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Effect of a High-fat Diet on 24-Hour Pattern of Circulating Adipocytokines in Rats

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We have shown a significant disruption of 24-h pattern of plasma pituitary, adrenal, and gonadal hormones in high-fat-fed rats. Our objective was to assess the effect of a high-fat diet (35% fat) on mean levels and 24-h pattern of several adipocytokines in rats. A normal diet–fed rats (4% fat) were used as controls. When body weight of high-[Q2] **fat-fed rats attained values about 25% higher than controls (after 66 days of treatment), the animals were killed at six different time intervals throughout a 24-h cycle. Plasma concentrations of insulin, adiponectin, interleukin (IL)-1, leptin, ghrelin, plasminogen activator inhibitor-1 (PAI-1), and monocyte chemoattractant protein-1 (MCP-1) were measured in a multianalyte profiling by using the Luminex-100 system. Tumor necrosis factor α (TNFα) and IL-6 were measured**[Q3] **by enzyme-linked immunosorbent assay. A significant hyperglycemia developed in high-fat-fed rats, together with a significant increase in plasma insulin. Mean levels of plasma adiponectin, IL-1, IL-6, TNFα, and leptin augmented, and ghrelin decreased, in high-fat-fed rats. The normal daily pattern of plasma insulin, adiponectin, IL-1, IL-6, TNFα, leptin, ghrelin, and MCP-1 became disrupted in high-fat-fed rats. The results indicate that a high-fat diet may bring about signs of insulin resistance and mild inflammation in rats, together with the disruption in daily variations of circulating insulin and ghrelin, and of several adipocytokines including leptin, adiponectin, IL-1, IL-6, TNFα, and MCP-1.**

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INTRODUCTION

There is a large body of evidence linking feeding regimens and food components with the circadian system (see refs. 1,2). A high-fat diet, that contributes to insulin resistance and inflammation (3), aggravates type 2 diabetes mellitus, stroke, and coronary artery disease (4) and can feed back to influence the biological clock (5). Indeed, circadian oscillation of many hormones involved in metabolism, such as corticosterone, insulin, glucagon, adiponectin, leptin, and ghrelin, becomes disrupted in the development of the metabolic syndrome and obesity (1).

Taking high-fat diet–fed rats as a model, we recently reported a significant disruption of 24-h pattern of plasma prolactin, luteinizing hormone, thyrotropin, testosterone, and corticosterone (6). Concomitantly, amplitude of the nocturnal pineal melatonin peak decreased by about half, underlying the significant effects that obesity has on the circadian apparatus. Indeed, via a number of secreted proteins called adipocytokines including hormones, cytokines, growth factors, complement factors, and matrix proteins, the adipose tissue participates in the regulation of body weight homeostasis, glucose and lipid metabolism, immunity and inflammation (see refs. 7–10). This prompted us to examine whether the significant disruption of 24-h hormonal pattern seen in high-fat-fed rats coexists with changes in the daily pattern of several circulating adipocytokines.

METHODS AND PROCEDURES

Animals and experimental design

Male Wistar rats (45 days of age) were maintained under standard conditions with controlled light (12:12-h light/dark schedule; lights on at 0800 hours) and temperature (22 ± 2 °C). Rats were divided into two groups: (i) normal diet *ad libitum* and (ii) high-fat diet *ad libitum*. Both control (4% fat) and high-fat (35% fat) diets were obtained from Harlan Iberica, Barcelona, Spain. Diets were balanced for protein as a percentage of energy intake and for essential vitamins and minerals. The fat source in both the control and high-fat diets was corn oil. The high-fat diet contained 4.8 kcal/g and the 4% fat control diet, 4.0 kcal/g. In place of fat (corn oil), the 4% fat diet contained a slightly greater amount of corn starch. Individual daily food intake was 17 ± 1 g (normal diet) and 19 ± 1 g (high-fat diet). The percentage of food intake at night was 71.4 ± 11.3% (normal diet) and 68.7 ± 9.4% (high-fat diet). No gross changes in physical activity timing were detected between groups.

When body weight of high-fat-fed rats attained values about 25% higher than controls (after 66 days of treatment), rats were killed by decapitation under conditions of minimal stress at six different time intervals (eight rats per group), every 4 h throughout a 24-h cycle,

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starting at 0900 hours. All experiments were conducted in accordance with the guidelines of the International Council for Laboratory Animal Science. Trunk blood was collected and plasma samples were obtained by centrifugation of blood at 1,500 *g* for 15 min. EDTA (6 g/100 ml) was used as an anticoagulant. Samples were stored at −70 °C until further analysis.

Hormone and adipocytokine assays

Plasma concentrations of insulin, adiponectin, interleukin-1, leptin, ghrelin, plasminogen activator inhibitor 1 (PAI-1), and monocyte chemoattractant protein-1 (MCP-1) were measured in a multianalyte profiling by using the Luminex-100 system and the XY Platform (Luminex, Oosterhout, the Netherlands) (11). Calibration microspheres for classification and reporter readings as well as sheath fluid were also purchased from Luminex. Acquired fluorescence data were analyzed by the MasterPlex QT software (Hitachi Software Engineering America, MiraiBio Group, South San Francisco, CA). All analyses were performed according to the manufacturers' protocols. Tumor necrosis factor α (TNFα) and IL-6 were measured by enzyme-linked immunosorbent assays with commercially available reagent sets (Quantikine HS Rat TNFα and Quantikine HS Rat IL-6 Immunoassays; R&D Systems, Minneapolis, MN)

Statistical analysis

Statistical analysis of results was performed by a Student's *t*-test, a one-way ANOVA or a two-way factorial ANOVA, as stated. For the factorial ANOVA, the analysis included assessment of the group effect (i.e., the occurrence of differences in mean values between normal and high-fat-diet rats), of time-of-day effects (the occurrence of daily changes), and of the interaction between the two factors (diet and time, from which inference about differences in timing and amplitude could be obtained). Post-hoc Bonferroni's multiple comparison tests in a one-way ANOVA were then employed to show which time points were significantly different within each experimental group to define existence of peaks. *P* values <0.05 were considered evidence for statistical significance.

RESULTS

Table 1 summarizes progression of body weight in the two groups of animals. Body weight of high-fat-fed rats attained values 23.8% higher than controls after 66 days of treatment.

The effect of a high-fat diet on the levels of glucose and insulin in plasma is summarized in **Figure 1**. A significant hyperglycemia developed in high-fat-fed rats ($F = 41.7$, $P < 0.00001$), glucose levels showing essentially similar daily patterns in both groups of animals, i.e., a nadir at the second part of the scotophase. A significant increase in plasma insulin occurred in high-fat-fed rats ($F = 73.2$, $P < 0.00001$), but this effect was not seen at all time points, e.g., at 0100 and 0500 hours, insulinemia did not differ between groups. Thus, a significant interaction "time × diet" was detectable in the factorial ANOVA ($F = 4.76$, $P < 0.001$).

Figure 2 shows the daily changes of plasma adiponectin, IL-1, IL-6, and TNFα in the two groups of animals. When analyzed as main factors in the factorial ANOVA, mean levels of adiponectin, IL-1, IL-6, and TNFα augmented in high-fat-fed rats ($F = 47.4$, $P < 0.00001$; $F = 4.68$, $P < 0.04$; $F = 8.66$, $P < 0.005$; and $F = 11.6$, $P < 0.001$, respectively). The daily pattern of adiponectin and TNFα differed significantly between groups as shown by a significant interaction "time × diet" in the factorial ANOVA (adiponectin: $F = 2.59$, $P < 0.04$; TNFα: $F = 8.38$, $P < 0.001$), i.e., the maximum adiponectin levels found in the

Table 1 Body weight (g) in Wistar male rats fed with normal or high-fat diet, as described in Methods and Procedures

Days on diet	Normal diet	High-fat diet
1	257.5 ± 35.1	263.9 ± 38.1
3	270.1 ± 65.9	294.2 ± 61.6
10	293.9 ± 67.2	331.9 ± 65.6*
14	305.9 ± 62.6	352.2 ± 58.4*
19	325.8 ± 65.1	374.5 ± 60.6*
31	345.8 ± 79.3	407.2 ± 68.1*
38	357.1 ± 59.6	419.9 ± 60.4*
45	368.0 ± 50.1	439.2 ± 62.3*
53	377.7 ± 63.7	455.2 ± 55.6*
60	386.0 ± 63.9	473.1 ± 67.7*
66	397.5 ± 73.6	492.5 ± 60.1*

Shown are the means ± s.d. ($n = 48$ rats/group).
*$P < 0.01$ vs. normal diet (Student's *t*-test).

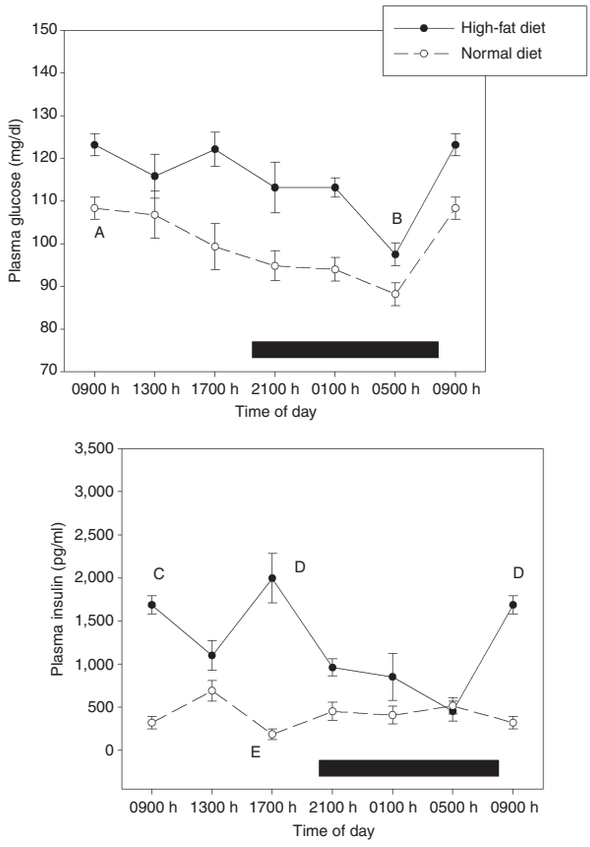

Figure 1 Twenty-four-hour changes in circulating levels of glucose and insulin in Wistar male rats fed with normal or high-fat diet, as described in Methods and Procedures. Groups of eight rats were killed by decapitation at six different time intervals throughout a 24-h cycle. Bars indicate scotophase duration. Shown are the means ± s.e.m. Letters indicate the existence of significant differences between time points within each experimental group after a one-way ANOVA followed by a Bonferroni's multiple comparison test. [A]$P < 0.05$ vs. 0500 hours. [B]$P < 0.01$ vs. 0900 hours, $P < 0.05$ vs. 1700 and 0100 hours. [C]$P < 0.01$ vs. 0500 hours. [D]$P < 0.02$ vs. 2100, 0100, and 0500 hours, $P < 0.05$ vs. 1300 hours. [E]$P < 0.01$ vs. 1300 hours. For further statistical analysis, see text.

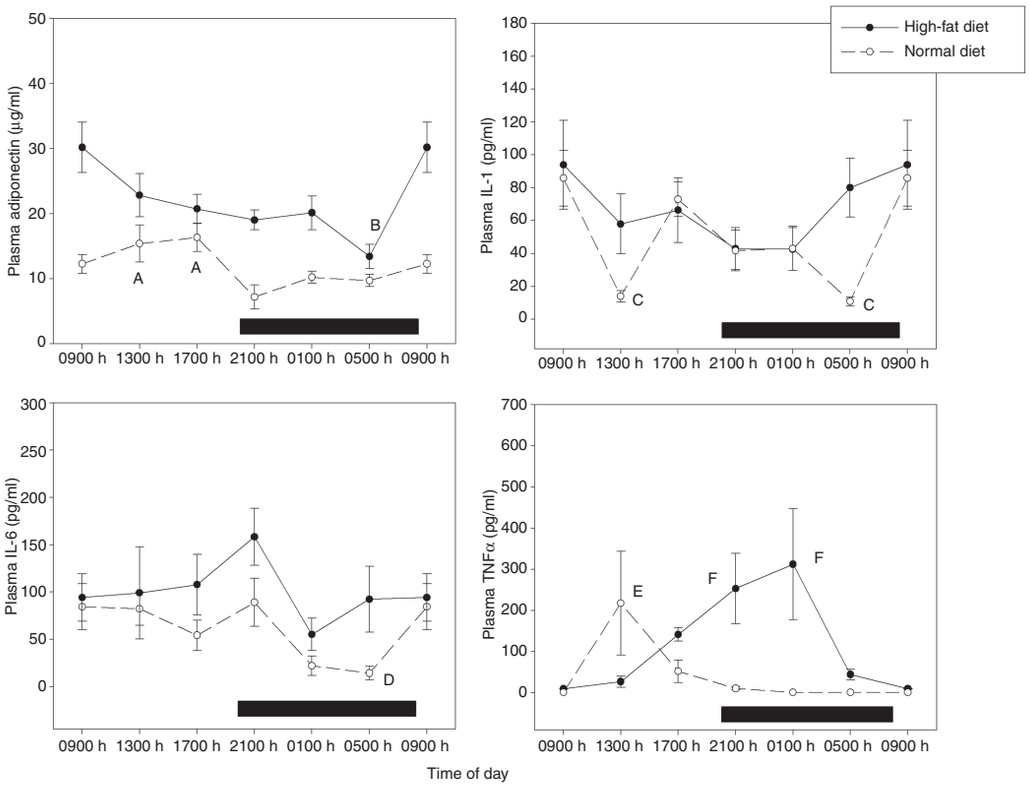

Figure 2 Twenty-four-hour changes in circulating levels of adiponectin, IL-1, IL-6, and TNFα in Wistar male rats fed with normal or high-fat diet, as described in Methods and Procedures. Groups of eight rats were killed by decapitation at six different time intervals throughout a 24-h cycle. Bars indicate scotophase duration. Shown are the means ± s.e.m. Letters indicate the existence of significant differences between time points within each experimental group after a one-way ANOVA followed by a Bonferroni's multiple comparison test. $^{A}P < 0.05$ vs. 2100 hours. $^{B}P < 0.05$ vs. 0900 and 1300 hours. $^{C}P < 0.02$ vs. 0900 and 1700 hours. $^{D}P < 0.05$ vs. 2100 hours. $^{E}P < 0.05$ vs. 0900, 2100, 0100, and 0500 hours. $^{F}P < 0.05$ vs. 0900 and 1300 hours. For further statistical analysis, see text. IL, interleukin; TNF, tumor necrosis factor.

afternoon was phase-advanced to early morning in obese rats whereas the nadir in TNFα levels seen in controls at night was not longer observed in high-fat-fed rats (**Figure 2**).

As depicted in **Figure 3**, high-fat-fed rats showed a significant increase in mean values of plasma leptin (expressed as total values or on body weight basis, **Figure 3a,b**, $F = 79.3$ and 59.9, $P < 0.00001$) and a significant decrease of mean values of total or active plasma ghrelin (**Figure 3c,d**, $F = 37.7$, $P < 0.00001$ and $F = 7.06$, $P < 0.02$). In rats kept under a normal diet, leptin values peaked at the middle of scotophase whereas in high-fat-fed rats, daily variations did not attain significance (**Figure 3**). The relatively higher values of ghrelin seen at night were not longer observed in high-fat-fed rats.

Figure 4 shows the daily changes of PAI-1 and MCP-1 in both groups of animals. Although PAI-1 levels did not show differences between groups, mean plasma MCP-1 levels augmented in high-fat-fed rats ($F = 17.8$, $P < 0.0001$). The increase was observed at certain time points only (light phase and early scotophase). Thus, a significant interaction "time × diet" was detectable in the factorial ANOVA ($F = 4.71$, $P < 0.002$).

DISCUSSION

As expected (12–15), in high-fat-fed rats, increased circulating levels of leptin and decreased plasma ghrelin occurred, together with signs of insulin resistance (i.e., hyperglycemia and increased insulin levels). Concomitantly, the increased mean levels of plasma IL-1, IL-6, TNFα, and MCP-1 suggested the occurrence of a moderate degree of inflammation in high-fat-fed rats. The normal daily pattern of plasma insulin, leptin, ghrelin, adiponectin, TNFα, and MCP-1, and to a lesser extent, that of IL-1 and IL-6, became disrupted in experimentally obese rats.

There is impressive information indicating that obesity is associated with a low-grade inflammation of the white adipose tissue that can subsequently lead to insulin resistance, impaired glucose tolerance, and diabetes (16–18). Inflammation in obesity is indicated by increased circulating levels of C-reactive protein and other biological markers of inflammation. The adipose tissue is in obesity characterized by an increased production and secretion of inflammatory molecules like TNFα and IL-6, which may have local and systemic effects (16–18). The amounts of TNFα and IL-6 are positively correlated with body fat and decrease in obese patients after weight loss (19,20). Among the biological actions of TNFα and IL-6, induction of insulin resistance is paramount; thus, fat cells are both a source of and a target of TNFα and IL-6. High-fat diets like that employed in the present study have been shown to produce a significant increase of TNFα, IL-1, and

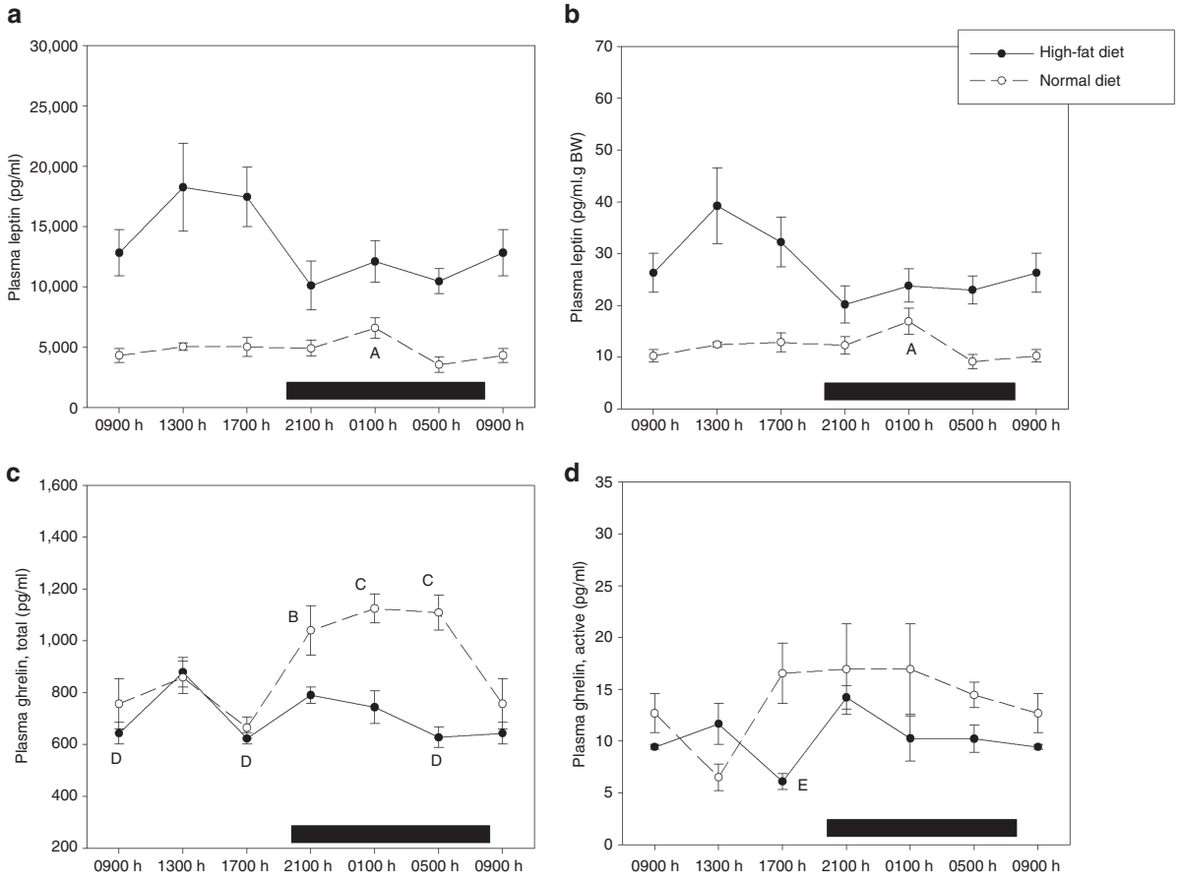

Figure 3 Twenty-four-hour changes in circulating levels of leptin (expressed as (**a**) total values or (**b**) per gram of BW), (**c**) total ghrelin, and (**d**) active ghrelin in Wistar male rats fed with normal or high-fat diet, as described in Methods and Procedures. Groups of eight rats were killed by decapitation at six different time intervals throughout a 24-h cycle. Bars indicate scotophase duration. Shown are the means ± s.e.m. Letters indicate the existence of significant differences between time points within each experimental group after a one-way ANOVA followed by a Bonferroni's multiple comparison test. $^{A}P < 0.05$ vs. 0500 hours. $^{B}P < 0.02$ vs. 1700 hours. $^{C}P < 0.02$ vs. 0900 and 1700 hours. $^{D}P < 0.02$ vs. 1300 hours. $^{E}P < 0.05$ vs. 2100 hours. For further statistical analysis, see text. BW, body weight.

IL-6 levels (9). The increased circulating levels of leptin and insulin and decreased plasma ghrelin herein reported in high-fat-fed rats confirm that excess fat accretion is associated with hyperleptinemia, hyperinsulinemia, and hypoghrelinemia in experimental animals and humans (9,12,14,15,21,22).

Ghrelin is a 28-amino-acid gastric peptide that in order to be active, requires n-octanoylation at serine 3 (ref. 23). As in humans (24), a correlation between circulating levels of total and active ghrelin was found in rats, with more defined effects of the high-fat diet on total ghrelin levels. The relatively higher concentration of ghrelin seen at night was not longer observed in high-fat-fed rats, a finding that could not be attributed to changes in timing of meal intake or physical activity that was essentially similar in the two diet groups.

Adiponectin is known to have an important anti-inflammatory and antiatherogenic effect that is apparently mediated by inhibition of inflammatory cytokines, blocking the activation of macrophages and posterior transformation to foam cells (see refs. 25,26). Although several studies point out to a decrease in plasma concentration of adiponectin in obesity, our present results indicate an increase in mean levels of adiponectin in high-fat-fed rats as well as a significant modification in its daily pattern in circulation. The data agree with the observations of Naderali *et al.* who reported that after 16 weeks of a high-fat diet, rats had significantly higher plasma levels of adiponectin, assayed at a single time point, as compared to chow-fed controls (27). Therefore, in dietary obese rats, a decrease in adiponectin mRNA levels reported in fat (27,28) does not translate to a parallel decrease in plasma adiponectin concentration.

In the stromovascular (nonadipocyte) fraction of adipose tissue from obese rodents, there is an increased number of bone marrow–derived macrophages (29). Indeed, white adipose tissue from obese animals expresses multiple genes usually attributed to macrophages. The mechanism for macrophage recruitment to adipose tissue has not been defined in detail but presumably includes chemotactic molecules like MCP-1, that is synthesized and secreted by preadipocytes and mature adipocytes in diet-induced obese mice (30). Our present results indicate that a high-fat diet augments the mean levels of MCP-1 and disrupts its 24-h pattern. Because MCP-1 impairs insulin-stimulated glucose uptake by cultured

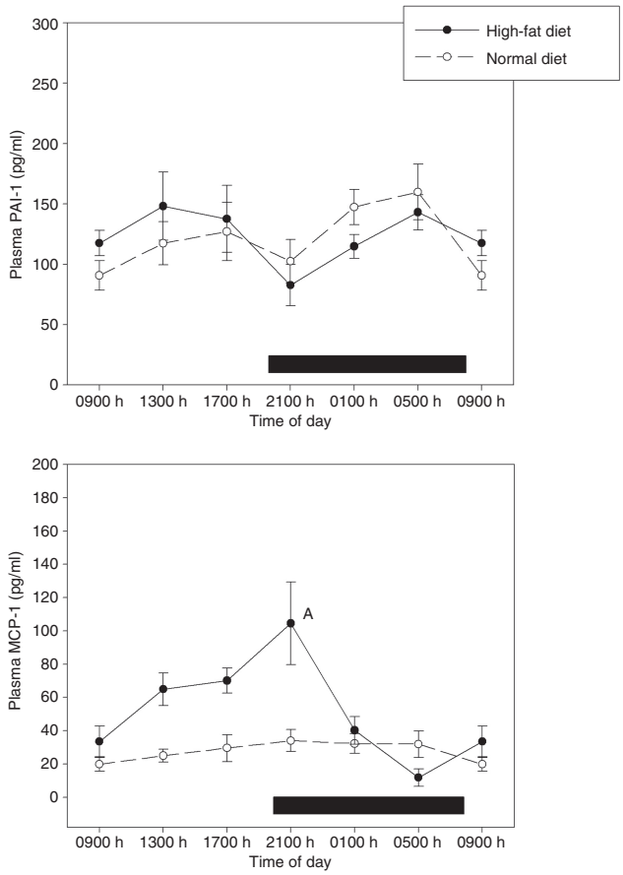

Figure 4 Twenty-four-hour changes in circulating levels of PAI-1 and MCP-1 in Wistar male rats fed with normal or high-fat diet, as described in Methods and Procedures. Groups of eight rats were killed by decapitation at six different time intervals throughout a 24-h cycle. Bars indicate scotophase duration. Shown are the means ± s.e.m. Letters indicate the existence of significant differences between time points within each experimental group after a one-way ANOVA followed by a Bonferroni's multiple comparison test. $^{A}P < 0.01$ vs. 0900 and 0500 hours. For further statistical analysis, see text. MCP-1, monocyte chemoattractant protein-1; PAI-1, plasminogen activator inhibitor-1.

adipocytes *in vitro* (31), the increased MCP-1 levels may contribute to the insulin resistance.

Our results on the lack of changes of circulating PAI-1 in obese rats were unexpected. PAI-1 is a prothrombotic factor secreted among other cells, by adipocytes that negatively regulate fibrinolysis by inhibiting tissue plasminogen activator (32). PAI-1 has been associated with insulin resistance, obesity, impaired glucose tolerance, and type 2 diabetes in cross-sectional studies (33). Perhaps the high-fat diet was not kept long enough to modify the mean levels or diurnal pattern of this adipocytokine.

Summarizing, what is novel from the present results in high-fat-fed rats is the disruption of the 24-h pattern of some plasma adipocytokines, remarkably adiponectin, TNFα, and MCP-1, and to a lesser extent, IL-1 and IL-6. Indeed, there is a large body of evidence that links feeding regimens, food components, and adipose tissue signals with the circadian system (1,2,5). A high-fat diet like that employed in the present study can feed back to influence the biological clock. We recently reported a significant reduction in the nocturnal pineal melatonin peak, a key marker of the central oscillator located in the suprachiasmatic nucleus, as well as a significant disruption of the 24-h pattern of several hormones, in high-fat-fed rats (6).

As the most relevant changes were observed in the circulating levels of adiponectin, TNFα, and MCP-1, the measurement of these adipocytokines in adipose tissue could be helpful for further understanding of these physiological processes involved. Measurement of the circulating levels of C-reactive protein under the present experimental conditions could also be helpful to define the intensity of inflammation. Likewise, because the presence of circadian clock genes has been reported in fat (34), further examination of clock gene expression in this tissue may give information on the metabolic implications of the present results.

Collectively, our previous observations and the present results suggest that a high-fat intake causing insulin resistance and signs of inflammation may disrupt the daily pattern of several hormones and adipocytokines, an indication that obesity has a significant effect on circadian organization of neuroendocrine and immune responses. The observational nature of the present study precludes any inference on the mechanisms involved. Further studies are needed to describe the physiologic consequence of the disrupted pattern of circulating adipocytokine levels in high-fat-fed rats.

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DISCLOSURE

The authors declared no conflict of interest.

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