Ghrelin, Leptin and Plasma Free Fatty Acid (FFA) Levels are Modulated in Adaptation to Fasting

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ABSTRACT

BACKGROUND: The hormones ghrelin and leptin play central roles in the regulation of appetite, energy expenditure and body weight. Changes in ghrelin and leptin, at least to some degree, are thought to underpin metabolic diseases such as the *metabolic syndrome*. This regulation is particularly highlighted in the response of a small mammal to a state of starvation in which ghrelin and leptin are modulated in order to increase survivability. The aim of this study was to examine the relationship between ghrelin, leptin, and plasma FFAs after fasting in mice.

METHODS: Ten male mice were subjected to a controlled 18-hour fast. After 18 hours, a terminal blood sample was obtained for the analysis of hormone and plasma FFA levels. These levels were compared to control mice that received a normal diet and to baseline levels prior to fast.

RESULTS: We found that the plasma ghrelin levels in the fasting mice were significantly higher than in control (1106 ± 72.57 vs. 282.3 ± 28.07 pg/ml; P<0.0001, STD err). In an antagonist manner, plasma leptin was significantly lower in fasting mice (3.2 [IQR 2.66-4.653] vs. 0.64 [IQR 0.458-1.105] ng/ml; P<0.0001). Plasma FFA doubled from control values of 4.438 ± 0.094 to a fasting average of 7.276 ± 0.413 µEq/L.

CONCLUSION: The levels of plasma ghrelin, which is an appetite inducer, are significantly increased during a fast. Plasma leptin, which produces satiety, is reduced. Plasma FFAs are increased approximately two-fold, probably as a result of mobilization of fat stores. These changes may help to prolong survival of the animal under harsh conditions.

Key words: Ghrelin, Leptin, Fasting, Adaptation

INTRODUCTION

Fasting and starvation present metabolic challenges to mammals, and require shifts in energy utilization in order to supply nutrients and energy-rich molecules to organ systems. The largest reserve of energy is stored in adipose tissue, which can be accessed when hepatic and muscle glycogen stores are depleted. Triacylglycerides (TAGs) stored in adipocytes are cleaved to produce non-esterified fatty acids (NEFA) or free fatty acids (FFA) resulting in sparing muscle protein, using glycerol for gluconeogenesis and formation of ketone bodies [1]. Ghrelin and leptin, two opposing hormones, play a significant role in regulating food intake and energy expenditure, through both neurological and endocrine processes.

Ghrelin is an orexigenic hormone which has the overall effect of increasing food intake (if possible) and decreasing energy expenditure. Furthermore, it upregulates lipolysis through the release of growth hormone. Ghrelin has been shown to decrease insulin; however, hyperinsulinemia appears to act as a negative feedback system for ghrelin secretion [3].

In contrast, leptin administration inhibits food intake and leads to a decrease in body weight.

Similar to ghrelin, leptin also has endocrine effects. Administration of leptin to fasting mice has been shown to increase the circulating levels of insulin, glucose and glucagon [5]. Leptin decreases glucocorticoid and increases thyroid hormone secretion, which can interfere with survival in the fasting animal [5]. Moreover, leptin acts as a feedback signal to the brain for the levels of energy remaining in storage [4, 5]

FFA flux is regulated by two key enzymes. Hormone-sensitive triacylglyceride lipase (HSL) is a cytosolic enzyme present in adipocytes that hydrolyzes internal TAGs to NEFAs and releases them into plasma [4]. Growth hormone increases HSL activity whereas insulin decreases it. The other enzyme is the membrane-bound lipoprotein lipase (LPL). LPL hydrolyzes plasma TAGs and sequesters FFAs into cells for lipogenesis. LPL activity is inhibited by growth hormone but upregulated by insulin [4]. As such, it may be that ghrelin and leptin act, through insulin, to increase or decrease fat mobilization through HSL and LPL, respectively.

The mouse is a small mammal and hence, it has a faster metabolic rate than humans and proportionally less adipose tissue. Therefore, an 18-hour fast is equivalent to starvation and is expected to greatly change the variables of interest. One simple measurement of judging the strength of the fast on metabolism is to observe weight change. We expect a significant reduction in weight in fasting mice as evidenced in previous studies on rodents [6, 7,8]. Mice have limited levels of glycogen which are all but depleted in a 24-hour fast [9]. Literature supports the rise in plasma FFAs in the fasting state in rodents, and this rise is approximately two-fold[8]. Furthermore, in mice, plasma FFAs reach a plateau at 6-12 hours and remain steady until near depletion of adipose tissue [7,8]. However in some studies, there has been no change in plasma FFAs in mice fasting for up to 48 hours [9]. Increase in ghrelin levels is well documented in the fasting state [1,3, 10]. Moesgaardet et al [6] report that after an 18 hour fast, plasma ghrelin levels went from a baseline of ~5 to ~9 ng/ml. Leptin is expected to decrease as reported in previous studies, and the reduction is expected to be in the range of 60-75% [11]. These three metabolic factors have not been measured in conjunction before. Furthermore, the number of such studies on mice has been limited with most researchers choosing to utilize rats due to easier surgical removal and measurement of liver, muscle and adipose tissue. We hypothesize that the endocrine hormone ghrelin increases vastly in the serum while leptin is suppressed in the fasting mice. Moreover, plasma FFAs (NEFA) increase in response to the energy restriction as the body weight is reduced.

MATERIALS AND METHODS

Animals

Twenty male mice, 8-9 weeks of age, of the strain C57BI/6 were fed normal diets prior to the experiment. The average weight of the mice was 24.8 ± 0.63 grams (95% CI. STD err). Permission to use these animals was obtained from the University of Queensland Ethics Committee (cert # SBMS/043/11/T). The mice were housed in appropriate enclosures, with free access to mouse chow and water, in a temperature and humidity controlled environment.

Experimental approach

The mice were assigned to one of two groups: fed *ad libitum* (control, n=10) and fasted (n=10). The pre-treatment weight of each mouse in each group was obtained. Mice in the control group had free access to standard mouse chow, consisting of ~70% carbohydrate content, and water. Mice in the fasting group were denied access to food, but not water, commencing approximately 9:00 PM the evening before sample collection; they fasted for approximately 18 hours. Mice were housed individually in their respective enclosures to minimize social stress. The following day, mice were weighed and then anesthetized with a single intraperitoneal injection of pentobarbital (32 mg/kg BW). The onset of complete anesthesia was tested using the pedal withdrawal reflex. Approximately 300-1000 µl of blood was obtained through cardiac puncture of the ventricle.

Blood samples were centrifuged for 2 minutes using a micro centrifuge to obtain 70-100 µl plasma as supernatant. The supernatant was removed and distributed into four tubes, ghrelin, leptin and FFAs (x2); about 25, 15, and 10µl of plasma was added to each tube, respectively, to meet requirements for the commercial ghrelin and leptin ELISA and FFA assay. 6µl phenylmethanesulfonylfluride (PMSF) was immediately added to the ghrelin sample to prevent deacylation of the active form. The samples were stored in cold temperature until assays were performed.

Measurement of plasma ghrelin, leptin and free-fatty acids

Acyl-ghrelin concentrations were measured using a commercial Linco® Rat/Mouse Ghrelin (active) ELISA kit (cat# EZRGA-90K). Eight standard solutions of known concentrations of acyl-ghrelin, ranging from 0 (blank) – 1900 pg/ml (assay range 25-2000 pg/ml), were used to generate a linear regression plot for assaying unknown samples

(see suppl.). Horseradish peroxidase and the substrate 3,3', 5,5' tetra-methylbenzidine produced absorbency at 450 nm, corrected from absorbency at 590 nm, in relation to ghrelin concentration. Specific steps were performed in accordance with the instructions enclosed with the kit. In a similar manner, plasma leptin was measured using a commercial Millipore® Mouse Leptin ELISA kit (cat# EZML-82K). Assay range was determined by standard solutions of leptin which were 0.2 – 20 ng/ml, plus 0 ng/ml (blank). However, 20 ng/ml standard leptin was excluded due to incongruity.

Non-esterified fatty acids (NEFAs) were measured using the Wako NEFA-C assay. Six standard solutions were used for the linear regression plot (0.125 – 1 µEq/L, plus 0 [blank]). Absorbency at 550 nm (blue-pigment) was used to detect quantity of hydrogen-peroxide which was yielded from the breakdown of FFAs.

Statistical analysis

A new variable, weight-change, was created from the difference between the pre- and post-treatment weight for both groups. This variable, and data for ghrelin, leptin and FFA, was tested for normality using the Shapiro-Wilk test, which all passed except for leptin. Hence, statistical comparison of fasted mice to control mice on weight-change, ghrelin and FFA values, was performed using Student's unpaired t-test. Comparison of leptin between the groups was performed using the Mann-Whitney non-parametric test. Exact P-values are reported with P<0.001 being highly significant (***). Results for weight-change, ghrelin and FFA presented as means ± S.E.M (*n*=10). Non-parametric data for leptin is presented as medians (IQR 25-75%).

RESULTS

In 18-hour starved mice, the average weight lost from baseline measurements prior to fasting, was significantly more than for mice that were fed *ad libitum* (P<0.0001, Fig. 1). Mice on fasting diet lost an average of 1.52 ± 0.15 g of body weight or a reduction of ~6%. This compares to a mean weight loss of 0.55 ± 0.05 g or ~2.3% reduction in control mice. The disparity in weight loss was much larger in fasted mice with about a 2.5 times larger standard deviation than control mice. Starting weight between the mice in the fasting group was not significantly different as compared to the starting weight of the control mice (P=0.0977).

Overall, plasma ghrelin and FFA concentrations were significantly higher in fasted mice than in control mice (P<0.0001, Fig. 2 and 4). Leptin concentrations were significantly lower in fasted mice than they were in mice fed *ad libitum (P*<0.0001, Fig. 3).

Mean plasma ghrelin concentration in fasting mice (1106 ± 72.57pg/ml) had an almost four-fold increase over control (282.3 ± 28.07 pg/ml, Fig. 2). The concentration of leptin, 3.2 (IQR 2.66-4.653) ng/ml, declined in fasted mice as compared to controls, 0.64 (IQR 0.458-1.105) ng/ml. On the other hand, plasma FFA levels rose approximately two-fold in the fasting state when compared to controls; from a control mean of 4.438 ± 0.094 to fasting mean of 7.276 ± 0.413 µEq/L (Fig. 4).

The assay quality control samples were within the expected range. The consistency of the replicates was such that the coefficient of variation was no larger than 0.3 for any of the measured variables. Similarly, as seen in supplementary figures, the linear regression plots also presented with small standard deviations; however, this was across two replicate samples.

DISCUSSION

We found that fasting for 18 hours produces a significant effect on the energy metabolism of mice as was elicited by the substantial changes in weight as well as regulatory hormones. Plasma FFA levels almost doubled indicating the mobilization of fat stores to cope with the likely depletion of carbohydrate reserves. Furthermore, the active form of plasma ghrelin was found to be greatly increased in the fasting mice. On the other hand, leptin levels were markedly lower in them. This study further adds support to what is known about processes occurring in fasting animals.

There was a significant decrease in weight in the fasting mice (1.52 ± 0.15 g, Fig. 1). There was also a less pronounced decrease in weight in the control mice (0.55 ± 0.05 g, Fig. 1). However, this is to be expected as the pre- and post-treatment weighing was not taken at the same time of the day and this is within daily fluctuation. The metabolic demands of mice mean that even in well-fed mice, body weight is significantly reduced a few hours after not eating due to small reserves of glycogen. The fasting mice lost more body weight than what could be considered normal. A reduction in weight of ~1-2 g or 6% of body weight is in agreement with the Moesgaardet et al [6] study on 18-hour fasted mice. Other studies report different amounts of weight loss but that is dependent on the length of the fast and proportion of fat to body weight. Obese mice survive longer in fasts (>4 days) and it is thought that this is due to greater energy reserves [9].

In agreement with the literature, acyl-ghrelin in the plasma showed a significant increase in the fasting state. There was an almost four-fold increase in active ghrelin (1106 ± 72.57 vs. 282.3 ± 28.07 pg/ml, P<0.0001; Fig. 2). In the study by Moesgaardet et al

[6] plasma ghrelin levels rose from a baseline of 5.2 to 8.8 ng/ml (a 1.7 fold difference) while fasting.

In another study on rats fasting for 24 hours, plasma ghrelin increased by four-fold over control values [1]. These previously reported increases in ghrelin levels in fasting animals are in accordance with our results. Ghrelin is an acute hormone and different factors may influence the levels of active ghrelin in blood plasma, such as the diet upon which the animal has been acclimatized. The ratio of acyl to deacyl-ghrelin is regulated by ghrelin O-acyl-transferase (GOAT) [2]. GOAT may be regulated by different factors, some of which are unknown. However, it is known that plasma ghrelin can increase while ghrelin expression can remain unchanged. This may be due to GOAT playing a role in short-term regulation of appetite by shifting ghrelin between the acylated and deacylated pool in the plasma. Overall, the results of this study appear to be well within the physiologically sound range.

Plasma leptin was found to be significantly lower in fasting mice than controls (0.64 vs. 3.2 ng/ml, $P<0.0001$; Fig. 3). Leptin has greater roles in long-term regulation of body weight because it is produced in relation to adipose tissue (the site of its synthesis) [12]. We found leptin in normal control mice to be 3.6 ± 0.3 ng/ml on average. This is similar to two other studies reporting control levels of approximately 3.3 and 3.9 with similar levels of deviation [12, 13] Furthermore, Yamada et al [13] reported a reduction in leptin during a fast from ~3.9 ng/ml to 1.5 ng/ml, which is remarkably similar to our findings.

As shown in Fig. 4, plasma FFA concentration was approximately two-fold higher in fasted mice than in controls. This observation is closest to what has been cited in literature. Several studies report that after deprivation of food, plasma FFA levels increase approximately two-fold, in both rodents and humans, and more or less remains steady for some time, until depletion of fat reserves [8, 9]. FFAs are tightly regulated by HSL and LPL, and ghrelin and leptin may contribute to maintaining FFA levels during starvation. For instance, it is observed that over a 6-day fast in rats, plasma FFAs reach a peak after which they decline slowly. Plasma ketone and glycerol levels also declined after reaching a peak, reflecting the mobilization and subsequent depletion of fat [7]. While ghrelin and leptin may be the causative agents in mobilizing fat stores during fasting, it is difficult to tease out precise interactions in our study. To our knowledge, this is the first study that examined the changes in these three variables in fasting mice. In order to further elucidate the interplay between ghrelin and leptin on FFA increase, changes in insulin, growth hormone and glucose should be observed. In addition, measuring the levels of ghrelin, leptin and FFA throughout the duration of a fast, rather than measuring endpoints, can allow clearer relationships to be charted.

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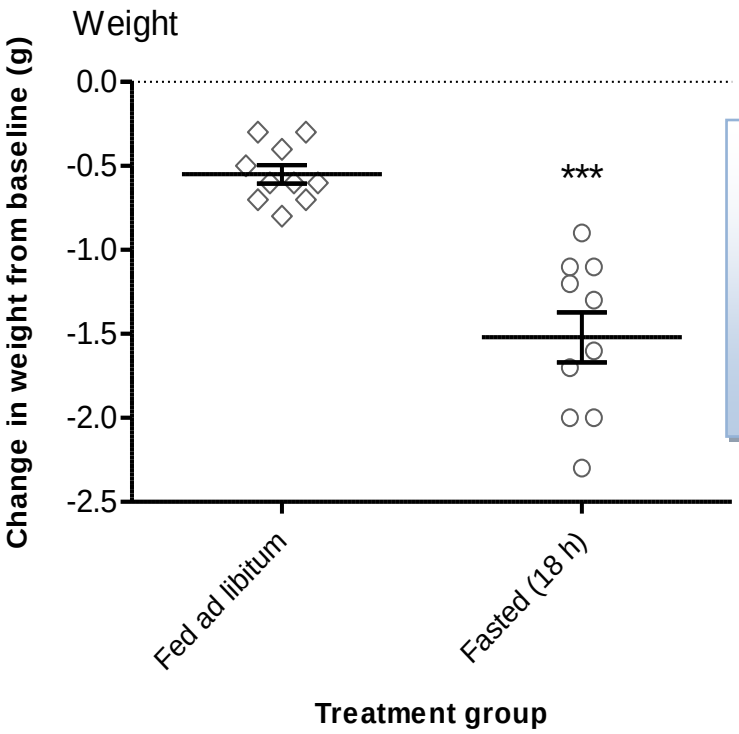

Figure 1: Change in weight from baseline (prior to treatment) in control (fed *ad libitum*) and 18 h fasted mice. Data presented as means ± S.E.M and individual measures are shown. Fasted (18 h) mean weight-loss of 1.52 ± 0.15 g was significantly (P<0.0001) different from mean weight loss of 0.55 ± 0.05 g in control mice.

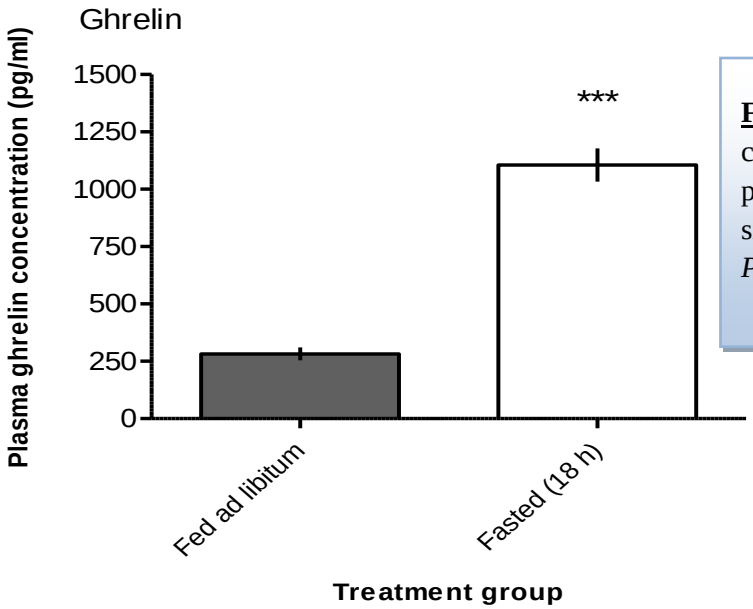

Figure 2: Plasma ghrelin concentrations in 18 h fasted mice compared to control mice. Shown as means ± S.E.M. Mean plasma ghrelin in fasted mice (1106 ± 72.57 pg/ml) was significantly higher than control (282.3 ± 28.07 pg/ml; P<0.0001).

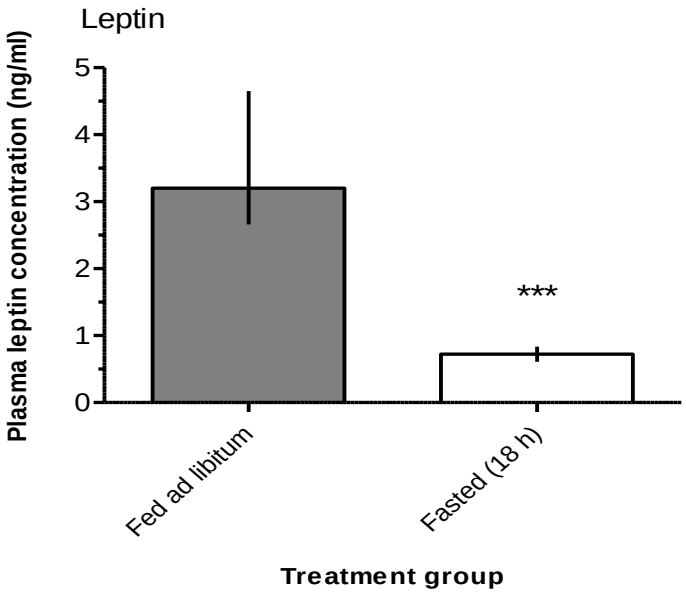

Figure 3: Plasma leptin concentrations in 18 h fasted mice compared to control mice, shown as medians and interquartile. Median plasma leptin was significantly lower in fasted mice (0.64 [IQR 0.458-1.105] ng/ml) than in control mice (3.2 ng/ml [IQR 2.66-4.653]; *P*<0.0001).

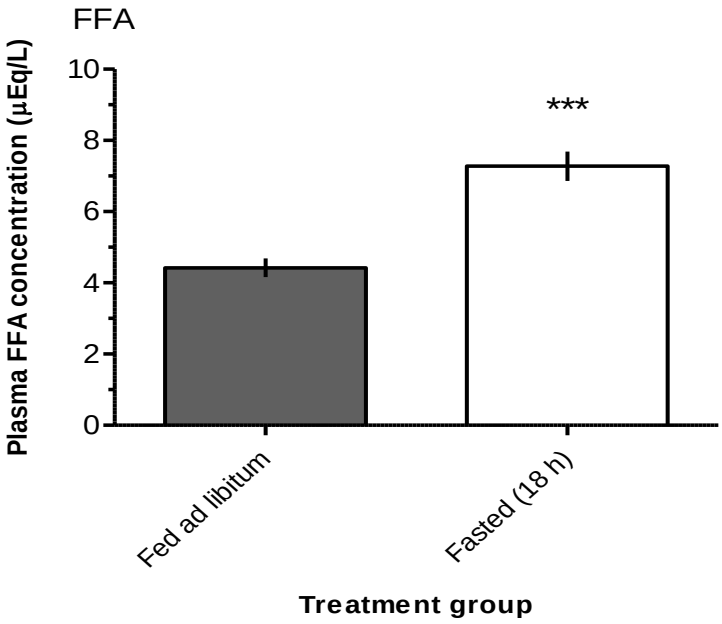

Figure 4: Plasma fatty-acid concentrations in 18 h fasted mice compared to control mice. Data shown as means ± S.E.M. In the fasting mice, FFA levels were about 2-fold higher than control: 7.276 ± 0.413 and 4.438 ± 0.094 µEq/L, respectively (*P*<0.0001).

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