ($EPI_{x\ \mu mol/L}$ + $INS\ z\ nmol/L$) at the respective concentration of insulin (z, 0 to 10 nmol/L insulin).

Unless noted, data are presented as mean ± SEM; n = 5 to 8 experiments. Statistical tests were performed using 2-tailed t tests and assuming equal variance on time-matched samples. Note that we did not attempt longitudinal comparisons (ie, LF at weeks 1 vs 4 vs 12 or HF at weeks 1 vs 4 vs 12) because the respective groups were studied at different times.

3. Results

Table 1 demonstrates that prolonged HF feeding leads to a progressive increase in adiposity. For example, after 1 week, there were no differences in either the body weight or the fat pad weight in mice fed the HF vs the LF diet. However, 4 weeks of HF feeding led to a ~12% increase in body weight ($P < .01$ vs LF diet) and a ~45% increase in epididymal fat mass (expressed as percentage of body weight, $P < .01$). As expected, the effect of the HF diet became more pronounced after 12 weeks of intervention; that is, mice fed the HF diet gained ~25% more body weight than those fed the LF diet ($P < .01$), with a ~100% increase in the amount of epididymal fat (expressed as percentage of body weight, $P < .01$). In parallel with changes in body mass and epididymal fat pad mass, we observed an increase in circulating concentrations of insulin with no change in circulating concentrations of glucose when comparing mice fed the HF vs LF diet (Table 1).

Fig. 1 demonstrates the sensitivity of intracellular lipolysis to epinephrine; data are expressed as glycerol released per microgram DNA, that is, the lipolytic rate per cell. In all cases, there was a sigmoidal response to epinephrine stimulation beginning at a dose of 0.025 μmol/L and reaching a plateau between 1.0 and 10 μmol/L. There were no differences between lipolytic profiles of mice fed the LF vs the HF diet except at 10 μmol/L of epinephrine.

Fig. 2 demonstrates the fractional lipolytic rates, that is, glycerol released per total triglyceride. There were no differences in the fractional degradation of the triglyceride pool unless mice were fed the HF diet for 12 weeks (Fig. 2C), where the trend is for slower rates of lipolysis in HF-fed mice.

Fig. 3 demonstrates the lipolytic potential of the epididymal fat depot, that is, the amount of glycerol that

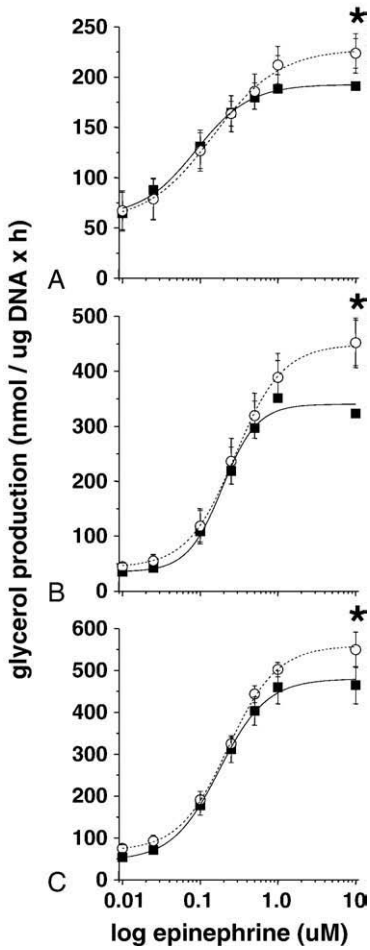

Fig. 1. Effect of diet-induced adiposity on the lipolytic activity of isolated adipocytes. The dose-response profile of lipolysis to epinephrine was determined by measuring the rate of glycerol production and normalizing to the amount of genomic DNA in mice fed an LF (closed symbols) vs an HF (open symbols) diet for 1, 4, or 12 weeks (*$P < .05$; A, B, and C, respectively) (see Eq. [1], "Materials and methods").

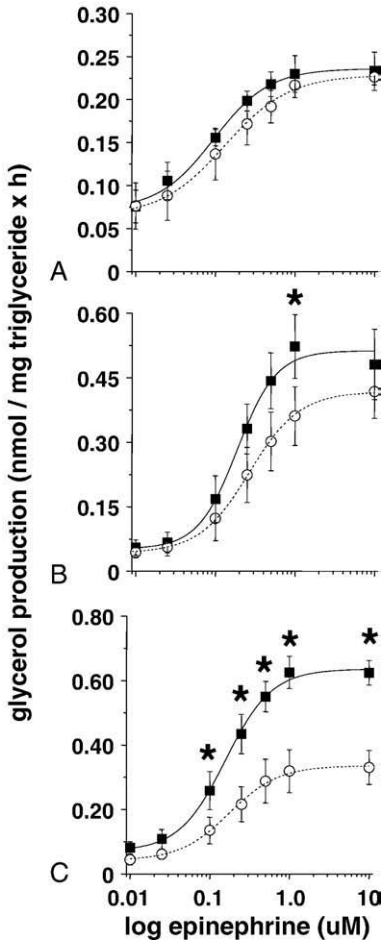

Fig. 2. Effect of diet-induced adiposity on the fractional rates of intracellular lipolysis. The dose-response profile of lipolysis to epinephrine was determined by measuring the rate of glycerol production and normalizing to the quantity of epididymal triglyceride in mice fed an LF (closed symbols) vs an HF (open symbols) diet for 1, 4, or 12 weeks (*$P < .05$; A, B, and C, respectively) (see Eq. [2], "Materials and methods").

would be released from the entire depot. After 1 week of HF feeding, there was no difference between the lipolytic potential regardless of whether mice were fed the LF vs the HF diet, whereas the lipolytic potential was markedly increased after 4 and 12 weeks of HF feeding. This observation of an increase in the lipolytic potential of the organ is consistent with the fact that HF feeding and diet-induced obesity in C57BL/6J mice lead to hypertrophy and hyperplasia [18-21].

The data shown in Fig. 1 were used to determine the dose of epinephrine that resulted in a 50% maximum activation of lipolysis (EC_{50}) for each group; this dose of epinephrine was used to examine the insulin-mediated suppression of epinephrine-stimulated lipolysis. Fig. 4 demonstrates the percentage of suppression of lipolysis by insulin in mice fed the HF vs the LF diet for 4 weeks (see "Materials and methods" for calculations). The rate of lipolysis (per adipocyte) was maximally suppressed at ~1 nmol/L insulin in adipocytes from LF-fed mice. Furthermore, insulin completely suppressed epinephrine-stimulated lipolysis in LF-fed mice; for example, ~90% to 95% suppression was observed at ~1 and 10 nmol/L insulin.

The data shown in Fig. 4, regarding HF-fed mice, demonstrate 2 points regarding an impairment in insulin-mediated suppression of epinephrine-stimulated lipolysis at most doses of insulin ($P = .10$ at 0.005 nmol/L insulin). First, it was not possible to completely suppress epinephrine-stimulated lipolysis in adipocytes from HF-fed mice; that is, rates of epinephrine-stimulated intracellular lipolysis could only be suppressed by ~75%. Second, the data demonstrate that the $K_{i\ 50}$ for insulin shifted from ~0.03 to ~0.09 nmol/L in LF- vs HF-fed mice. Note that we did not observe any impairment in insulin action on total lipolysis after 1 week of HF feeding, whereas 12 weeks of HF feeding had an effect that was comparable to what we observed after 4 weeks of HF feeding (not shown). By comparing the rates of FFA and glycerol release, we could estimate rates of FFA reesterification;

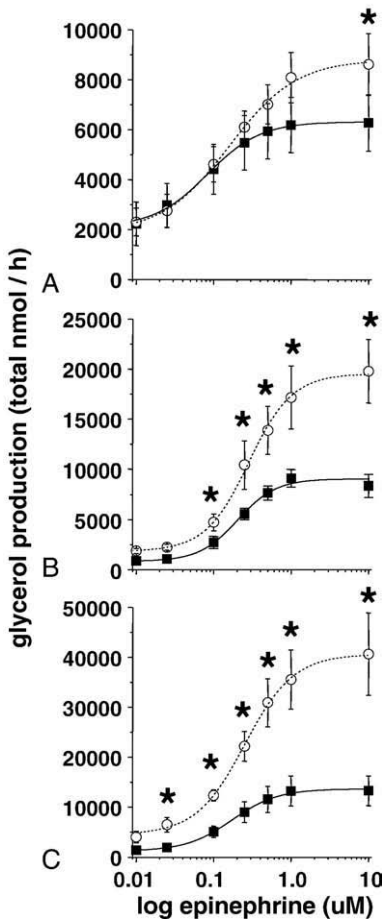

Fig. 3. Effect of diet-induced adiposity on the lipolytic potential of the epididymal fat depot. The dose-response profile of lipolysis to epinephrine was determined by measuring the rate of glycerol production per microgram DNA and multiplying by the total amount of genomic DNA in mice fed an LF (closed symbols) vs an HF (open symbols) diet for 1, 4, or 12 weeks (*$P < .05$; A, B, and C, respectively) (see Eq. [3], "Materials and methods").

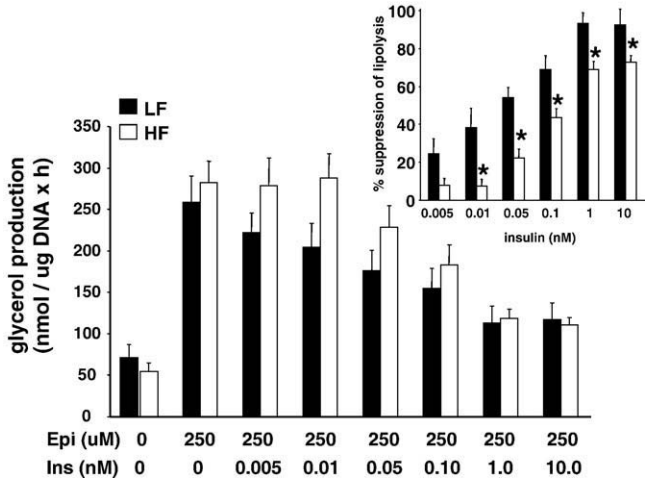

Fig. 4. Effect of diet-induced adiposity on insulin-mediated inhibition of epinephrine-stimulated lipolysis. The dose-response profile of lipolysis to epinephrine ± insulin was determined by measuring the rate of glycerol production per microgram DNA in mice fed an LF (closed bars) vs an HF (open bars) diet for 4 weeks. The rate of glycerol release was determined as a marker of lipolysis (main panel); the inset contains data calculated as the percentage of suppression of lipolysis (*$P < .05$) (see Eq. [5], "Materials and methods").

stimulation with epinephrine (250 μmol/L) led to ratios of 2.6 ± 0.3 and 2.7 ± 0.4 in LF and HF mice, respectively, whereas the addition of insulin (250 μmol/L epinephrine + 10 nmol/L insulin) decreased the ratios to 2.1 ± 0.2 and 2.3 ± 0.2 in LF and HF mice, respectively. Although the directional changes in reesterification are in agreement with the general effects of the respective hormones—that is, the addition of epinephrine decreased the rates of reesterification (the ratio of FFA to glycerol approached 3:1), and the addition of insulin stimulated the rates of reesterification (the ratio of FFA to glycerol decreased)—we did not observe differences between the dietary groups.

4. Discussion

Our overall objective was to investigate the effect(s) of diet-induced changes in fat mass on the response of intracellular lipolysis to hormonal stimuli [22,23]. Efforts were directed toward quantifying (1) the stimulation of lipolysis by epinephrine (a general stress hormone that activates β- and α_2-adrenergic receptors [24-26], unlike isoproterenol [27-29], cyclic adenosine monophosphate analogs [30,31], or other selective agents [32]) and (2) the suppression of epinephrine-stimulated lipolysis by insulin [3,10,23,24,32,33]. The ability to use an ex vivo model allowed us to normalize data in different ways and to therefore draw conclusions that are generally not possible during in vivo studies. Attention was directed toward studies of epididymal fat because this depot generally changes in the same direction and magnitude as total body fat during HF feeding [34] and we have data from in vivo studies that suggest that changes in epididymal fat lipolysis reflect alterations in whole-body lipolysis [35,36]. Thus, the general lessons learned from our in vitro studies might be applicable to the in vivo setting.

Surwit and colleagues [18,20,37,38] have established the use of HF feeding in C57BL/6J mice as a model of diet-induced adiposity that is associated with the development of hypertension, insulin resistance, glucose intolerance, and type 2 diabetes mellitus (which can be corrected by switching to an LF diet [39]). Others have reported specific time-dependent changes in metabolic and hormonal parameters that result after HF feeding in C57BL/6 mice. For example, Almind and Kahn [40] demonstrated rapid weight gain and hyperinsulinemia in as early as 2 weeks of HF feeding, whereas Lin et al [41] demonstrated early leptin resistance in mice fed an HF diet. Park et al [42] demonstrated decreased glucose uptake, insulin resistance, and lipid accumulation in nonadipose tissues in as early as 1 to 3 weeks in C57BL/6J mice fed an HF diet. Our data regarding the time course for the onset of diet-induced adiposity are consistent with the literature; for example, we observed hyperinsulinemia after 4 weeks of HF feeding that was associated with a substantial increase in epididymal fat mass (Table 1). However, in our practical experience with this model, we have found an inconsistent phenotype regarding the development of hyperglycemia and "type 2 diabetes mellitus." For example, in earlier studies where we fed the respective diets for ~8 weeks, fasting blood glucose was ~2-fold greater in HF- vs LF-fed mice (ie, ~12 vs ~7 mmol/L, respectively; unpublished observations). It is not immediately obvious as to why we did not observe a stronger effect of HF feeding on blood glucose concentration in the current study (Table 1). One possible explanation is that mice were semifasted the night before the study, which could blunt any subtle differences between LF- and HF-fed mice [43]. Another explanation is a variability in the time-dependent metabolic profile between studies. The fact that we did not observe differences between fasting glucose concentrations does not limit our study; this observation suggests that overt impairments in carbohydrate homeostasis may develop later in the progression of HF-diet–induced adiposity.

Our first aim was to determine whether the activation of intracellular lipolysis by epinephrine is altered in diet-induced adiposity. We found that the stimulation of intracellular lipolysis via increasing doses of epinephrine led to a progressive increase in the rate of glycerol production (a marker of triglyceride hydrolysis, Fig. 1). Regardless of the group that was studied, we observed a sigmoidal dose-response curve that spanned the physiologic range of epinephrine [44,45]. These data demonstrate that the initial development of diet-induced adiposity is not characterized by an inability to mobilize stored lipids. For example, the addition of epinephrine resulted in a comparable stimulation of lipolysis in virtually all paired groups (Fig. 1 and Table 2); in all cases, parallel increases in the release of FFAs were observed (not shown).

Table 2
Characteristics of adipocytes during epinephrine stimulation

Dietary intervention	Intracellular lipolysis (nmol glycerol/μg DNA x h)		EC_{50} (μmol/L)
	Initial rate	Maximal rate	
1 wk			
LF	62 ± 9	193 ± 8	0.09 ± 0.01
HF	55 ± 17	228 ± 21	0.14 ± 0.07
4 wk			
LF	33 ± 6	349 ± 31	0.20 ± 0.04
HF	43 ± 8	449 ± 44	0.28 ± 0.08
12 wk			
LF	46 ± 4	480 ± 35	0.18 ± 0.04
HF	69 ± 6	558 ± 31	0.22 ± 0.03

The C57BL/6J mice were fed a high-carbohydrate, LF diet vs a low-carbohydrate, HF diet for 1, 4, or 12 weeks. Adipocytes were isolated, and the release of glycerol was used to determine the rates of intracellular lipolysis and the sensitivity to epinephrine (EC_{50}, expressed as micromoles per liter). In all cases, data are shown as mean ± SEM; n = 5 to 8.

Our observations regarding the stimulation of lipolysis by epinephrine (Fig. 1 and Table 2) differ from the work of Collins et al [37,46] who reported markedly decreased lipolytic rates in both HF (diet-induced) and genetic models of obesity. In those studies, a common molecular mechanism was suggested, that is, a decrease in the expression of β-adrenergic receptors. There are certain differences worth noting regarding our experimental design and that of Collins et al [37,46]. First, they fed an HF diet with a caloric density of 5.5 kcal/g for 16 weeks, whereas in our studies, mice were fed an HF diet with a caloric density of 4.2 kcal/g for up to 12 weeks. Consequently, it is possible that the reduced fat content and/or the shorter duration of feeding affected the phenotype. In addition, Collins et al considered the expression of adrenergic receptors and adenylate cyclase activity as end points, whereas we have measured the release of glycerol; it may be that a given change in a signal transducer(s) (eg, receptors and/or small molecules) does not lead to an identical change in biochemical flux. Our data (Fig. 1) are supported by Hubbard and Matthew [47] who found that the rates of norepinephrine-stimulated lipolysis were initially proportional to the cell number and that the reductions in lipolytic stimulation only became apparent as adipocytes became larger. Taken together, these observations suggest that (1) intracellular lipolytic responses do not change until later in the development of obesity and (2) the development of certain forms of obesity may cause "adrenergic resistance," contrary to the interpretation that reduced mobilization of lipids is the cause of excess lipid accumulation.

Our second aim was to determine whether insulin resistance develops in the adipocyte during the development of diet-induced adiposity. As expected, incubation with insulin led to a dose-dependent reduction in the rate of epinephrine-stimulated lipolysis in adipocytes from mice fed an LF diet. Namely, physiologic concentrations of insulin (~1 nmol/L) completely suppressed intracellular lipolysis in the LF-fed group (Fig. 4, inset); that observation is consistent with other reports regarding the dose-response relationship between insulin and the suppression of lipolysis [21,32,42,48]. However, HF feeding leads to marked impairments in insulin action (Fig. 4). For example, we observed a decrease in the ability of insulin to maximally suppress lipolysis and an increase in the $K_{i\ 50}$; that is, the dose-response curve was shifted down and to the right (Fig. 4, inset). It should be noted that, in all cases, similar effects were observed regarding the release of FFAs. The development of impaired insulin action in adipose tissue parallels the development of hyperinsulinemia; for example, we observed a reduction in insulin sensitivity in adipocytes after 4 and 12 weeks of dietary intervention but not after 1 week, which corresponds to the times when we observed an elevation in plasma insulin concentrations (Table 1). It is important to note that the insulin resistance appears to be specific for the degradation of triglycerides because similar rates of reesterification were observed in the presence of epinephrine ± insulin.

As noted earlier, the use of an ex vivo model allowed us to normalize the data against different end points. Thus far, we have considered the interpretation of data when normalized per DNA, allowing us to examine adipocyte function, albeit in a presumably mixed cell population containing small, medium, and large adipocytes. Although we were not able to determine the range of cell size(s) within a given depot, it is evident that the overall ability to stimulate lipolysis is not altered (Fig. 1), whereas the overall ability to inhibit lipolysis becomes impaired (Fig. 4). Because diet-induced changes in adiposity are characterized by an increase in cell number and cell size, it is of interest to consider the meaning of alternative interpretations. For example, investigators have concluded that rates of lipolysis are lower in obese vs lean subjects, implying that obesity may be characterized by an inability to mobilize stored lipid [12]. Those conclusions are often reached in in vivo studies of lipolysis because data (ie, rates of glycerol production) are normalized against total adipose mass. Our data agree with that concept when we normalize the rate of lipolysis against the total amount of triglyceride (which is proportional to the total mass of the tissue). For example, the *fractional rate of triglyceride degradation* (Eq. [2]) is lower in adipocytes from HF-fed mice (Fig. 2C). However, we believe that it is misleading to conclude that lipolytic rates are truly lower in our study because the absolute amount of triglyceride being hydrolyzed is comparable per cell (Fig. 1).

The ability to remove the entire adipose depot and to determine the total adipocyte DNA (a measure of the total number of adipocytes) allowed us to estimate the lipolytic potential of the organ (Fig. 3). The greater total DNA content in HF-fed mice is consistent with the known effect of HF feeding on adipocyte hyperplasia in C57BL/6J mice [18,21]. Based on these observations, we believe that in vivo studies of lipolysis require a careful (re)interpretation because those

studies often do not account for the fact that increased adipose mass in obesity is partially affected by hyperplasia. Presumably, a more accurate measure of adipose function is obtained when one expresses rates of flux against DNA (a marker of cell number). Therefore, contrary to remarks regarding "lower rates of lipolysis in obesity," our data suggest that absolute rates of lipolysis are increased in the early stages of diet-induced obesity because there is an increase in the total number of cells per epididymal fat depot (Fig. 3) and because those cells respond normally to epinephrine (Fig. 1).

In summary, HF feeding in mice, which leads to changes in fat mass, promotes greater rates of lipolysis. This is mediated by an increase in the number of fat cells and not by a primary abnormality in their sensitivity to stimulation by epinephrine. In addition, HF feeding leads to a primary defect in insulin-mediated suppression of epinephrine-stimulated lipolysis in adipocytes. The combined influence of these 2 alterations on the control of intracellular lipolysis suggests a mechanism(s) that (partly) explains the increased exposure of target organs to lipid-derived energy substrates, for example, FFAs and glycerol.

We conclude by briefly raising a question/hypothesis regarding the interaction(s) between pool sizes and hormone concentrations, the latter of which may be the target of a pharmaceutical agent. Our work implies that if a therapeutic intervention lowers the concentration(s) of a stress hormone(s) in an obese subject to the concentration (s) seen in a healthy lean subject, the physiologic effect may be less than ideal. For example, as the adipocyte pool increases, the stress hormone(s) will exert a more pronounced effect. In addition, at a given concentration of insulin, it is expected that one will observe an increased lipolytic flux in cases where the pool of adipocytes is increased. Clearly, however, our data must be carefully interpreted as applying during the initial development of obesity, as other factors will likely come into play later in the progression of disease.

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