Reappraisal of the optimal fasting time for insulin tolerance tests in mice

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ABSTRACT

Objective: Most studies routinely use overnight or 6 h of fasting before testing metabolic glucose homeostasis in mice. Other studies used empirically shorter fasting times (<6 h). We attempted to determine the shortest fasting time required for optimal insulin responsiveness while minimizing metabolic stress.

Methods: A course of fasting for up to 24 h (0, 2, 4, 6, 12, and 24 h) was conducted in C57B/6J male mice. Body weight, metabolic parameters, and insulin tolerance were measured in each experimental group. The organs were collected at the same time on separate occasions and glycogen and metabolic gene expression were measured in the liver and skeletal muscle.

Results: Our data show that blood glucose levels do not significantly change during a 6 h fast, while plasma insulin levels decrease to similar levels between 2 h and 6 h of fasting. During overnight (12 h) and 24 h fasts, a robust decrease in blood glucose and plasma insulin was observed along with a profound depletion in liver glycogen content. Insulin tolerance was comparable between baseline and 6 h fasts while 4 h and 6 h fasts were associated with a greater depletion of liver glycogen than 2 h fasts, impacting the glucose counter-regulatory response. Fasting induced progressive weight loss that was attenuated at thermoneutrality. Fasting longer than 4 h induced major body weight loss (>5%) and significant changes in catabolic gene expression in the liver and skeletal muscle.

Conclusion: Collectively, these data suggest that 2 h of fasting appears optimal for the assessment of insulin tolerance in mice as this duration minimizes major metabolic stress and weight loss.

Keywords: Fasting; Blood glucose; Insulin tolerance; Weight loss; Catabolic genes; Muscle atrophy

1. INTRODUCTION

Fasting is a common procedure used to reset metabolism and reduce variability in physiological parameters in experimental studies of mice [1,2]. Fasting refers to a state in which the animal is completely deprived of food but has ad libitum access to water. For instance, a primary screen to evaluate whether a treatment or genetic manipulation alters glucose homeostasis is the measurement of fasting glucose and insulin levels [3,4]. Fasting mice reduces the range of baseline glucose readings and can reveal significant differences between experimental groups that would not reach significance in non-fasted animals [1]. In larger species, animals are typically fasted overnight, as in humans, to obtain a more consistent baseline glucose concentration [5]. In mice, overnight fasting was routinely used prior to glucose tolerance tests and was recommended as a standard operating procedure until recently [6,7]. However, long periods of fasting in small mammals induce profound catabolic states and weight loss, particularly at housing temperatures below thermoneutrality [8]. In addition, although mice may eat up to 60% of their daily food intake at night, they usually nibble throughout the day and rarely undergo a true fast to maintain a constant body core temperature and avoid torpor. Therefore, daytime fasting likely produces profound changes in mouse physiological parameters and behavior. Shorter fasting times are now considered more physiological to reduce metabolic stress in mice. Consequently, 6-h fasts were recommended by the National Institute of Health Mouse Metabolic Phenotyping Center (MMPC) Consortium in 2010 to conduct metabolic tests of glucose homeostasis in mice [3]. Since then, 6-h fasts have been widely and routinely used by most investigators in the field of metabolism to determine whether fasting glucose levels fall within a normal range. Other investigators used empirically shorter fasting times (2–5 h) [9,10]. To the best of our knowledge, no study has yet determined the shortest optimal fasting time required to conduct insulin tolerance tests while minimizing the impact on metabolic parameters and body weight in mice. The purpose of this study was to more exhaustively describe the metabolic impact of fasting during a 24 h course in young lean mice. Our data indicate that a short fasting duration such as 2 h should be recommending prior to conducting insulin tolerance tests in mice to minimize metabolic stress and weight loss.
2. MATERIALS AND METHODS

2.1. Mice
All of the experimental procedures were approved by our institutional animal care and use committee CEEA122 (protocol# 2016122311033178) and conducted according to Inserm guidelines and the 2010/63/UE European Directive for the care and use of laboratory animals. Three-month-old male C57BL/6J mice were housed at room temperature (21 °C) in a pathogen-free barrier facility (12 h light/dark cycles) and fed a standard chow diet (Ssniff Spezialdiäten GmbH, Soest, Germany). A first cohort of mice (cohort 1) was randomly assigned to 6 experimental groups (n = 5 per group): 0 not fasted, 2 h fast (2), 4 h fast (4), 6 h fast (6), 12 h overnight fast (12N), and 24 h fast (24). The entire protocol started at 7 a.m. on one day and ended at 7 a.m. on the following day except for the 12N group, which was starved overnight from 7 p.m. to 7 a.m. The mice were investigated in two experimental sessions: one session to assess body weight and conduct the ITT, and one week later, another session for blood measurements and organ collection. Two other mice cohorts (cohort 2, n = 6, and cohort 3, n = 5) were not fasted and used for longitudinal measurements of blood glucose and ketones, plasma triglycerides, glycerol, NEFA, insulin, and body weight at the exact same times of the day (7 a.m., 9 a.m., 11 a.m., 1 p.m., and 7 p.m.) as cohort 1 to assess the potential variability and daytime circadian rhythm of the measured parameters (Supplementary Figures 1 and 3). A fourth cohort (cohort 4) of mice was acclimated at thermoneutrality in a chamber with controlled variability and daytime circadian rhythm as blood glucose levels measured throughout the day in the light/dark cycles) and fed a standard chow diet (Ssniff Spezialdiäten GmbH Hilden, Germany) following the manufacturer’s protocol. The RNA quantity was determined on a Nanodrop ND-1000 (Thermo Fisher Scientific, Rockford, IL, USA). Reverse-transcriptase PCR was conducted using the MultiScribe Reverse Transcriptase method (Applied Biosystems, Foster City, CA, USA). Quantitative real-time PCR (qRT-PCR) was performed in duplicate using a ViiA 7 real-time PCR system (Applied Biosystems). All of the expression data were normalized by the 2^(-ΔΔCt) method using 18S as a housekeeping gene. For TaqMan chemistry, the primers used were 18S (TaqMan assay ID: Hs99999901_s1), Bnip3 (TaqMan assay ID: Mm01275600_g1), MyoD1 (TaqMan assay ID: Mm00440387_m1), and Pck1 (TaqMan assay ID: Mm00440636_m1). The SYBR chemistry primer sequences are listed in Supplementary Table 1.

2.5. Determination of total glycogen
Liver and tibialis anterior muscle samples were weighed and homogenized in acetate buffer (0.2 M and pH 4.8). After centrifuging the samples at 12,000 g for 10 min, supernatant was transferred into clean tubes and divided in two aliquots. An aliquot of each homogenate was mixed with amyloglucosidase (Sigma) and incubated at 55 °C for 15 min. The other was mixed with water and incubated at 4 °C for 15 min. The glucose content was determined using a spectrophotometer. The samples were analyzed in duplicate, and the results were determined as μg of glycogen per mg tissue.

2.6. Statistical analyses
All of the statistical analyses were conducted using GraphPad Prism 8.0 for Windows (GraphPad Software Inc., San Diego, CA, USA). Normal distribution and homogeneity of data variance were tested using Shapiro–Wilk and F tests, respectively. One-way ANOVA followed by Tukey’s post hoc tests were conducted to determine differences between groups. Two-way ANOVA and Bonferroni’s post hoc tests were used when appropriate. All of the values in the figures are presented as means ± SEM. Statistical significance was set at p < 0.05.

3. RESULTS

3.1. Influence of fasting on glucose homeostasis
Fasting up to 6 h did not significantly change blood glucose levels (Figure 1A) while reducing plasma insulin levels by ~50% (p = 0.01) (Figure 1B). Blood glucose variability decreased during 2, 6, 12N, and 24 h fasts (SD = 12.4 at 0 h, SD = 7.1 at 2 h, SD = 8.9 at 6 h, SD = 9.6 at 12N, and SD = 5.0 at 24 h). The plasma insulin level during 0 h fasting (7 a.m.) was 0.71 ± 0.08 ng/ml and rapidly decreased to ~0.33 ± 0.09 ng/ml within 2 h of fasting (p = 0.007) (Figure 1B). Long-term fasting for 12 and 24 h markedly lowered blood glucose (Figure 1A) and plasma insulin (~70% compared to baseline levels) (Figure 1B). These changes could not be attributed to circadian rhythm as blood glucose levels measured throughout the day in the non-fasted mice did not significantly change (Supplementary Figure 1A). The ability of insulin to lower blood glucose, that is, insulin responsiveness, assessed during an insulin tolerance test (ITT) was similar between 0, 2, 4, and 6 h fasts, particularly during the first 15 min (Figure 1C). Blood glucose levels decreased by approximately 20–25% during this first phase in the four experimental groups (Figure 1D and E). However, blood glucose levels remained significantly lower at 60 min after insulin injection in the 6 h fast group (Figure 1C and D). In contrast, blood glucose levels during the ITT were dramatically lower in the 12N and 24 h groups (Figure 1C), and insulin responsiveness was impaired in these two groups compared to the 6 h fast (Figure 1E–F).

3.2. Influence of fasting on liver metabolism
We next investigated the impact of the fasting duration on the liver glycogen content. We observed a time-dependent depletion of liver...
glycogen content starting at 4 h fasting and maximal at 12 h (Figure 2A). At 2 h fasting, liver glycogen was not significantly different from baseline. There was a 44% depletion in the liver glycogen content at 6 h fast that reached a steady state at 12 h fast with an 81% depletion of the total glycogen pool in the liver at 12N and 24 h fasts (Figure 2B). Notably, there was a strong positive correlation between the liver glycogen content and blood glucose (Supplementary Figure 1B) and insulin (Supplementary Figure 1C) levels. Interestingly, this considerable liver glycogen depletion was associated with an upregulation of liver Pck1 gene expression at 12N and 24 h fasts that reached statistical significance at 24 h fast (Figure 2C). No significant change in liver G6pc gene expression was noted (Figure 2D). In contrast, we observed a significant upregulation of liver Ppara (Figure 2E) and Cpt1a (Figure 2F) mRNA levels from 6 h fast onward. This was accompanied by an upregulation of Bdh1 mRNA levels (Supplementary Figure 2A), the rate-limiting enzyme of β-hydroxybutyrate production, and a time-dependent upregulation of the PPARα-responsive hepatokine Fgf21 (Supplementary Figure 2B), both reaching statistical significance at 12N and 24 h fasts. This occurred in light of no significant change in the liver Cctβ (Supplementary Figure 2C) and Lpl (Supplementary Figure 2D) mRNA levels.

Interestingly, liver Ppara gene expression was strongly correlated with the total liver glycogen content (Supplementary Figure 2E).

3.3. Fasting induces robust weight loss and metabolic changes at room temperature

Fasting induced a time-dependent weight loss that reached ~10% at 12N and ~14% at 24 h fasts (Figure 3A–B). At 2 h fasting, there was already a significant although moderate (3.5%) body weight loss (Figure 3B). At 6 h fast, body weight loss (5.5%) was significantly higher than at 2 h fast (Figure 3B). In parallel to body weight changes, there were profound changes in systemic lipid metabolism. Increased circulating NEFA levels were observed after 2 h, 12 h, and 24 h fasting (Figure 3C). Interestingly, plasma NEFA levels were strongly inversely correlated to the total liver glycogen content (Supplementary Figure 3). Similarly, fasting augmented circulating glycerol levels from 2 h onward, although it did not reach statistical significance at 6 h fast (Figure 3D). Consistent with the upregulation of Ppara (Figure 2E) and other lipid metabolism genes in the liver (Figure 2F and Supplementary Figure 2), we noted a robust 3-fold induction of circulating ketone body levels at 12N and 24 h fasts (Figure 3F), consistent with the observed upregulation of liver Bdh1 at 12N and 24 h (Supplementary Figure 2A).
Importantly, body weight (Supplementary Figure 4A) measured in the non-fasted mice in cohorts 2 and 3 did not significantly change throughout the day. Blood ketone levels were slightly elevated at 1 p.m. but remained within a low physiological range (<0.4 mM) (Supplementary Figure 4B). Similar to body weight, plasma NEFA (Supplementary Figure 4C), glycerol (Supplementary Figure 4D), triglycerides (Supplementary Figure 4E), and insulin (Supplementary Figure 4F) did not change throughout the day. We also noticed that 12 h overnight fasting (12N) induced a greater percentage of weight loss than 12 h of daytime fasting (9.94/0.77 vs 6.12/0.49%, \( p = 0.0031 \) for 12N and 12D, respectively).

3.4. Fasting-induced weight loss is attenuated at thermoneutrality
Because mice must constantly fight against thermal stress to maintain a constant body core temperature, we hypothesized that fasting-induced weight loss and metabolic stress would be minimized at thermoneutrality. In the mice housed at room temperature, we observed a time-dependent weight loss both in absolute terms (Figure 4A) and percentages (Figure 4B). Blood glucose (Figure 4C) and ketones (Figure 4D) decreased with fasting and followed a similar pattern as in the mice housed at room temperature. However, blood glucose variability remained greater at each time point compared to room temperature (SD = 17.4 at 0 h, SD = 20.4 at 2 h, SD = 21.7 at 4 h, SD = 15.4 at 6 h, SD = 10.3 at 12N, and SD = 14.7 at 24 h). Importantly, in agreement with our hypothesis, both the absolute (Figure 4D) and percentage of body weight loss (Figure 4E) were significantly lower in the mice housed at thermoneutrality.

3.5. Fasting activates atrophy signaling and promotes muscle atrophy
Since we observed significant muscle atrophy and reduction in the soleus muscle mass at 12N and 24 h fasts (Supplementary Figure 5A), we investigated the impact of the fasting duration on skeletal muscle mass and atrophy. This was related to a marked upregulation of major atrophy genes such as Murf1 (Figure 5A and Supplementary Figure 5B) and Atrogin-1 (Figure 5B and Supplementary Figure 5C) after 12N and 24 h fasts. Actrb2b, a gene involved in hypertrophy, was downregulated by fasting from 2 h onward, although it reached statistical significance only at 4 h fast (Figure 5C and Supplementary Figure 5D). In addition, Bip3 and Drp1, two genes involved in autophagy and
mitochondrial fission, respectively, were upregulated after 12N and 24 h fasts (Figure 5D−E and Supplementary Figure 5E). More strikingly, we observed a rapid and robust suppression of MyoD1, a key transcription factor of myogenesis, mRNA levels after 4 h of fasting and onward (Figure 5F and Supplementary Figure 5F). Consistent with the marked metabolic stress and muscle atrophy, we also observed a significant downregulation in the total muscle glycogen content at 12N and 24 h fasts (Supplementary Figure 6).

4. DISCUSSION

Fasting is widely used in metabolic research to reduce variability of various physiological readouts such as blood glucose levels in mice [1,3,12,13]. However, unlike in humans, small mammals such as mice have large body surface area and constantly fight to maintain a constant body core temperature when they are housed below thermoneutrality [14]. Consequently, mice are very sensitive to food deprivation even during the daytime despite the common belief that mice eat mostly during the night. This study demonstrated that the widely and routinely used 6 h fast model prior to assessing glucose homeostasis in mice induces major metabolic stress and weight loss (>5%) that significantly alters mice physiology and is a major frailty factor [15]. The major difference between humans and mice is that humans reduce their blood glucose levels and variability after an overnight fast of 12 h in the absence of measurable weight loss. Thus, using anthropomorphism, researchers have extrapolated this to mice. However, mice exhibit higher metabolic rates than humans to maintain euthermaia. This species difference renders mice highly susceptible to fasting, and as reported in our study, 12 h fasting provokes major weight loss (~10%) and catabolic processes in the liver and skeletal muscles. This metabolic stress is inevitably associated with induction of muscle atrophy and profound changes in systemic lipid metabolism,
severely hindering insulin tolerance. Therefore, fasting longer than 4 h prior to investigating glucose homeostasis in mice is not physiological and should be proscribed. Thus, a 2 h fast minimizes weight loss and should be used prior to conducting insulin tolerance tests to maximize research results and attenuate the discomfort of mice to ensure greater animal welfare.

Our data show that fasting rapidly downregulated plasma insulin levels by approximately 40% within 2 h of fasting to maintain steady blood glucose levels. The plasma insulin levels at 0 h fast (7 a.m.) were already approximately half of those classically found in the fed mice (>1 ng/ml) [16]. This was paralleled by a time-dependent depletion of liver glycogen reflecting an elevated rate of hepatic glucose output during fasting to maintain steady blood glucose levels [17]. It is reasonable to posit that the observed sustained decrease in blood glucose levels at 12 h overnight and 24 h fasts was directly related to robust liver glycogen depletion (>80%), since mice genetically engineered to maintain high liver glycogen content during 36 h fasts are capable of maintaining their blood glucose levels within a normal physiological range despite significant weight loss [16]. Since the decrease in circulating insulin during fasting derepresses Pck1 expression through Foxo1 in the liver [17,18], we observed an increase in liver Pck1 expression at 12N and 24 h fasts. Although G6pc is also considered an insulin-regulating gene in the liver, we did not observe significant changes in liver G6pc mRNA levels during fasting. PPARα, a nuclear hormone receptor activated by FA, is a transcription factor that is central to the metabolic shift from glucose to fat utilization initiated by fasting [19,20]. Upon activation of PPARα, FA oxidation is stimulated and increased amounts of acetyl-CoA are produced. A large proportion of acetyl-CoA derived from FA b-oxidation is utilized for ketogenesis [21]. Liver Ppara and Cpt1a mRNA levels were significantly upregulated from 6 h fasting onward, thus reflecting a major metabolic switch occurring in the liver to activate FA b-oxidation and ketogenesis [19]. The metabolic switch was also visible at the systemic level with a rapid induction of whole-body lipolysis reflected by elevated circulating levels of NEFA and glycerol within 2 h of fasting. More strikingly, we noted a rapid and sustained reduction in circulating TG levels after 2 h

Figure 4: Body weight loss (A), percentage of body weight loss (B), blood glucose (C), and blood ketone bodies (D) were measured in the mice after 0, 2, 4, 6, 12N, or 24 h of fasting (n = 5/group). All of the measurements were conducted in the mice housed at thermoneutrality (30°C). *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 vs time point 0. Body weight loss (E) and percentage of body weight loss (F) were compared in the mice housed at 21°C vs 30°C. *p < 0.05, **p < 0.01, and ****p < 0.0001 vs 21°C.
of fasting, possibly due to substantial uptake by peripheral organs through lipoprotein lipase [22]. Consistent with liver activation of Ppara, Cpt1a, and Bdh1, circulating levels of ketone bodies increased by >3-fold after 12 h of fasting. Ketone bodies such as β-hydroxybutyrate can be used as fuel by a number of peripheral organs to spare glucose utilization during fasting [23].

Although fasting expectedly promoted a metabolic switch in the liver and at the systemic level, it inevitably induced substantial weight loss. Thus, mice are very sensitive to food deprivation even during the daytime and particularly at temperatures below thermoneutrality [8]. To maintain a constant body core temperature, mice must nibble throughout the day, even during the daytime. In line with this, our data showed that the mice housed at thermoneutrality lost less weight in response to fasting than the mice housed at room temperature. Indeed, when investigating the mice feeding patterns over 24 h, it was found that mice had an average of 36 food bouts during one day and night, each lasting approximately 3 min. The average interval between food bouts was 34 min [24]. In addition, fed mice maintained at thermoneutrality ate approximately 50% less than mice kept at 23 °C, suggesting that mice exhibit high metabolic rates under standard animal care facility temperatures [8]. Consistent with these studies, our data showed that the non-fasted mice housed at room temperature in our animal care facility (21 °C), therefore under thermal stress, had very stable body weights across the photophase (daytime) from 7 a.m. to 7 p.m. This finding suggests that mice eat during the daytime to maintain a stable body weight and defend their body core temperature. In contrast, due to their high metabolic rate and need to maintain euthermia under thermal stress, mice experience rapid and profound weight loss during fasting. In line with other studies [3,25], we demonstrated that lean mice experience major weight loss within 6 h of fasting that reached an ethical limit of 10% weight loss after 12 h overnight fasting. In addition to a major metabolic switch observed in the liver and at the systemic level, one detrimental effect of fasting is the loss of lean body mass and particularly skeletal muscle mass. Skeletal muscle is a key insulin-sensitive tissue that can contribute up to 85% of glucose disposal in response to insulin stimulation [26]. In this study, we found that both 12 h and 24 h fasting were associated with a significant loss of muscle mass and blunted insulin responsiveness. A downregulation in total muscle glycogen content at the same time points likely contributed to water loss and muscle mass loss. In addition, muscle wasting occurs as a physiological response to fasting triggered by low insulin levels in which the loss of muscle proteins primarily results from increased degradation of cell proteins [27]. Two major protein degradation pathways, the ubiquitin-
proteasome and autophagy-lysosome systems, are activated during muscle atrophy and variably contribute to loss of muscle mass [28]. Fasting for 12 h and 24 h promotes robust induction of Atrogin-1 and Murf-1, two key canonical genes encoding E3-ubiquitin ligases involved in muscle atrophy in a variety of pathophysiological states [29,30]. Fasting also induces a weaker transcriptional induction of Bnip3, a key player in autophagy, and Dmp1, a major player in mitochondrial fission, after 12 h and 24 h of fasting. Muscle atrophy primarily involves a loss of intracellular and myofibrillar proteins [30,31]. Among these, myosin-related proteins and myogenic factors such as MyoD1 have been identified as atrogin-1 targets [29]. Food deprivation was associated with the rapid suppression of myogenic transcription factor MyoD1, which plays a major role in muscle differentiation and the transcriptional activation of heavy and light myosin chains [32]. For practical reasons inherent to experimental research, the fasting started at night when mice eat the majority of their daily food. In this study, we investigated lean chow diet-fed mice while high fat diet-fed mice are typically used in metabolic research to assess insulin sensitivity and susceptibility to type 2 diabetes. Based on previous reports summarized in [1], body weight loss appears more dependent on fasting time than initial body weight in mice. Thus, our observations could be extrapolated to obese HFD-fed mice.

5. CONCLUSION

Collectively, our data demonstrated that laboratory mice, in contrast with humans, experience rapid and time-dependent weight loss during fasting. We showed that the widely and routinely used 6 h fast model prior to assessing glucose homeostasis is sub-optimal due to major weight loss (~5%) and liver glycogen depletion. Thus, 2 h of fasting during the photophase period should be conducted prior to assessing insulin responsiveness in mice to minimize metabolic stress induced by weight loss. We believe that these findings will maximize the scientific outcomes of experimental studies carried out in mice while ensuring greater animal welfare.

CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2020.101058.

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