Leptin secretion after a high-fat meal in normal-weight rats: strong predictor of long-term body fat accrual on a high-fat diet


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Leptin secretion after a high-fat meal in normal-weight rats: strong predictor of long-term body fat accrual on a high-fat diet. Am J Physiol Endocrinol Metab 290: E258–E267, 2006; doi:10.1152/ajpendo.00609.2004.—The objective of this study was to investigate meal-related endocrine changes in lean and their differential weight gain on a high-calorie diet. Early weight gain or dietary fat preference and long-term obesity (4, 7, 46, 70). Dietary paradigms, which have particular relevance to human obesity, have also allowed investigators to define subpopulations of outbred strains on high-calorie mixed diets or on pure macronutrient diets. Although these studies have revealed significant correlations between measurements of early weight gain or dietary fat preference and long-term body fat accrual, the OP rats identified by these markers generally reach a higher body weight or require significant dietary fat exposure before they are ready for investigation along with OR rats (11, 15, 26, 34, 45, 54, 64).

A simple blood collection, using a chronic cardiac catheter or perhaps a tail vein puncture, may be the easiest way of probing an animal in a truly preobese state and on a low-fat diet. This technique can reveal small endocrine changes in response to acute challenges and thus may allow one to detect subtle differences in animals at normal weight that are ultimately different in their propensity toward obesity. Early studies using this approach have revealed an exaggerated cephalic phase insulin response to a glucose challenge in OP rats compared with OR rats (5, 47). Also, in more recent investigations, OP rats are found to exhibit elevated levels of norepinephrine in response to glucose injection (30). In clinical studies, normal-weight offspring of parents who are obese show greater insulin levels after infusions of β-endorphin (13). This evidence gives promise to the possibility that subtle endocrine responses that are markers of long-term body fat accrual can be detected in a preobese state in response to an experimental challenge.

Leptin secretion after a high-fat meal may provide a more natural challenge that can identify animals, of similar body weight, that subsequently show a differential propensity toward obesity on a chronic high-fat diet. In already-obese animals compared with their lean counterparts, basal or fasting levels of hormones and metabolites are generally different. The obese exhibit hyperinsulinemia, often associated with elevated leptin, glucose, triglycerides, galanin; neuropeptide Y; and then investigated for mechanisms that contribute to their excessive body fat accrual on a high-fat diet.

In three separate experiments, the HFM-induced rise in leptin was found to be the strongest, positive correlate ($r = +0.58, +0.62$ and $+0.64$) of long-term body fat accrual. The lowest (2–5 ng/ml) vs. highest (6–9 ng/ml) scores for this post-HFM leptin measurement identified distinct OR and OP subgroups, respectively, when they were similar in body weight (340–350 g), premeal leptin (2.6–3.4 ng/ml), and meal size (40 kcal). Subsequent tests in these normal-weight OP rats revealed a distinct characteristic compared with OR rats, namely, exaggerated HFM-induced rise in expression of the orexigenic peptide galanin in the paraventricular nucleus. Thus, with this HFM-induced leptin measurement, OP rats can be identified while still at normal weight and then investigated for mechanisms that contribute to their excessive body fat accrual on a high-fat diet.

Insulin; triglycerides; galanin; neuropeptide Y...
MATERIALS AND METHODS

particularly responsive to the consumption of a high-fat diet
sion of the orexigenic peptide galanin (GAL), known to be
and OP subgroups at normal weight with differential expres-
pared with lean rats. They identified the measurement of leptin
on a low-fat diet but subsequently exhibited differential weight
of obese vs. those that remained lean while on a chronic high-fat
typical metabolic disturbances typically seen in obese subjects.
Building on these investigations, the present study used blood collection techniques to examine meal-induced endo-
acidine changes. This was performed first in rats that had become
vs. those that remained lean while on a chronic high-fat diet,
and then in OP vs. OR rats that were still of normal weight on
a low-fat diet but subsequently exhibited differential weight
while switched to a high-fat diet. The results of these experi-
ments demonstrated HFM-induced endocrine disturb-
ances in normal-weight OP animals compared with OR rats,
which were similar to those seen in already-obese rats
pared with lean rats. They identified the measurement of leptin
after an HFM as the strongest positive correlate of long-term body fat accrual in animals with normal premeal levels. More-
over, they showed this measurement to identify distinct OR
and OP subgroups at normal weight with differential expres-
ion of the orexigenic peptide galanin (GAL), known to be
particularly responsive to the consumption of a high-fat diet
(27, 28).

MATERIALS AND METHODS

Animals and diets. Adult, male Sprague-Dawley rats (220–240 g; Charles River Breeding Labs, Kingston, NY) were individually housed (22°C, with lights off at 13:00 PM for 12 h) in a fully accredited American Association for the Accreditation of Laboratory Animal Care facility, according to institutionally approved protocols as spec-
ified in the NIH Guide to the Use and Care of Animals and with the approval of the Rockefeller University Animal Care Committee. All animals were given 7–10 days to acclimate to laboratory conditions, during which time they were maintained ad libitum on lab chow and water. All protocols fully conformed to the “Guiding principles for research involving animals and human beings” (1). The rats were maintained ad libitum either on lab chow or on a high-fat diet. The constituents of this high-fat diet (5.15 kcal/g) described in detail elsewhere (15, 16) were 50% fat, consisting of 80% lard (Armour), and 20% vegetable oil (Wesson); 25% carbohydrate, consisting of 30% dextrin, 30% cornstarch (ICN Pharmaceuticals), and 40% su-
crose (Domino); and 25% protein, consisting of casein (Bioserv, Frenchtown, NJ) with 0.03% 1-cysteine hydrochloride (ICN Pharma-
cueuticals), each supplemented with 4% minerals (USP XIV Salt Mixture Brigs, ICN Pharmaceuticals) and 3% vitamins (Vitamin Diet Fortification Mixture, ICN Pharmaceuticals). Food intake mea-
surements were taken weekly to confirm a stable feeding pattern.

Surgery and blood sampling procedures. Experiments 1 and 2 used rats with chronically implanted cardiac catheters. Rats weighing 325–345 g were anesthetized using pentobarbital sodium. Surgeries for implantation of the cardiac catheter were then performed with minor modifications to the techniques described by Steffens (57) and Gerozissis et al. (19). These modifications included different internal diameters (0.01–0.03 mm) and lengths (25–120 mm) of the silicone catheter. The incision of the jugular vein was made using a Student Vannas Spring Scissors, and a 5-mm² reinforced Silastic silicone sheet was sutured to the surrounding tissue to anchor the catheter in place. In addition, a smaller catheter, one with a 0.012-mm ID, was employed, as it provided the best compromise between the ease of entry into the jugular vein and the tightness of fit. Instead of the Perspex cylinder apparatus, the catheter adapter assembly consisted of 0.2-mm tubing, in which the other end of the 0.012-mm tubing was inserted, and this 0.2-mm tubing was then inserted into 0.3-mm tubing. This assembly was pulled through under the skin from the skull to the chest and fitted into a bent 20-gauge metal tube. This tube was secured with dental cement onto the skull by means of four cap screws, and it was protected by a plastic cylinder cemented around the metal tube. All animals were given 7–10 days to recover and resume stable weight gain. For the blood sampling tests, a polyethylene tube (length 200 mm, ID 0.20 mm) was connected to a counterweighted, rotating cantilever beam, which allowed the rats to move freely during the collection period. Blood samples (0.4 ml) were withdrawn both before and after a meal as described below, the samples were centrifuged at 3,000 g for 15 min, and serum was separated for measure-
mence of hormones and metabolites. To prevent hemorrhagic disturb-
ance, the total volume of blood withdrawn was replaced with an
isotonic saline solution (39).

In experiments 3–5, blood was collected using a tail vein puncture technique. For this procedure, the rats were gently placed in a plastic restrainer (Harvard Apparatus), and their tails were wrapped with a warm towel for ~10 s to facilitate blood flow. The tail vein was punctured with a 21G1 needle, and blood was allowed to drip from the other, cut-off end into a 5-ml glass tube over a period of ~45 s. Approximately 1 ml of blood was collected, yielding 0.5–0.6 ml of serum, which was stored at –80°C until use. This procedure is simple and quick, with the rats restrained for less than 1 min. They become very accustomed to this process, and the minimal stress it may cause is clearly transient, as indicated by stable levels of CORT measured at 30 and 60 min after the tail puncture.

Test procedures. In each experiment, the rat’s body weight was recorded weekly, except in experiment 4 where daily measurements were taken during the first week on a high-fat diet. In addition, endocrine measurements in relation to an HFM challenge, from blood collected via the chronic catheter or tail vein puncture, were exam-
ined. For these meal tests, the rat’s chronic lab chow diet was removed from the cage 2 h before the start of the nocturnal feeding cycle to prevent random eating just before the test. Water was available ad libum. Then, at dark onset, the rats were given a high-fat diet (50% fat) for a 2-h period. This interval was chosen because it is close to a natural intermeal interval (54) and found to reveal consistent, post-
meal changes in hormones and metabolites (28, 65). To control for the size of the test meal, each rat was given 40 kcal of the high-fat diet, and those rats that consumed fewer than 38 kcal in the 2-h interval (generally 15–20% of the total group) were eliminated from the experiment. By use of this paradigm, the rats were given seven HFM tests separated by 1–2 days. The first three were pretests designed to adapt the rats to the diet and test procedures without any blood collections. For the actual experiment, there were four additional HFM tests that were preceded and/or followed by a blood collection. In the rats implanted with a chronic cardiac catheter (experiments 1 and 2), blood samples were collected from all animals both 2 h before (premeal) and 2 h after (postmeal) the HFM. For the tests using the tail vein puncture (experiments 3–5), one premeal test was conducted at the outset to confirm that the rats had normal basal levels of the hormones and metabolites before the HFM, and this was followed by three postmeal tests with blood collected 2 h after the HFM. The blood samples collected by either method were assayed for the hormones leptin, insulin, and CORT, and the metabolites glucose, TG, and NEFA. The data calculated for each rat reflect an average of the scores for the three HFM (postmeal) blood collection tests. The validity of each HFM test was confirmed across the entire group by showing strong positive correlations, ranging from $r = +0.70$ to $r = +0.88$, between the scores for the different tests.
In experiment 1, the rats (n = 21) were first placed for 3 wk on a high-fat diet (50% fat) and then subsequently given these acute tests with the HFM. During the first and second weeks of this 3-wk period, they were implanted with the chronic cardiac catheters and given time to recover from the surgery. At the end of the 3 wk, the four HFM blood collection tests were conducted, and then the rats were killed by rapid decapitation shortly before dark onset, 3 h after food was removed. Their unilateral fat depots from three regions (inguinal, retroperitoneal, and epididymal), as well as the mesenteric fat pad, were dissected, weighed, and summed to provide a measurement of body fat. The rats were rank ordered and differentiated into lean and obese subgroups (lower and upper tertiles, respectively) based on the weight of their dissected fat pads at death, and the data obtained for the hormones and metabolites were compared with the data for the middle group of rats eliminated from the analysis. Experiment 2 used the same procedures, except that the four HFM tests with blood collected via the catheter implant were performed at the beginning of the experiment, while the rats (n = 21) were still maintained on lab chow. After these meal tests, the rats were placed for 3 wk on a chronic high-fat diet, then killed and subgrouped on the basis of their low (OR) vs. high (OP) fat pad weights, with the middle group of rats eliminated from the analysis. In experiments 3 and 4, the rats were similarly maintained on lab chow during the four HFM tests, although their blood was collected via tail vein puncture, in the first test before the meal and in the next three tests after the meal. They were then placed for 3 wk in experiment 3 (2 sets of rats, n = 30–36/group) or 5 wk in experiment 4 (n = 70) on the high-fat diet and killed for measurements of fat pad weights. The fat pad and endocrine data obtained in these two experiments were analyzed in both a retrospective and a prospective manner. In the retrospective analysis, the rats were subgrouped on the basis of their ultimate fat pad weights (lowest and highest tertile with the middle group omitted) and compared with respect to their initial endocrine measurements. In the prospective analysis, the rats were subgrouped on the basis of their endocrine measurements (lowest and highest tertile) and then compared in terms of their fat pad weights. In experiment 5, the rats (n = 30) were maintained on lab chow, given the four HFM tests, and then killed immediately after the fourth test, 2 h after presentation of the meal. Their trunk blood was collected, and brains were rapidly removed, immediately dissected, and frozen in liquid nitrogen for measurement of the hypothalamic peptides by use of real-time quantitative PCR.

Hormone and metabolite determinations. Serum from trunk blood or tail vein was assayed for insulin and leptin with RIA kits from Linco Research (St. Charles, MO). Levels of CORT were determined by RIA with a kit from Diagnostic Products. The metabolites glucose, TG, and NEFA were measured with an E-Max Microplate Reader using a glucose Trinder Reagent Kit (Sigma, St. Louis, MO), TG Assay Kit (Sigma), or NEFA C Kit (Wako Chemicals, Richmond, VA), respectively. The hormone and metabolite assays were performed at different times for the different experiments. The intra- and interassay coefficients of variation (%) are as follows: TG assay 1.25 and 1.6; glucose assay 5.6 and 8.4; NEFA assay 1.1 and 2.1; CORT assay 7.1 and 7.2; leptin assay 4.1 and 3.0; and insulin assay 4.3 and 8.5.

Real-time quantitative PCR. Immediately after the animals were killed, the brains removed for peptide measurements using real-time quantitative PCR were placed in a matrix with the ventral surface facing up, and three 1.0-mm coronal sections were made, with the middle optic chiasma as the anterior boundary. The sections were placed on a glass slide, and two hypothalamic areas, the paraventricular nucleus (PVN, Bregma −1.3 to −2.1 mm) and the arcuate nucleus (ARC, Bregma −2.56 to −3.3 mm), were rapidly microdissected under a microscope, using the fornix and third ventricle as landmarks. The PVN was dissected as a reversed isosceles triangle, 1.0 mm bilateral to the ventricle and between the fornix structures, as described (10). For the ARC, the area adjacent to the bottom of the third ventricle was dissected parallel to the border of the ventricle, with the width of 0.1 mm at the top gradually widening to 0.3 mm at the bottom. These dissections were immediately frozen in liquid nitrogen and stored at −80°C until processed.

As previously described (10), total RNA from pooled microdissected PVN or ARC samples was extracted with TRIzol reagent. RNA was treated with RNase-free DNase I to remove any contaminating genomic DNA before reverse transcription. cDNA and minus RT were synthesized using an oligo(dT) primer with or without SuperScript II RT. The SYBR Green PCR core reagents kit (Applied Biosystems) was used, with β-actin as endogenous control. PCR was performed in MicorAmp Optic 96-well reaction plates (Applied Biosystems) on an ABI PRISM 7700 Sequence Detection system (Applied Biosystems), with the condition of 2 min at 50°C, 10 min at 95°C, and then 40 cycles of 15 s at 95°C and 1 min at 60°C. Each study consisted of four independent runs of PCR in triplicate, and each run included a standard curve, nontemplate control, and negative RT control. The levels of GAL and neuropeptide Y (NPY) gene expression were quantified relative to the level of β-actin by using standard curve method. The primers, designed with ABI Primer Express v. 1.5a software based on published sequences, were 1) β-actin: 5′-GGC-CAACCGTGAAAGATGA-3′ (forward) and 5′-CACACGCTG-GATGCCACTGT-3′ (reverse); 2) GAL: 5′-TTCCACACTGGT-CAAAGT-3′ (forward) and 5′-TTGCCGACAGGGTTGCAA-3′ (reverse); and 3) NPY: 5′-CACAGAAATGGCCCCAGAAA-3′ (forward) and 5′-GTCAGGAGCAAGTTTCTTCC-3′ (reverse). The concentrations of primers were from 100 to 200 nM, and all reagents, unless indicated, were from Invitrogen.

Data analysis. All values are expressed as means ± SE. Comparisons between groups were tested using either a two-way ANOVA followed by a Bonferroni post hoc test for multiple comparisons between groups or an unpaired Student’s t-test when appropriate. Correlations between within-group measures were performed using a Pearson’s product moment correlation. The criterion for the use of the term “significant” in the text was that P for a given test be <0.05.

RESULTS

These experiments had three objectives: 1) in experiments 1 and 2 to collect blood via a chronic cardiac catheter before and after an HFM (40 kcal), to compare endocrine changes in rats that were already obese (vs. lean) on a high-fat diet or that were normal weight but prone (vs. resistant) to obesity on this diet; 2) in experiments 3 and 4 to collect blood via a tail vein puncture after an HFM in normal-weight rats, to determine whether this simpler procedure could reveal HFM-induced endocrine changes similar to those seen with the chronic cardiac catheter and predictive of a rat’s propensity toward dietary obesity; and 3) in experiment 5 to use the most reliable HFM-induced endocrine measure for identifying OP and OR subgroups and then characterize these rats at normal weight with additional measures of circulating hormones and hypothalamic peptides.

Experiment 1: HFM-induced endocrine changes in already-obese rats with chronic cardiac catheters. Rats in this experiment (n = 21), with chronic cardiac catheters allowing blood collections before and after an HFM (40 kcal), were given their meal tests after being maintained for 3 wk on a high-fat diet. Based on the weight (g) of their four dissected fat pads at death, they were rank ordered and divided into two subgroups (lowest and highest tertile, n = 7/group) designated lean (15–19 g) or obese (26–32 g), respectively, which had significantly different body weights (441 ± 8 vs. 506 ± 10 g, P < 0.001) but similar 24-h food intake (105 ± 5 vs. 115 ± 7 kcal) at the time of the meal tests. As expected, the obese
with lean group had significantly higher pre- as well as post-HFM measurements of leptin, insulin, TG, and glucose and higher postmeal measures of NEFA but not of CORT levels (Table 1). Also, by comparing the postmeal with premeal scores, the results in Table 1 revealed a significant HFM-induced rise in leptin, insulin, and lipids, with little change in glucose and a decline in CORT, reflecting the well-known circadian rhythm of this steroid (62).

Most informative were the clear differences between the lean and obese rats in the magnitude of their HFM-induced endocrine changes (Table 1). When the postmeal scores were compared with premeal scores, the differences observed for the obese rats in their measures of leptin, insulin, and TG were significantly greater than those obtained for the lean rats. This larger response was revealed in a two-way ANOVA by the significant interaction effects for leptin and insulin (1, 24 F 4.53, P 0.001), producing significantly higher postmeal levels of both hormones compared with premeal levels in the obese rats (40–55% vs. lean 15–30%). On the other hand, the NEFA and glucose levels did not show comparable increases in the obese rats, which were remarkably similar to those seen in the already-obese rats. When the postmeal scores were compared with the premeal scores (Fig. 1), the OP group showed greater shifts than the OR group in their measurements of leptin [F(1, 24) = 10.54, P < 0.001], insulin [F(1, 24) = 4.68, P < 0.05], and TG [F(1, 24) = 4.53, P < 0.05], producing significantly higher postmeal levels of all three hormones compared with the OR group.

Despite their normal premeal levels and equivalent meal size (40 kcal), the OP rats revealed HFM-induced changes that were remarkably similar to those seen in the already-obese rats. When the postmeal scores were compared with the premeal scores (Fig. 1), the OP group showed greater shifts than the OR group in their measurements of leptin [F(1, 24) = 10.54, P < 0.001], insulin [F(1, 24) = 4.68, P < 0.05], and TG [F(1, 24) = 4.53, P < 0.05], producing significantly higher postmeal levels of all three hormones compared with the OR group.

**Table 1. Measurement of hormones and metabolites before and after HFM at dark onset in lean and obese rats with a chronic cardiac catheter (experiment 1)**

<table>
<thead>
<tr>
<th></th>
<th>Leptin, ng/ml</th>
<th>Insulin, ng/ml</th>
<th>TG, mg/dl</th>
<th>NEFA, mlq/l</th>
<th>Glucose, mg/dl</th>
<th>CORT, ng/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lean (n = 7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-HFM</td>
<td>6.9 ± 0.3</td>
<td>1.5 ± 0.2</td>
<td>110 ± 7</td>
<td>0.9 ± 0.1</td>
<td>143 ± 2.2</td>
<td>224 ± 17</td>
</tr>
<tr>
<td>Post-HFM</td>
<td>8.1 ± 0.8†‡</td>
<td>2.1 ± 0.2†</td>
<td>134 ± 9†</td>
<td>1.3 ± 0.2‡</td>
<td>139 ± 2.6</td>
<td>176 ± 16†</td>
</tr>
<tr>
<td>Obese (n = 7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Pre-HFM</td>
<td>8.7 ± 0.9*</td>
<td>2.4 ± 0.3*</td>
<td>134 ± 12*</td>
<td>1.0 ± 0.1†</td>
<td>151 ± 2.7*</td>
<td>221 ± 11</td>
</tr>
<tr>
<td>Post-HFM</td>
<td>12.0 ± 1.5†‡</td>
<td>3.8 ± 0.1*†‡</td>
<td>198 ± 11*‡</td>
<td>1.9 ± 0.3*†</td>
<td>144 ± 1.7*</td>
<td>180 ± 14†</td>
</tr>
</tbody>
</table>

Values are means ± SE. TG, triglycerides; NEFA, nonesterified fatty acids; CORT, corticosterone; HFM, high-fat meal. *P < 0.05, significant main effects for comparisons of obese to lean subgroups. †P < 0.05, significant main effects for comparisons of pre- to postmeal scores in lean or obese rats. ‡P < 0.05, significant interaction effects (group × meal) comparing obese to lean rats in terms of magnitude of difference between their post- and premeal scores.

**Fig. 1.** Hormones and metabolites 2 h before and after a high-fat meal in normal-weight rats maintained ad libitum on a chow diet and designated obesity resistant (OR) and obesity prone (OP) on the basis of their subsequent body fat accrual (lowest vs. highest tertile, respectively, n = 7/group) after 3 wk on a 50% fat diet. NEFA, nonesterified fatty acids; CORT, corticosterone. Values are means ± SE. *P < 0.05 for comparisons between postmeal OR and OP rats.
of these hormones and metabolite (Fig. 1). The greatest HFM-induced increase and greatest difference between OP and OR rats were seen in the levels of leptin. Relative to premeal levels, postmeal leptin was significantly elevated by 60% in the OP group vs. only 8% in the OR group, leading to 75% higher levels postmeal in the OP rats, as shown in Fig. 1. A smaller group difference was seen with the measurements of insulin and TG. Compared with premeal levels, both insulin and TG post-HFM were elevated by ~40% in the OP rats compared with 20–25% in the OR rats and thus were only 30–35% higher post-HFM in the OP compared with OR (Fig. 1). As in experiment 1, no group differences were seen in the postmeal levels of NEFA (1.7 ± 0.17 vs. 2.0 ± 0.15), glucose (140 ± 8 vs. 150 ± 10), and CORT (195 ± 10 vs. 210 ± 6), as well as in their percent shift relative to premeal scores. Thus the OP rats at normal weight showed early signs of obesity, responding similarly to the obese rats of experiment 1 in their endocrine changes induced by a fat-rich meal.

Experiment 3: HFM-induced leptin as the strongest correlate of ultimate body fat accrual. The purpose of experiment 3 was to determine whether the results obtained with the cardiac catheter procedure, both the HFM-induced endocrine changes and the greater responsiveness in OP rats, were reproducible using a simpler tail vein puncture procedure with blood collected after the HFM. With preliminary evidence indicating that they were, the specific objectives were to determine whether these HFM endocrine effects were 1) closely correlated with ultimate body fat accrual on a high-fat diet and 2) sufficiently robust to distinguish subjects at normal weight that had increased propensity toward obesity. Two larger groups of rats (n = 30–36/group) were tested in a similar manner to those in experiment 2, except that blood was collected via tail vein puncture 2 h before the HFM in the first test and 2 h after the HFM in three subsequent tests, as described in MATERIALS AND METHODS. After these four meal tests (40 kcal), the rats were placed on the high-fat diet for 3 wk. They were ranked ordered on the basis of their fat pad weights and then separated into two OR and OP subgroups (n = 11–12/group), the lowest (18–21 g) and highest (30–33 g) tertile, respectively, with the data for the middle group eliminated from the analysis. The OR and OP rats in each group had similar body weights, averaging 340 g at the time of the HFM blood collection tests, but showed a 55-g difference in their weights after 3 wk on the high-fat diet (465 ± 30 vs. 510 ± 41 g, P < 0.001). Also, as revealed in the initial, premeal test, their basal levels of hormones and metabolites were also normal, equivalent to those indicated for the normal-weight rats in experiment 2.

These two groups (group 1 and group 2) receiving tail vein punctures yielded results similar to each other and also to those obtained with the cardiac catheter technique. This indicates that the HFM-induced endocrine changes are reproducible and that the simpler tail vein puncture may be a valid method for identifying OR and OP rats. Once again, despite their similar meal size, premeal measurements, and body weight, the OR and OP rats showed significant mean differences and interaction effects in their HFM-induced changes in measurements of leptin, insulin, and TG but not of NEFA, glucose, and CORT. Moreover, the greatest difference once again was detected in their post-HFM measurements of leptin. When correlated across the entire group, these postmeal leptin scores were significantly positively correlated with their fat pad weights after 3 wk on the high-fat diet for group 1 (r = +0.58, P < 0.05) and group 2 (r = +0.64, P < 0.01). This relationship in these normal-weight rats was not detected for basal leptin levels, which generally fell within a tight range (2.5–3.5 ng/ml). Moreover, with the rats subgrouped in the retrospective analysis into OR/low-body-fat and OP/high-body-fat groups as in experiment 2, the post-HFM leptin scores of the OP rats were significantly higher than those of the OR rats in both group 1 (+74%, P < 0.001) and group 2 (+80%, P < 0.001), averaging 5.8 ± 0.5 vs. 3.3 ± 0.3 ng/ml, respectively. Although the post-HFM measurements were also significantly higher in the OP vs. OR rats for insulin (1.9 ± 0.3 vs. 1.3 ± 0.2 ng/ml, P < 0.05) and TG (128 ± 13 vs. 91 ± 15 ng/ml, P < 0.05), this increase was consistently smaller (40–45%) than that obtained for leptin, in agreement with the weaker correlations of these measurements to fat pad weights (r = +0.48 and r = +0.51, P < 0.05).

A prospective analysis of these data demonstrated that, consistent with experiment 2, this measurement of leptin can accurately identify rats at normal weight with clear differences in their ultimate body fat accrual. When rank ordered and subgrouped on the basis of their post-HFM scores, the rats in both groups with low leptin (2.5–3.9 ng/ml) vs. high leptin (4.2–8.1 ng/ml) levels were similar in their measurements of body weight, premeal leptin levels, and meal size at the time of the HFM tests. They were significantly different, however, in their adiposity after 3 wk on the high-fat diet, with the high-leptin group accumulating 60% heavier fat pads (18 ± 2 vs. 29 ± 3 g, P < 0.001). Consistent with their weaker correlations, the postmeal measurements of insulin and TG were less effective in distinguishing subgroups with clear differences in their fat pad weights. These results once again show leptin levels 2 h after an HFM, collected via a tail vein puncture, as the strongest early marker of ultimate body fat accrual on a high-fat diet, which can identify OP subgroups with >50% heavier fat pads than OR subjects.

Experiment 4: validation of HFM-induced leptin in differentiating distinct OR and OP subgroups. To further validate this positive relationship between post-HFM leptin at normal weight and long-term body fat accrual, experiment 4 performed one final test in a larger group of rats (n = 70) and after longer subsequent exposure (5 wk) to a high-fat diet. The procedures were the same as in experiment 3. The rats, while on lab chow, first received the four HFM tests (40 kcal) with tail vein blood collections (1 premeal and 3 postmeal) and were then placed for 5 wk on the high-fat diet. The OR and OP subgroups (n = 23/group), differentiated by their low (20–32 g) vs. high (40–55 g) body fat, were, during the HFM tests, equal in body weight (335 ± 7 vs. 342 ± 11 g) and basal leptin levels during the initial test (2.8 ± 0.5 vs. 2.9 ± 0.4 pg/ml). After 5 wk on the high-fat diet, however, the OP rats weighed 60 g more than the OR rats (571 ± 8 vs. 510 ± 5 g, P < 0.001). In contrast to a weak correlation between basal leptin and ultimate body fat measures (r = +0.21), the post-HFM leptin scores were significantly positively related to fat pad weights (r = +0.62, P < 0.001; Fig. 2), and they were 75% higher in the OP (6.3 ± 0.6 ng/ml) compared with the OR (3.6 ± 0.4 ng/ml, P < 0.001) subgroups. The reliability of postmeal leptin as a marker of long-term body fat accrual was once again revealed by the prospective analysis, which identified nearly distinct OR and OP subgroups. The rats with low HFM-induced leptin levels

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(3.4 ± 0.4 ng/ml, ranging from 2.5 to 4.5 ng/ml) had low fat pad weights (26 ± 1.7 g) and were thus considered OR. In contrast, the OP rats with high leptin levels after the HFM (7.0 ± 0.7 ng/ml, ranging from 6 to 9 ng/ml) had 70% more body fat (44 ± 1.9 g, \( P < 0.01 \)). Once again, significant but weaker correlations to fat pad weights were obtained for meal-induced changes in insulin and TG, resulting in smaller differences between the subgroups identified by these endocrine measures.

Together with experiment 3, the results of this experiment establish a well-defined and simple experimental paradigm that, with a measurement of post-HFM leptin, permits one to identify rats at normal weight (330–350 g) that are prone to obesity. In recent reports, the measurement of weight gain during the first 5 days on a high-fat diet was also found to be a strong, positive correlate of long-term body fat accrual (15, 68). The present group, with body weight measurements taken during the first week on the diet, permitted a direct comparison between these strong markers of adiposity. These measurements of initial weight gain and HFM-induced leptin were found to be positively correlated but only moderately so (\( r = +0.52, P < 0.01 \)). This suggests that the subgroups distinguished by their post-HFM leptin levels are similar, but not identical, to those defined by their 5-day weight gain on a chronic high-fat diet.

Experiment 5: phenotype of normal-weight OP rats identified by post-HFM leptin levels. With post-HFM leptin able to identify nearly distinct OR and OP subgroups at normal weight, experiment 5 took an initial step toward phenotyping these subgroups with measurements of two hypothalamic peptides involved in eating and body weight regulation. These were GAL in the PVN, which is generally found to be positively related to dietary fat and body fat accrual on a high-fat diet (2, 27), and NPY in the ARC, which is more specifically controlled by leptin (40). Using the same procedures as described in experiments 3 and 4 and MATERIALS AND METHODS, rats \( (n = 30) \) on a chow diet and at normal body weight (340–350 g) were given four HFM tests at dark onset with tail vein blood collections performed 2 h before (1 premeal) or 2 h after (3 postmeal) the HFM. They were subgrouped \( (n = 5/\text{group}) \) as OR with low post-HFM leptin \( (3.2 ± 0.3 \text{ ng/ml}) \) or OP with high post-HFM leptin \( (6.5 ± 0.5 \text{ ng/ml}) \). The rats were then killed immediately after the final 2-h meal test, their trunk blood was collected, and their brains were dissected and prepared for measurements of peptide expression using real-time quantitative RT-PCR.

At the time the animals were killed, these subgroups after the HFM tests were similar in body weight \( (355 ± 5 \text{ vs. } 360 ± 8 \text{ g}) \) and their dissected fat pad weights \( (17 ± 2 \text{ g} \text{ vs. } 19 ± 3 \text{ g}) \). They also had similar basal levels of insulin \( (1.3 ± 0.2 \text{ vs. } 1.4 ± 0.1 \text{ ng/ml}) \) and TG \( (80 ± 5 \text{ vs. } 82 ± 9 \text{ mg/ml}) \) in trunk blood. Despite their equal meal size \( (40 \text{kcal}) \), these subgroups were significantly different in their expression of GAL (Fig. 3). This effect was seen in the PVN, where the OP group with high post-HFM leptin had 25% higher GAL mRNA (relative to \( \beta\)-actin mRNA) compared with the OR group with low post-HFM leptin. In contrast to this peptide, these normal-weight subgroups showed no difference in their measurements of NPY mRNA in the ARC (Fig. 3).

DISCUSSION

The results of these experiments demonstrated that OP rats at normal body weight show very early signs of obesity in their HFM-induced changes in leptin, insulin, and TG. The rise in leptin after an HFM challenge was found to be the strongest correlate of long-term body fat accrual. This measurement identified distinct OR and OP subgroups at normal weight and revealed in OP rats a greater sensitivity to dietary fat, as reflected by an exaggerated HFM-induced rise in PVN GAL expression.

Premeal, basal hormone levels in obese and OP rats. With measurements in trunk blood, basal levels of leptin, insulin, TG, and glucose are generally elevated in rats that become obese on a high-fat diet compared with their lean counterparts (8, 64). In blood collected via the chronic cardiac catheter, a similar pattern was observed here in obese vs. lean rats on a high-fat diet, with levels of NEFA and CORT showing little or...
no difference. Before being placed on the high-fat diet, however, the normal-weight OP rats on lab chow were similar to the OR subjects in their basal (premeal) levels of these hormones and metabolites. There are few published studies of this nature that have tested at normal weight subjects that are believed to be prone to obesity. As shown here, these studies in rats (31) and humans (20, 48, 49) show generally normal endocrine profiles in OP compared with OR subjects on a low-fat diet. Disturbances in basal or fasting levels of OP rats generally become evident after approximately 1 wk on a high-fat diet as body fat significantly rises (28).

**HFM-induced rise in leptin, insulin, and lipid levels.** The focus of this report is on meal-induced changes in hormones and metabolites. The chronic cardiac catheter technique permitted direct comparisons between samples collected both before and after an HFM within the same test. In normal-weight rats maintained on lab chow, the 2-h HFM relative to premeal levels at dark onset produced a significant rise in leptin, insulin, TG, and NEFA, with no change in glucose. In the few studies that have made such comparisons between pre- and postmeal levels, similar meal-induced changes in normal-weight subjects have been described in rats (66) and humans (41, 52, 58). The decline in CORT levels during the first 2 h of the dark cycle presumably reflects the natural, circadian-related shift in this steroid (62). One objective of the present study was to determine whether the tail vein puncture, with blood collected only after the meal except for an initial premeal test, could reveal in normal-weight animals a meal-induced endocrine pattern similar to that seen with the chronic catheter. This was found to be the case. Compared with their basal, premeal scores determined on the first test, a significant increase in post-HFM levels of leptin, insulin, and lipids was clearly seen in blood collected from the tail vein. This validates the utility of this simpler method of blood collection for revealing subtle, meal-induced endocrine responses in subjects with normal basal levels of hormones and metabolites.

**Greater HFM-induced rise in leptin, insulin, and TG in obese and OP rats.** The major findings of this study were the clear difference between normal-weight OP and OR rats in the magnitude of their meal-induced endocrine changes and the similarity of the OP pattern to that seen in already obese rats compared with their lean counterparts. With blood collected via cardiac catheter both before and after a meal, the obese subjects with already elevated premeal levels of the hormones and metabolites exhibited a twofold greater rise after the HFM in leptin, insulin, and TG compared with lean subjects. No group differences, however, were detected in their meal-induced changes in NEFA, glucose, and CORT. There are apparently no such studies comparing meal-related endocrine patterns in rats of different body weight. However, there is some evidence in humans (21, 24), albeit not consistently demonstrated (14, 43, 51), that levels of insulin and lipids after a fat-rich meal compared with premeal baseline show a greater increase in the obese than in the lean subjects.

Having established these endocrine changes in already-obese subjects, it is particularly noteworthy that a similar pattern was seen in OP subjects at normal body weight. In three separate experiments, the normal-weight OP rats, like the obese rats, showed a significantly exaggerated HFM-induced rise in leptin, insulin, and TG levels compared with the OR rats. No group differences were seen in NEFA, glucose, or CORT. Also, these postmeal measurements of leptin, insulin, and TG levels were strongly positively correlated with ultimate body fat accrual after 3–5 wk on a high-fat diet. These results were robust, obtained whether blood was collected via the cardiac catheter implant or via the simpler, tail vein puncture procedure. Also, the exaggerated changes seen in OP rats appear to be dependent on the fat in the meal, as they were not evident in preliminary tests conducted with a low-fat meal. There are only a few animal or clinical studies of meal-related effects in OP subjects. Consistent with the present results is a study in humans describing elevated post-HFM leptin levels in normal-weight, postobese human subjects with normal premeal leptin levels (49). There are also reports in normal-weight rats showing cephalic phase insulin secretion to be exaggerated in OP compared with OR subjects on a high-calorie diet (6, 47). Whereas basal leptin levels are known to rise in direct proportion to body fat (3, 63), there are apparently no animal studies suggesting that leptin at normal weight, whether at basal or postmeal levels, correlate with a predisposition to obesity. This possibility is consistent with evidence that OP rats are less sensitive to the anorectic effects of centrally administered leptin (33). Thus subtle, meal-related changes in leptin, insulin, and TG seen in OP subjects with normal basal levels may be early indications of a resistance to these hormones as well as a disturbance in fat metabolism.

**Prediction of obesity by high post-HFM leptin levels.** The main objective of this series of experiments was to identify in rats an early endocrine marker that can reliably and accurately predict future weight gain on a chronic high-fat diet. There are numerous clinical studies that have sought to establish predictors of obesity. These investigations in obese or postobese subjects have revealed positive relationships to fat mass with measurements of basal insulin levels or sensitivity (44, 50, 71). This is similarly seen with basal levels of leptin in some (9, 49, 53) but not all (37, 50) reports. Furthermore, in normal-weight rats, measurements of fasting levels of TG or of glucose-stimulated plasma norepinephrine levels are found to be strong correlates of future weight gain on a high-calorie diet (25, 30). Having demonstrated and confirmed in the present study specific meal-related endocrine changes in OP rats, a critical next step was to test the validity of this early correlate in terms of its ability to identify distinct OR and OP subgroups. The results clearly showed the measurement of leptin after an HFM to be the strongest and most consistent correlate of long-term body fat accrual. It was also the most effective in accurately identifying OR and OP rats. With minimal error, low leptin levels after the HFM (~3.5 ng/ml (range 2–5 ng/ml) predicted a low amount of body fat accumulation (16–26 g). This is in contrast to the high leptin levels after the HFM averaging 6.8 ng/ml (6–9 ng/ml), which predicted the greatest accumulation of body fat (30–44 g) on a chronic high-fat diet that was 70% higher than in the low-leptin group. This postmeal difference in leptin between the OR and OP subgroups, similar across all three experiments, occurred despite their equal meal size, body weight, and basal levels of leptin. It also identified subgroups that were essentially distinct, with only 6% of the total group incorrectly categorized. This shows the importance of a specific stimulus, a 2-h HFM consumed at dark onset, in revealing a strong correlate of body fat accrual. Both insulin and TG levels after an HFM were positively related to body fat, and the subgroups distinguished by these measures revealed a signifi-
cant difference in their body fat. However, these relationships were not as strong as with leptin, and there was greater overlap in the OR and OP subgroups identified by these measurements.

Mechanisms underlying increased HFM-induced leptin in normal-weight OP rats. With the finding that HFM-induced leptin in normal-weight rats is a strong predictor of future body fat accrual, we sought to understand the significance of this endocrine marker in terms of its etiology and causal relation to a rat’s susceptibility. Basal levels of leptin are known to be strongly positively correlated with body fat on a high-fat diet (17). However, there are conditions in which leptin levels can be dissociated from body fat (28, 67), suggesting that its secretion may be induced by other factors besides increased adiposity. In our preliminary studies, we found that a high-carbohydrate meal fails to reveal the positive relationship between meal-induced leptin and long-term propensity toward obesity. This suggests that dietary fat is a critical factor in this association with leptin release. Other studies show that leptin is stimulated by insulin, depending on dose and nutritional status (12, 18, 35), and by accelerated fat synthesis when insulin levels are elevated (56). This evidence is consistent with the present finding that insulin and TG levels, like leptin level, are significantly elevated after the HFM in OP as well as obese rats, although they are not as closely related to ultimate body fat accrual. The elevated release of leptin after a meal may have some relation to the greater, HFM-induced suppression of fat oxidation in OP subjects (20, 48), which may reflect an early resistance to the effects of leptin. Thus, in addition to identifying a marker of susceptibility, the present experiments with a 2-h HFM revealed endocrine changes that precede any detectable shifts in basal hormone levels or rise in body fat and thus may contribute to the hyperleptinemia as well as hyperinsulinemia and hypertriglyceridemia seen in obese subjects. In understanding the mechanisms that underlie these mealrelated endocrine changes, it would be helpful to determine whether the OR and OP subgroups differ in their temporal patterns. The interpretation of results would be altered by patterns showing, for example, an earlier peak of leptin in the OR rats vs. a delayed and flattened secretory response in the OP rats. Disturbances in hypothalamic peptides that accompany elevated HFM-induced leptin in OP rats. With HFM-induced leptin able to identify normal-weight OP rats with considerable accuracy, the final experiment examined these subjects to determine whether they exhibit any disturbance in hypothalamic peptides that may contribute to their propensity toward obesity. In OP subjects showing elevated HFM-induced leptin compared with OR rats, a significantly higher expression of the GAL gene was observed in the PVN. This elevated GAL levels are strongly positively correlated with body fat on a high-fat diet (17). However, there are conditions in which leptin levels can be dissociated from body fat (28, 67), suggesting that its secretion may be induced by other factors besides increased adiposity. In our preliminary studies, we found that a high-carbohydrate meal fails to reveal the positive relationship between meal-induced leptin and long-term propensity toward obesity. This suggests that dietary fat is a critical factor in this association with leptin release. Other studies show that leptin is stimulated by insulin, depending on dose and nutritional status (12, 18, 35), and by accelerated fat synthesis when insulin levels are elevated (56). This evidence is consistent with the present finding that insulin and TG levels, like leptin level, are significantly elevated after the HFM in OP as well as obese rats, although they are not as closely related to ultimate body fat accrual. 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Disturbances in hypothalamic peptides that accompany elevated HFM-induced leptin in OP rats. With HFM-induced leptin able to identify normal-weight OP rats with considerable accuracy, the final experiment examined these subjects to determine whether they exhibit any disturbance in hypothalamic peptides that may contribute to their propensity toward obesity. In OP subjects showing elevated HFM-induced leptin compared with OR rats, a significantly higher expression of the GAL gene was observed in the PVN. This elevated GAL expression and peptide in the PVN GAL (10, 27, 28). Although not directly tested in the present report, each of these published studies suggests that this change in GAL gene expression is likely to result in an increase in GAL peptide synthesis and release. Further tests will be needed to determine whether this trait of OP rats reflects a greater GAL sensitivity or a change in responsiveness to leptin.

In either case, the increase in GAL expression and peptide in OP rats is likely to contribute to their early development of obesity on a high-fat diet. This is supported by evidence that acute injection of GAL has a stronger feeding-stimulatory effect on a high-fat diet compared with a low-fat diet, and it has metabolic effects that include a reduction in energy expenditure and sympathetic nervous system activity and a stimulation of carbohydrate over fat metabolism (29, 36, 38, 42, 72). Also, chronic GAL injection increases body weight and body fat accrual, most strongly on a high-fat diet (23, 72).

In contrast to GAL, there was no difference in NPY mRNA in the ARC of OP compared with OR rats. In two other reports, the expression of this peptide was found to be elevated in normal-weight OP subjects identified by their urinary norepinephrine levels (31, 32). The absence of this effect here after an HFM may be related to the elevated leptin in OP rats, which is known to have a potent inhibitory effect on NPY expression and to prevent an increase in NPY in already-obese rats (31, 40). In contrast to NPY, GAL, which appears to be less sensitive to leptin, invariably rises under various conditions where leptin levels are elevated (28, 29). Thus, in response to an HFM, measurements of PVN GAL expression are more revealing of significant differences between OR and OP subjects, with the latter showing greater responsiveness to the fat content of a meal.

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GRANTS

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