Nocturnal free fatty acids are uniquely elevated in the longitudinal development of diet-induced insulin resistance and hyperinsulinemia

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It has traditionally been believed that the development of insulin resistance associated with obesity is due to an increase in the level of circulating free fatty acids (FFA) resulting from an impairment of insulin’s ability to suppress lipolysis in adipose tissue (4, 7, 21). Increased FFA levels have been shown to decrease insulin’s ability both to suppress hepatic glucose output and to promote peripheral glucose uptake, which can then result in an increase in fasting glucose (14, 15). It has traditionally been thought that this increase in fasting glucose resulting from insulin resistance is responsible for compensatory hyperinsulinemia. Thus increasing FFA by lipid infusion results in development of insulin resistance and a compensatory increase in insulin levels (9) in addition to causing mild fasting hyperglycemia due to stimulation of both glycogenolysis and gluconeogenesis (40). However, studies in several different animal models as well as in humans have not consistently demonstrated increases in fasting FFA or glucose during the development of insulin resistance and hyperinsulinemia during obesity (16, 20, 22, 38, 41). Studies conducted in our own laboratory (25, 31) using the fat-fed dog model have found development of insulin resistance with concomitant increases of 90–150% in basal insulin with no significant changes in either fasting FFA or glucose. Elevated levels of fasting FFA and fasting hyperglycemia do not appear to be the cause for development of insulin resistance and subsequent upregulation of insulin, at least in the obese, insulin-resistant dog model. Moreover, we have found no changes in the fasting levels of other factors [glucagon-like peptide-1 (GLP-1), cortisol, growth hormone, and others] typically associated with obesity and insulin resistance. However, because the majority of studies, including our own, exclusively examine plasma levels in the morning after an overnight fast, this does not address the possibility that an elevation of FFA, glucose, or other related factors at times of day other than in the morning might contribute to insulin resistance and/or compensatory hyperinsulinemia. We postulate that FFA and/or glucose at times other than in the morning may contribute to insulin resistance and/or upregulation of insulin when insulin resistance is induced by a high-fat, hypercaloric diet in the dog model. We have measured putative signals overnight that could be responsible for metabolic changes seen with fat feeding.

MATERIALS AND METHODS

Animals

Male mongrel dogs (n = 8, 29.8 ± 1.4 kg) were housed in the Keck School of Medicine of University of Southern California (USC) vivarium under controlled kennel conditions (12:12-h light-dark cycle). Animals were accepted into the study following physical examination and a comprehensive blood panel. Chronic catheters were surgically implanted 7–10 days before the beginning of the study: one was inserted in the jugular vein and advanced to the right atrium for sampling of central venous blood, and a second catheter was inserted in the femoral vein and advanced to the vena cava for tracer, insulin, and somatostatin infusion. All catheters were led to the neck subcutaneously and exteriorized. Catheters were flushed with heparinized saline (10 U/ml) at least twice a week, and the exteriorization site was cleaned with hydrogen peroxide (4%). Dogs were accustomed to laboratory procedures and were used for experiments only if judged to be in good health as determined by visual observation, body temperature, and hematocrit. On the morning of each experiment, 19-gauge angiocatheters (Allegiance Healthcare, Ontario, CA) were inserted percutaneously into the saphenous vein for glucose infusion. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked ‘‘advertisement’’ in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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experimental protocol was approved by the USC Institutional Animal Care and Use Committee.

**Diet**

Dogs were fed a weight-maintaining standard diet of one can of Hill’s Prescription Diet (10% carbohydrate, 9% protein, 8% fat, 0.3% fiber, and 73% moisture; Hill’s Pet Nutrition, Topeka, KS) and 825 g of dry chow (36.6% carbohydrate, 26.4% protein, 14.7% fat, and 2.9% fiber; Wayne dog food; Allied Mills, Chicago, IL) for a period of 2–3 wk before any experiments were conducted to ensure weight stabilization. This standard diet consisted of 3,885 kcal/day: 27.4% carbohydrates, 19% protein, and 53.6% fat. To acclimate the animals to the feeding protocol that would be used during the 24-h plasma profiling experiments (see below), throughout the study dogs were presented with their meal at 9:00 AM and given 1 h to eat, after which the meal was removed.

**Magnetic Resonance Imaging**

During weeks 0 and 6 of the high-fat diet, magnetic resonance imaging (MRI) scans were performed on the dogs as previously described (25). Thirty 1-cm axial abdominal images (T1 slices, TR 500, TE:14) were obtained using a General Electric 1.5-Tesla Horizon magnet (version 5.7 software). Of the 30 images obtained, ~20 of these images were used for analysis of total trunk body fat, depending on the relative torso length of the animal. Images were analyzed using Scion Image (Windows 2000 version Beta 4.0.2; Scion, Frederick, MD), which quantifies fat tissue (pixel value 121–254) and other tissue (20–120) in each slice. Fat volume was calculated by dividing the number of pixels counted as fat by the ratio of the total number of pixels (256 × 256) and the number of pixels (34.9 × 34.9) cm for a 1-cm image. Total trunk fat and tissue were estimated as the integrated fat or tissue across all 20 slices. Percent fat was calculated as the total trunk fat divided by the total trunk tissue. Omental fat was defined as fat within the peritoneal cavity in an 11-cm region of the thorax, using the slice at the level where the left renal artery branches from the abdominal aorta as a midpoint landmark. Percent omental fat was calculated as the omental fat divided by the total tissue area in these same slices.

**Euglycemic Hyperinsulinemic Clamps**

The euglycemic hyperinsulinemic clamps were performed as previously described (25) during weeks 0 and 6 of the high-fat diet. Animals were familiarized with the Pavlov sling at least 1 wk before the first experiment. At ~7:00 AM on the day of the clamp, animals were brought to the laboratory and placed in the Pavlov sling. A 19-gauge angiocatheter was placed in a saphenous vein and secured. Approximately 30 min later (t = −120 min), a primed continuous infusion of high-performance liquid chromatography-purified [3-3H]glucose (25 µCi + 0.25 µCi/min infusion; DuPont-NEN, Boston, MA) was started. After tracer equilibration, basal samples were taken at −30, −20, −10, and −1 min. At time t = 0 min, a somatostatin infusion (1.0 µg·min⁻¹·kg⁻¹, Bachem California, Torrance, CA) was started to suppress endogenous insulin and glucagon secretion and was continued for the duration of the experiment. Porcine insulin was infused (0.75 mU·kg⁻¹·min⁻¹; Eli Lilly, Indianapolis, IN) into the femoral vein to attain hyperinsulinemia. Glucose was clamped at basal by a variable glucose infusion labeled with [3-3H]glucose (2.0 µCi/g) to minimize fluctuations in plasma specific activity. Blood samples were drawn from the jugular catheter every 10 min from −30 to 60 min, every 15 min from 60 to 120 min, and then every 10 min from 120 to 180 min.

**Twenty-Four-Hour Plasma Profiling**

To obtain a plasma profile over a 24-h period, we utilized the following protocol during both week 0 and week 6 of the study. The precise profiling protocol was identical at week 0 and week 6. At ~5:00 AM on the day of each experiment, the animal was brought into the laboratory. The jugular vein catheter was exposed and secured at the neck to allow blood sampling. The animal was housed individually in a kennel (5 × 5 ft) for the duration of the experiment. Animals were unrestrained in the kennel throughout the experimental protocol. Starting at 6:00 AM, blood samples were drawn at 1-h intervals for the 24-h period until 6:00 AM the following morning. The dogs were presented with the standard diet meal (see Diet) at 9:00 AM and given until 10:00 AM to eat, whereupon the meal was removed. All uneaten food was weighed and recorded, and the exact same meal by weight and composition was given during the week 6 experiments to negate any acute effects of differences in food consumption on the experimental outcome. The animals consumed ~40% of the meal during the week 6 experiment, and there was no significant difference in percent meal consumption during the week 6 experiment.

**Sample Collection and Storage**

Samples for assay of insulin, d-[3-3H]glucose, cortisol, and growth hormone were taken in tubes precoated with lithium fluoride and heparin (Brinkmann Instruments, Westbury, NY). The tubes for insulin and glucose also contained 50 µl of EDTA. Samples for determination of C-peptide and glucagon were collected in precoated lithium fluoride/heparin tubes containing 25 µl of EDTA and 50 µl of Trasylol (10,000 KIU/ml; Serological Proteins, Kankakee, IL). GLP-1 samples were taken in tubes precoated with lithium fluoride and heparin containing 50 µl of EDTA and 25 µl of dipeptidyl peptidase IV inhibitor (Linco Research, St. Charles, MO). Samples for FFA, glycerol, and triglyceride assays were taken in tubes with EDTA and paraxon to inhibit lipase activity. All samples were immediately centrifuged, and plasma was separated and stored at −80°C for further analysis.

**Assays**

Glucose was measured with a YSI 2300 autoanalyzer (Yellow Springs Instruments, Yellow Springs, OH). FFA (NEFA C; Wako Pure Chemical Industries, Richmond, VA), glycerol, and triglycerides (serum triglyceride/glycerol determination kit; Sigma Chemical, St. Louis, MO) were measured using colorimetric methods, utilizing commercially available kits. Insulin was measured using an ELISA originally developed for human serum or plasma (Linco Research) and adapted for dog plasma by using a dog standard kindly provided by Novo-Nordisk. The method is based on two murine monoclonal antibodies that bind to different epitopes of insulin but that do not bind to proinsulin. Cortisol, glucagon, growth hormone, and canine C-peptide were measured using a radioimmunoassay kit, and active GLP-1 was measured using ELISA (cortisol RIA kit from Diagnostic Products, Los Angeles, CA; all other kits from Linco Research).

Samples for [3H]glucose tracer assay were deproteinized using barium hydroxide and zinc sulfate. The supernatants were then evaporated in a vacuum, reconstituted in water, and counted in Ready Safe scintillation fluid (Beckman liquid scintillation fluid; Beckman Instruments, Fullerton, CA). Tracer infuses were processed identically to plasma samples.

**Calculations**

The time courses of endogenous glucose production and glucose disappearance during the euglycemic hyperinsulinemic clamp were calculated using Steel’s model with a labeled glucose infusion as
previously described (17). Derivatives of all time course data were calculated with OOPSEG (10). Basal was defined as the average of four samples taken every 10 min from $t = -30$ to 0 min, and steady state was defined as the average of four samples taken from $t = 150$ to 180 min. Insulin sensitivity ($S_I$) was calculated using the equation

$$S_I = \frac{\Delta G_{inf}}{\Delta t \times G}$$

where $\Delta G_{inf}$ is the difference in glucose infusion rate at steady state from basal, $\Delta t$ is the difference in plasma insulin at steady state from basal, and $G$ is the steady-state plasma glucose concentration.

During the 24-h protocol, the total integrated area under the curve (AUC) was calculated using the trapezoidal rule. Insulin secretion rates were estimated using measured C-peptide levels and a two-compartment model for C-peptide distribution, as described previously (33). C-peptide sampling occurs from the central compartment, from which it diffuses into a peripheral compartment. Estimates of the kinetic parameters associated with our analysis were acquired from previously published data for canine C-peptide distribution kinetics (33, 35, 36).

Statistical Analyses

All experimental data are expressed as means ± SE. Repeated-measures ANOVA with Bonferroni posttests was used to compare all time course data before and after fat feeding. Paired Student’s $t$-tests were used to identify the significantly different time point pairs and to compare all fasting metabolic parameters, 24-h averages, and 24-h AUC between weeks 0 and 6. The $t$-tests were performed using Microsoft Excel XP, and all ANOVAs were performed using GraphPad InStat 3.0 (GraphPad Software, San Diego, CA).

RESULTS

Body Composition

As expected, body weight increased in response to the increased caloric content starting at week 1 (Fig. 1A). By week 6, animals had gained an average of 3 kg (from 27.6 ± 1.4 to 30.3 ± 2.0 kg, $P < 0.05$). Weight increase was reflected in a substantial increase in body fat as assessed by MRI. Total trunk fat increased from $1,081 \pm 133$ cm$^3$ at week 0 to $1,907 \pm 304$ cm$^3$ at week 6 ($P = 0.006$), an approximate 76% increase in total trunk adiposity over the 6-wk period.

Comparing the contribution of the omental and subcutaneous fat depots to total body fat (Fig. 1, B and C), we found that within the defined axial region (11 cm) of the trunk, omental fat volume increased by $51 \pm 16\%$ (week 0: 370 ± 37 vs. week 6: 557 ± 87 cm$^3$, $P < 0.05$), whereas subcutaneous fat volume increased by $90 \pm 17\%$ (week 0: 245 ± 33 vs. week 6: 453 ± 65 cm$^3$, $P < 0.05$).

Fasting Metabolic Parameters

Despite an impressive 76% increase in total trunk fat (see Body Composition) and increased body weight, neither fasting free fatty acids (week 0: 0.50 ± 0.06 vs. week 6: 0.58 ± 0.04 mM; $P = 0.24$) nor fasting glucose (week 0: 99 ± 2 vs. week 6: 95 ± 1 mg/dl; $P = 0.12$) was changed by the high-fat diet. This lack of a change in fasting levels of FFA or glucose recapitulates previous results with a lesser fat diet (25, 31). However, despite unchanged fasting levels of FFA, there was

![Fig. 1](http://ajpendo.physiology.org/)
a significant increase in insulin resistance after 6 wk of a hypercaloric, high-fat diet such that whole body insulin sensitivity decreased by ~30% (week 0: 7.7 ± 0.8 vs. week 6: 5.5 ± 0.3 × 10^{-4} \text{dl/pM}^{-1}\cdot \text{mg}^{-1}\cdot \text{kg}^{-1}; P = 0.008) in association with the increased adiposity. This decrease in insulin sensitivity was due to a significant reduction in insulin’s ability to suppress endogenous glucose output to stimulate glucose uptake and a tendency for a decrease in sensitivity was due to a significant reduction in insulin’s ability with the increased adiposity. This decrease in insulin sensitivity nearly doubled from 30% (week 0) to 60% (week 6; P < 0.05). Thus, as previously reported for a moderate-fat diet, insulin resistance and compensatory fasting hyperinsulinemia after high-fat feeding occurs with no significant changes in either FFA or glucose.

**Twenty-Four-Hour Plasma Profile**

**Insulin.** We considered the possibility that increases in FFA, glucose, or other related factors at alternative times of the day might have played a role in the development of insulin resistance and compensatory hyperinsulinemia. The insulin response to a meal (10:00 AM to 6:00 PM) was profoundly enhanced after fat feeding (Fig. 3A). The total AUC for plasma insulin for the 8-h period following the 9:00 AM meal increased by 223 ± 44% after 6 wk of fat feeding (P = 0.01). This increased insulin response following the meal resulted in a significant increase in both the average insulin concentration and the total AUC over the 24-h period (Table 1) such that average insulin was increased by 70.1 ± 25.8% (P < 0.05) and the total AUC was increased by 69.8 ± 26.1% (P < 0.05) compared with week 0.

**C-peptide.** Unlike the marked increase in plasma insulin levels, the 24-h profile of C-peptide was not changed after fat feeding (Fig. 3B). The average plasma concentration for the 24-h period remained consistent before and after the diet, as did the total AUC (Table 1).

Insulin secretion rates, calculated from deconvolution of plasma C-peptide concentrations, were unchanged after 6 wk of high-fat diet (Fig. 3C), suggesting that a decrease in insulin clearance was a major contributor to the compensatory increase in plasma insulin after fat feeding. This finding confirms previous results for the fat-fed dog model, which showed that decreases in insulin clearance as well as increases in insulin secretion can act to contribute to hyperinsulinemia (25, 31).

**Glucose.** Recapitulating fasting glucose levels, there was absolutely no discernible increase in glucose throughout the 24-h observation period after a 6-wk high-fat diet (Fig. 4A). The average glucose for the 24-h period before fat feeding was unchanged, as was the total AUC (Table 1), indicating that increased glucose could not be a signal for either insulin resistance or compensatory hyperinsulinemia in the fat-fed dog model.

**FFA, glycerol, and triglycerides.** In sharp contrast to 24-h glucose, we observed a dramatic increase in both the 24-h average and the total AUC for FFA (Fig. 4B). The 24-h average for FFA was increased by 48.4 ± 6.7% after the fat diet (P < 0.001), as was the total AUC (increased by 48.7 ± 7.1%; P < 0.005), because of an elevation in plasma FFA concentrations beginning at 5:00 PM, which peaked at 3:00 AM. There was an increase in FFA at 6:00 AM on the second morning of the 24-h protocol not observed at week 0 that may be due to the acute effects of altered meal composition given to the fat-fed animals during the 24-h experiment (see MATERIALS AND METHODS).

It was of interest to ask whether this return to the control diet only on the day of the experiment was responsible for the overall increase in FFA observed during the late postprandial and nocturnal periods. To discount this possibility, we presented a fat-supplemented meal to a separate set of 6-wk fat-fed dogs (n = 9) on the day of nocturnal measurements. To confirm the phenomenon of increased nocturnal FFA, we assessed FFA levels from 6:00 to 8:00 AM (when the rise begins) and from 2:00 to 4:00 AM (when FFA are maximal). There was a very similar increase in FFA levels after 6 wk of fat feeding whether or not we returned to the control diet for 1 day (Fig. 5). In the present study, FFA levels between 6:00 and 8:00 PM increased by 0.37 ± 0.07 mM at week 0 to 0.59 ±

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**Fig. 2.** Time course data of glucose uptake (A), glucose production (B), and free fatty acids (FFA; C) during the clamp at week 0 (○) and week 6 (●) of the high-fat diet. *P < 0.05 vs. week 0, paired t-test.
0.08 mM at week 6. Similarly, when dogs were fed the fat-supplemented meal on the day of the week 6 experiments, FFA levels also increased from 0.25 ± 0.04 mM at week 0 to 0.59 ± 0.03 at week 6 (P < 0.001). The changes in overnight FFA also showed remarkably similar results. In the present study, when dogs were fed the control diet on the day of the week 6 experiments, average FFA levels from 2:00 to 4:00 AM increased from 0.45 ± 0.04 mM at week 0 to 0.80 ± 0.05 mM at week 6. When dogs were fed the fat-supplemented meal on the day of the week 6 experiments, FFA levels were still increased during this time frame from 2:00 to 4:00 AM (week 0: 0.47 ± 0.07 vs. week 6: 0.76 ± 0.04 mM; P < 0.001). It was clearly not the switch to the control diet for a single day that was responsible for the large increase in FFA at night.

Glycerol concentrations showed a similar increase in nocturnal levels despite unchanged fasting levels. At week 6, plasma glycerol (Fig. 4C) began to increase at 5:00 PM and remained elevated such that both the 24-h average and the total AUC were increased by 41.2 ± 11.7 and 42.0 ± 11.8%, respectively (P < 0.005; Table 1). Triglyceride levels (Fig. 4D and Table 1) remained unchanged during fasting and throughout the 24-h observation period. Increases in both FFA and glycerol without a corresponding change in triglyceride levels suggest that the increase in FFA was due to increased lipolysis.

Cortisol, growth hormone, and glucagon. Cortisol, growth hormone, or glucagon did not exhibit a change in overall plasma concentrations over the 24-h period after 6 wk of fat feeding. (Fig. 6, A–C). In addition, the secretory pattern of growth hormone as derived from the 15-min sampling period between 12:00 and 3:00 AM was unchanged (data not shown).

Table 1. Twenty-four-hour averages and AUC before and after high-fat feeding

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<tr>
<th></th>
<th>24-h Average</th>
<th>24-h AUC</th>
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<tbody>
<tr>
<td></td>
<td>Before fat</td>
<td>After fat</td>
</tr>
<tr>
<td>Insulin, pM</td>
<td>64 ± 8</td>
<td>97 ± 9*</td>
</tr>
<tr>
<td>C-peptide, pM</td>
<td>277 ± 20</td>
<td>231 ± 27</td>
</tr>
<tr>
<td>Insulin-to-C-peptide ratio</td>
<td>0.23 ± 0.02</td>
<td>0.41 ± 0.03*</td>
</tr>
<tr>
<td>Glucose, mg/dL</td>
<td>98 ± 2</td>
<td>95 ± 2</td>
</tr>
<tr>
<td>Free fatty acids, mM</td>
<td>0.40 ± 0.04</td>
<td>0.59 ± 0.05†</td>
</tr>
<tr>
<td>Glycerol, mM</td>
<td>0.12 ± 0.01</td>
<td>0.16 ± 0.02†</td>
</tr>
<tr>
<td>Triglycerides, mM</td>
<td>0.41 ± 0.02</td>
<td>0.45 ± 0.03</td>
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Values are means ± SE of 24-h average metabolic levels (concentration at units provided) and 24-h area under the curve (AUC; results for each are units-h). *P < 0.05; †P < 0.005 vs. week 0; paired t-test.

Fig. 3. Twenty-four-hour plasma profile of insulin (A) and C-peptide (B) at week 0 (○) and week 6 (●). C: insulin secretion as calculated by C-peptide deconvolution analysis at week 0 (solid line) and week 6 (hatched line). Vertical dashed lines represent times of meal presentation and meal removal. *P < 0.05 vs. week 0, paired t-test.

Fig. 4. Twenty-four hour plasma profiles at week 0 (○) and week 6 (●). A: plasma glucose concentrations did not show a significant increase from basal throughout the 24-h period, and there was no significant difference in plasma glucose levels at any time point before and after fat feeding. Plasma FFA (B) and plasma glycerol levels (C) over the 24-h period showed a similar increase at week 6 of the study that became significant at 5:00 PM and continued until the morning, whereas triglyceride levels (D) were unchanged by fat feeding. Vertical dashed lines represent times of meal presentation and meal removal. *P < 0.05 vs. week 0, paired t-test.
GLP-1. GLP-1 is well known as a β-cell growth factor and insulinotropic hormone, but we observed no increase in the 24-h pattern of active GLP-1 as a result of increased adiposity. In fact, GLP-1 had a tendency to decrease both at basal (week 0: 5.9 ± 0.4 vs. week 6: 4.6 ± 0.7 pM; P = 0.10) and over the 24-h experimental protocol (Fig. 6 and Table 1). Thus, of all the potential factors related to insulin resistance and hyperinsulinemia during obesity, only the 24-h FFA pattern was elevated by the high-fat diet.

DISCUSSION

Of the many possible factors influencing development of the metabolic syndrome, FFA have been implicated as an important component in the development of insulin resistance, particularly in obesity (8, 14). Elevated levels of fasting FFA levels are often thought of as a characteristic marker for insulin resistance in obesity, and it has been suggested that these FFA may be responsible for reduced insulin action at both skeletal muscle and liver. Ensuing insulin resistance would then result in elevated glucose, which signals for a compensatory increase in circulating insulin concentrations. Thus it has long been assumed that modest fasting and/or postprandial plasma glucose is responsible for increases in plasma insulin in the insulin-resistant state. As an example, in Pima Indians, a population highly at risk for diabetes, glucose increases longitudinally with insulin resistance (46). In contrast, other populations at risk for diabetes are able to maintain normal glucose levels despite insulin resistance (16, 22), indicating that elevated fasting glucose may not be the primary signal responsible for hyperinsulinemia with insulin resistance but, rather, that glucose only becomes elevated when hyperinsulinemia is unable to fully compensate for insulin resistance. And although fasting levels of FFA can be elevated in obesity and insulin resistance, this is not always the case. Studies conducted in humans as well as animals have found relatively normal levels of both FFA and glucose at fasting despite obesity, insulin resistance, and hyperinsulinemia (16, 20, 22, 38). The latter data lead to the question of whether these metabolites are responsible for insulin resistance and hyperinsulinemia during obesity. Studies conducted in our own laboratory (25, 31) using the fat-fed dog model have shown significant insulin resistance and increases of 90–150% in basal insulin with no measurable change in fasting FFA or glucose. In the present study, we sought to examine whether there were any alterations in FFA or glucose over the 24-h day as well as other factors, including cortisol, growth hormone, and GLP-1, that could potentially serve as contributors to the development of insulin resistance and compensatory hyperinsulinemia.

Fig. 5. Average plasma FFA concentrations at week 0 (open bars) and week 6 (hatched bars) at basal, between 6:00 and 8:00 PM, and between 2:00 and 4:00 AM, when the control meal was given on the day of the experiment (A) and when the fat-supplemented meal was given on the day of the experiment (B). *P < 0.05 vs. week 0, paired t-test.

Fig. 6. Twenty-four-hour plasma profiles of cortisol (A), growth hormone (B), glucagon (C), and glucagon-like peptide-1 (GLP-1; D) at week 0 (○) and week 6 (●). Vertical dashed lines represent times of meal presentation and meal removal. *P < 0.05 vs. week 0, paired t-test.
In the current study, eight normal dogs were fed a hyper-
caloric, high-fat diet with an increase in fat content of ~20% 
for a period of 6 wk. There was significant accumulation 
of total trunk body fat due to increases in both visceral 
and subcutaneous fat depots with a concomitant increase in 
body weight. The dogs exhibited a decrease in insulin sensitivity 
and developed fasting hyperinsulinemia. However, despite 
increased adiposity, insulin resistance, and fasting hyperinsulin-
emia, the animals did not develop an elevation in fasting FFA 
or fasting hyperglycemia. Neither fasting FFA nor glucose 
appears to be the cause for development of insulin resistance 
and compensatory hyperinsulinemia in this obese dog model. 
More impressive was virtually exact reproducibility of the 24-h 
glucose pattern in lean vs. fat-fed animals in contrast to the 
elevation in 24-h insulin profile after fat feeding. Similarly, 
although circadian rhythms were present, there were no 
changes in the 24-h profiles of growth hormone, cortisol, or 
even GLP-1 before compared with after fat feeding. In contrast 
to all other variables measured, there was a profound and 
highly significant increase in the 24-h profile of FFA after fat 
diet, due almost entirely to the nocturnal rise in FFA, which 
 began at ~5:00 PM and continued throughout the night into 
the early morning. These results nominate nocturnal elevation 
of FFA to be potentially responsible for development of insulin 
resistance and subsequent increase in plasma insulin availabil-
ity during diet-induced obesity in the dog model.

There has been much evidence supporting the existence of 
differences between the diurnal metabolic profiles of obese and 
nonobese individuals as well as the discrepancy between the 
diurnal patterns of healthy vs. insulin-resistant subjects. It has 
been reported that a diurnal variation in FFA exists (32) as well 
as a fall in hepatic glucose output which occurs during sleep 
that is highly synchronized with a decrease in FFA levels in 
normal weight individuals (13). It has also been shown that the 
rate of appearance of FFA at night is increased in type 2 
diabetics (30) as well as an overnight elevation in plasma FFA 
that is correlated with the overnight increase in hepatic glucose 
output in type 2 diabetes (44). In addition, it has been demonstra-
ted that a reduction in overnight FFA levels by acute 
pharmacological blockade of lipolysis results in a reduction of 
insulin levels (1). This suggests that the elevated nocturnal 
FFA observed with fat feeding may be pivotal in the develop-
ment of hepatic insulin resistance in addition to being a 
potential signal for insulin upregulation, even when fasting 
FFA are unchanged.

The data of the current study linking elevated nocturnal FFA 
with insulin resistance and compensatory hyperinsulinemia are 
indeed correlative in nature. Nevertheless, the suggestion that 
FFA themselves may play an important role in the metabolic 
syndrome is supported by a wealth of data showing that 
chronic infusion of FFA decreases insulin sensitivity and that 
FFA can upregulate insulin levels by both increasing insulin 
secretion and decreasing hepatic insulin extraction. Acute elev-
ation of FFA by intravenous infusion enhanced both basal 
and glucose-stimulated insulin secretion, whereas long-term infu-
sion of FFA has been shown to attenuate pancreatic insulin 
secretion (9, 12). Others have shown that an acute elevation of 
FFA can cause hyperinsulinemia without a change in insulin 
secretion (5). In addition, FFA impair the hepatic clearance 
of insulin, which would increase the fraction of secreted insulin 
delivered to the systemic circulation (42, 47). In this study, 
there was development of hyperinsulinemia without a similar 
change in C-peptide concentrations, suggesting that hepatic 
clearance of insulin was indeed responsible for basal hyper-
insulinemia and the upregulated insulin levels in this model of 
insulin resistance. Although the majority of the difference in 
plasma insulin levels occurs when FFA are in fact similar, it is 
possible that the chronic effect of elevated FFA at night may 
impair hepatic insulin extraction by means of increased triglyc-
eride deposition in the liver, resulting in subsequent hyper-
insulinemia (43). Our group has shown previously that dogs 
fed a diet with an elevated fat content exhibited a 45% increase 
in liver triglyceride (24, 25). In addition, because we did not 
measure the time course of changes in nocturnal FFA during 
fat feeding, we cannot discount the possibility that potential 
earlier elevations in nocturnal FFA led to temporal augmenta-
tion of insulin secretion by direct stimulation of the pancreatic 
ß-cells. Previous studies completed in our laboratory have 
shown that in dogs fed a lesser fat diet, compensation for 
insulin resistance occurs in a longitudinal manner that includes 
both enhanced insulin secretion and decreased insulin clear-
ance (31), suggesting that there may be a temporal effect of 
FFA in mediating hyperinsulinemia via insulin secretion vs. 
insulin clearance. Studies examining the longitudinal time 
course for the development of elevated nocturnal FFA and 
insulin resistance during high-fat feeding in conjunction with 
the direct examination of insulin secretion vs. hepatic insulin 
 extraction are currently under investigation in our laboratory.

Although the present studies were not designed to determine 
the origin of the highly significant increase in the nocturnal 
levels of FFA, we can speculate as to potential mechanisms. 
Lipolysis of endogenous FFA stored as triglycerides (mainly 
adipose tissue) is a major determinant of increased FFA in 
plasma (4). However, in the present study, likely lipolytic 
candidates such as growth hormone and cortisol as well as 
glucagon were ruled out. It is also possible that the sensitivity 
to lipolytic factors is altered in the obese, insulin-resistant 
animal. So-called “adipokines,” which have been implicated in 
the development of insulin resistance and hyperinsulinemia 
(34), may also be responsible for increased lipolytic activity 
during obesity. In particular, IL-6 has been shown to be 
elevated in obesity and has also been shown to display a 
diurnal pattern of secretion, with IL-6 levels peaking at ~1:00 
AM or later (39). Moreover, it has been demonstrated that IL-6 
can elicit lipolytic effects in adipose tissue both in vitro and in 
vivo (27, 28), implying that IL-6 may be a mechanism 
involved in modulating FFA levels. Low adiponectin levels 
have also been associated with insulin resistance and obesity 
(3) and have been implicated as a possible regulatory factor 
involved in FFA release (45). In addition, adiponectin was 
recently shown to exhibit ultradian pulsatility as well as a 
diurnal variation that declines significantly at night (19) and is 
absent in obesity and insulin resistance (11), suggesting that 
decreased levels of adiponectin may also be involved in me-
diating the nocturnal elevation of FFA observed in this study. 
Another possible mechanism for increased FFA is catechol-
amine-stimulated lipolysis. Efferent signals from the brain via 
sympathetic nervous system innervation of adipose tissue have 
been shown to be of great significance in the mobilization of 
FFA stores from adipose tissue (6). Moreover, Landsberg (26) 
found increases in sympathetic activity in obesity in humans, 
and it has been suggested that sympathetic activity is increased
at night in at-risk patients (33a). Our group (23) recently presented evidence for cyclic lipolysis that can be blocked by the β3 receptor antagonist bupranolol, indicating an important role for the central nervous system in regulating the provision of lipid fuels. It is tempting to suggest that the central nervous system plays an intermediate role in the development of insulin resistance and subsequent upregulation of plasma insulin in response to fat feeding by modulating sympathetic nervous system control of FFA levels at night. In addition to increased lipolytic release of intracellular FFA stores, a proportion of fatty acids generated by intravascular triglyceride hydrolysis also contributes to plasma FFA (18, 29), and this can be increased in obesity (37). In this study, although FFA levels showed a similar suppression following the meal before and after fat feeding, FFA levels at week 6 began to increase during the late postprandial period despite unchanged triglyceride levels. This late postprandial rise in FFA levels suggests that there may have been an increase in the proportion of fatty acids released from triglyceride-rich lipoproteins that further contributed to the nocturnal increase in FFA. Further studies utilizing tracer techniques are needed to address this issue.

It is also possible that the sensitivity to lipolytic factors is altered in the obese, insulin-resistant animal. Although lipolytic hormones such as growth hormone, cortisol, and glucagon were unchanged by fat feeding during the 24-h experiments, we observed elevated FFA levels the morning following the experiment (i.e., 6:00 AM on day 2). Although it is possible that the experimental paradigm per se may have contributed to the elevated FFA observed on day 2 of the experiment, it is of note that this effect was only observed after 6 wk of fat feeding.

It is generally agreed that the defect in insulin sensitivity as well as a defect in pancreatic function in combination are responsible for the chronic hyperglycemia seen in diabetes. To effectively prevent the onset of diabetes, it is crucial to understand the underlying mechanisms responsible for development of insulin resistance and subsequent hyperinsulinemic compensation in normal individuals and how these mechanisms might fail in those at risk for diabetes. Our results indicate that during obesity induced by a high-fat diet, there is development of central nervous system origins of the sympathetic nervous system outflow to white adipose tissue. Ann J Physiol Regul Integr Comp Physiol 275: R291–R299, 1998.

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