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Temporal Partitioning of Adaptive Responses of the Murine Heart to Fasting

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Abstract

Recent studies suggest that the time of day at which food is consumed dramatically influences clinically-relevant cardiometabolic parameters (e.g., adiposity, insulin sensitivity, and cardiac function). Meal feeding benefits may be the result of daily periods of feeding and/or fasting, highlighting the need for improved understanding of the temporal adaptation of cardiometabolic tissues (e.g., heart) to fasting. Such studies may provide mechanistic insight regarding how time-of-day-dependent feeding/fasting cycles influence cardiac function. We hypothesized that fasting during the sleep period elicits beneficial adaptation of the heart at transcriptional, translational, and metabolic levels. To test this hypothesis, temporal adaptation was investigated in wild-type mice fasted for 24-hr, or for either the 12-hr light/sleep phase or the 12-hr dark/awake phase. Fasting maximally induced fatty acid responsive genes (e.g., *Pdk4*) during the dark/active phase; transcriptional changes were mirrored at translational (e.g., PDK4) and metabolic flux (e.g.,

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DISCLOSURES

None.

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glucose/oleate oxidation) levels. Similarly, maximal repression of myocardial p-mTOR and protein synthesis rates occurred during the dark phase; both parameters remained elevated in the heart of fasted mice during the light phase. In contrast, markers of autophagy (e.g., LC3II) exhibited peak responses to fasting during the light phase. Collectively, these data show that responsiveness of the heart to fasting is temporally partitioned. Autophagy peaks during the light/sleep phase, while repression of glucose utilization and protein synthesis is maximized during the dark/active phase. We speculate that sleep phase fasting may benefit cardiac function through augmentation of protein/cellular constituent turnover.

Keywords

autophagy; chronobiology; heart; metabolism; nutrition

INTRODUCTION

Time-of-day-dependent oscillations in biological processes are essential for maintenance of homeostasis, as well as cellular and organ function. In association with behavioral patterns, such as sleep/wake and fasting/feeding cycles, mammals exhibit daily rhythms in various circulating factors (hormones and nutrients), neural stimulation (e.g., autonomic and sympathetic tone), and cardiovascular parameters (e.g., blood pressure and heart rate) (12, 20, 33). Disruption of these oscillations, through behavioral, environmental, and/or genetic means, invariably precipitates pathology. In humans, sleep deprivation and shift work both adversely impact neurohumoral factor oscillations, and significantly increase the risk of developing cancer, obesity, diabetes, and cardiovascular disease (CVD) (6, 15, 19, 36). This has led to speculation that maintenance/restoration of biological rhythms (through pharmacological or behavioral interventions) may be effective in the prevention/treatment of various pathologies. One such strategy includes time-of-day-restricted feeding. For example, restricting food intake only to the active period attenuates high fat diet induced body weight gain, adiposity, insulin resistance, and hyperlipidemia in rodents; this is associated with an augmentation of biological rhythms at tissue-specific (e.g., transcriptional, translational) and whole body (e.g., neurohumoral, behavioral) levels (1, 3, 4, 16). Similar cardiometabolic benefits have been observed in humans following restriction of caloric intake prior to the evening (22, 26). Recently, we have reported that restricting food intake to the active period (particularly towards the early portion of the active period) attenuates high fat diet induced cardiac dysfunction (25, 35). Collectively, these observations suggest that prevention of food intake during the inactive/sleep phase may be beneficial for various cardiometabolic parameters, including contractile function of the heart. However, the mechanisms mediating cardioprotection remain largely unknown.

Considerable information is available regarding daily oscillations in cardiac processes; this is particularly true for cardiac metabolism. Upon awakening, cardiac contractility increases in association with foraging for food, predation avoidance, and reproduction. Accordingly, myocardial oxidative metabolism must increase at this time, in order to meet demands for energy. During the initial stages of foraging, catabolic processes active during the sleep phase fast would therefore be expected to remain active, providing substrates for continued

search of food. Once the forage for food is successful, anabolic processes should become more active, thereby facilitating storage of excess nutrients. Consistent with these concepts (reviewed in (41)), we have previously reported that myocardial glucose utilization increases during the active period in *ad libitum* fed mice, likely meeting the energetic demands of the heart in response to elevated workload (8). Myocardial triglyceride synthesis also increases towards the end of the active period in *ad libitum* fed mice, thereby facilitating storage of excess nutrients (possibly in anticipation of the upcoming sleep phase fast) (34). More recently, we have found that protein synthesis increases at the end of the active phase, which, we hypothesize, promotes replacement of damaged proteins in anticipation of the next active period (41). However, despite appreciation that organisms likely evolved to anticipate periods of food scarcity, the temporal regulation of many of these metabolic processes during fasting is less well understood, particularly in the mouse.

The purpose of the present study was to examine the temporal adaptation of the murine heart to fasting. We hypothesized that fasting during the sleep period elicits beneficial adaptation of the heart at transcriptional, translational, and metabolic levels. These studies revealed that in terms of glucose utilization and protein synthesis, the murine heart appears to exhibit greater responsiveness to fasting during the dark/active phase. In contrast, fasting-mediated alterations in autophagic markers are greatest during the light/sleep phase. Collectively, these observations support the concept that responsiveness of the heart to fasting is temporally organized. We speculate that sleep phase fasting may benefit cardiac function through augmentation of protein/cellular constituent turnover.

MATERIALS AND METHODS

Mice

We used wild-type mice, either on the C57Bl/6J (24-hr fasting time course) or FVB/N (12-hr time-of-day-dependent fasting) background. All mice were male, were 16–20 weeks old (at the time of euthanasia), and were housed at the Animal Resource Program at the University of Alabama at Birmingham (UAB), under temperature-, humidity-, and light- controlled conditions. A strict 12-hour light/12-hour dark cycle regime was enforced (lights on at 6AM; Zeitgeber Time [ZT] 0). The light/dark cycle was maintained throughout these studies; as such, physiologic diurnal variations were investigated in mice (as opposed to circadian rhythms). All mice had free access to water. When in the fed state, mice were provided a standard rodent chow. At the time of tissue and blood collection, mice were anesthetized with pentobarbital. All animal experiments were approved by the Institutional Animal Care and Use Committee of UAB.

Fasting Protocols and Non-Invasive Mouse Monitoring

For the 24-hr fasting time course study, a wire bottom floor was placed in the microisolator cage, thus preventing consumption of feces or bedding; *ad libitum* fed mice were also placed on a wire bottom floor, but were allowed access to chow. For the 12-hr time-of-day-dependent fasting study (as well as a sub-set of the 24-hr fasting time course studies), mice were housed in a computer-controlled Comprehensive Laboratory Animal Monitoring System (CLAMS; Columbus Instruments Inc., Columbus, OH), which enforced time-of-

day-dependent food access in the absence of direct human intervention. More specifically, mice were allowed access to food either during the 12-hr dark phase (i.e., light phase [LP] fasted mice) or during the 12-hr light phase (i.e., dark phase [DP] fasted mice); these feeding/fasting regimes were enforced for a 9 day period, thereby allowing sufficient equilibration time. The CLAMS also continuously assessed food intake, physical activity (beam breaks), energy expenditure (indirect calorimetry), and respiratory exchange ratio (RER), as described previously (3). In all studies, mice were singly housed and acclimatized to their housing conditions for at least 1 week prior to initiation of the experimental protocol.

Humoral Factor Analysis

Blood was collected at the time of euthanization, placed in EDTA-containing tubes, and centrifuged at 3,000g for 10 minutes at 4°C; resultant plasma was stored at –80°C prior to assessment of insulin, non-esterified fatty acids (NEFA), and glucose levels using commercially available kits. Insulin was measured using a sensitive rat insulin RIA kit (EMD Millipore Corporation, Billerica, MA; catalog number SRI-13K). NEFA were measured on the Stanbio Sirrus analyzer (Stanbio Laboratories, Boerne, TX) using reagents from Wako Diagnostics, Mountain View, CA (catalog numbers are 999-34691, 995-34791, 991-34891, and 993-35191). Glucose was measured on the Stanbio Sirrus analyzer (Stanbio Laboratory, Boerne, TX) using a glucose oxidase reagent (manufactured by Stanbio Laboratory; catalog number 1071-250). Glucose levels were also determined in a drop of blood (prior to plasma preparation) through use of a FreeStyle Lite glucometer (Abbott Diabetes Care Inc., Alameda, CA).

Quantitative RT-PCR

RNA was extracted from hearts using standard procedures (5). Candidate gene expression analysis was performed by quantitative RT-PCR, using methods described previously (13, 17). Specific Taqman assays were designed for each gene from mouse sequences available in GenBank, and have been reported previously (7, 9, 34, 39, 40). All quantitative RT-PCR data were normalized to the housekeeping gene *cyclophilin* (this gene did not differ between experimental groups). Quantitative RT-PCR data is presented as fold change from the trough value in a specified group.

Immunoblotting

Qualitative analysis of protein expression and posttranslational modifications (e.g., lipidation, phosphorylation) was performed as described previously (8). Lysates (5–30 µg) were separated on bis-acrylamide gels by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to a PVDF membrane and initially stained with ponceau for assessment of total protein loading. Membranes were subsequently probed with antibodies against PDK4 (Abcam #Ab38242), mTOR (Cell Signaling 2983), p-mTOR (Ser2448; Cell Signaling 2971), S6 (Cell Signaling 2317), p-S6^{Ser240/244} (Cell Signaling 2215), LC3 (Cell Signaling 12741), p62 (Abnova H00008878-M01), or anti-calsequestrin (Abcam 3516). Following visualization, bands were quantified with the freely available software, ImageJ (NIH). Proteins were normalized to calsequestrin, while phosphoproteins were normalized to their respective total proteins. Importantly, due to the nature of time course studies, in

order to minimize the contribution that position on the gel might have on outcomes, samples were randomized on gels; samples were re-ordered post-imaging, only for the sake of illustration of representative images (note, all bands for representative images for an individual experiment were from the same gel).

Working mouse heart perfusions

Myocardial substrate utilization and contractile function were measured *ex vivo* through isolated working mouse heart perfusions, as described previously (2, 8, 34, 35). All hearts were perfused in the working mode (non-recirculating manner) for 30 minutes with a preload of 12.5 mmHg and an afterload of 50 mmHg. Standard Krebs–Henseleit buffer was supplemented with 8 mM glucose, 0.4 mM oleate conjugated to 3% BSA (fraction V, fatty acid-free; dialyzed), 0.66 mM β -hydroxybutyrate, 0.22 mM acetoacetate, 10 μ U/ml insulin (basal/fasting concentration), 0.05 mM L-carnitine, and 0.13 mM glycerol. Metabolic fluxes were assessed through the use of distinct radiolabeled tracers: [U- ^{14} C]-glucose (0.12 mCi/L from MP Biomedicals; glucose oxidation); and 2) [9,10- ^{3} H]-oleate (0.067 mCi/L from Sigma-Aldrich; β oxidation). Measures of cardiac metabolism (*e.g.*, oxygen consumption) and function (*e.g.*, cardiac power) were also monitored. At the end of the perfusion period, hearts were snap-frozen in liquid nitrogen and stored at -80°C prior to analysis. Ketone bodies were added to the perfusate because: 1) during the basal state, the mouse heart utilizes quantitatively appreciable levels of ketone bodies; and 2) circulating ketone bodies are elevated in the mouse during fasting. It is important to note that previous studies have shown that similar perfusion conditions provide alternative substrates, such that fatty acid oxidation rates are attenuated (29, 32).

Myocardial Glycogen Levels

Glycogen content was assayed using a spectrophotometric-based assay, as described previously (25).

In vivo Protein Synthesis

Mice were injected via tail vein with 150 mM phenylalanine plus [2,3,4,5,6- ^{3} H]-phenylalanine (0.18 mCi/kg), according to the flooding dose technique (23). Myocardial protein was precipitated and washed with 10% trichloroacetic acid, neutralized with 1 M NaOH, and counted in a scintillation counter (Beckman Coulter).

Statistical analysis

Statistical analyses were performed using two-way ANOVA, as described previously (2, 3). Briefly, analyses were performed on SPSS statistical software (IBM, Armonk, NY) to investigate main effects of time and/or nutritional status, followed by Bonferroni post hoc analyses for pair-wise comparisons (indicated in Figures). In all analyses, the null hypothesis of no model effects was rejected at $p < 0.05$.

RESULTS

Identification of the natural fasting period in mice

We initially sought to establish a fasting protocol that mimics the scenario in which the mouse is unsuccessful in its forage for food upon awakening. To identify the natural trough of food intake, 20 wild-type C57Bl/6J mice were housed in CLAMS cages for a 2-wk period, to assess feeding/fasting cycles non-invasively. Figure 1A revealed that lowest food intake was observed 4-hrs into the light phase (i.e., ZT4); thereafter food intake began to rise, peaking at the light-to-dark transition (i.e., ZT12). Interestingly, a second trough in food intake is observed approximately 8-hrs into the dark phase (i.e., ZT20; Figure 1A). Time-of-day-dependent oscillations in food intake were mirrored by rhythms in physical activity, energy expenditure, and RER (Figure 1B–D).

Temporal adaptation of the mouse to fasting at the whole body level

We next investigated temporal whole body adaptation of a sub-set of mice to fasting, when initiated at ZT4; *ad libitum* fed mice served as controls. As anticipated, fasting increased physical activity (Figure 2Aii), while decreasing both energy expenditure (Figure 2Aiii) and body weight (Figure 2B) in mice. In addition, RER significantly decreased within 1-hr following food withdrawal, and remained lower than *ad libitum* fed mice for the remainder of the fasting period (Figure 2Aiv). Plasma insulin levels declined steadily following food withdrawal (Figure 2Ci), associated with a rise in plasma NEFA levels (Figures 2Cii). In contrast, both plasma and blood glucose levels remained relatively similar between fed and fasted mice, exhibiting time-of-day-dependent oscillations regardless of feeding status (Figure 2Ciii–iv). Collectively, these data are consistent with whole body adaptation to this fasting protocol, with maintenance of glucose homeostasis.

Temporal adaptation of the murine heart to fasting at transcriptional, translational, and metabolic levels

Prior studies have reported that fasting induces fatty acid responsive genes, thus facilitating myocardial fatty acid oxidation (11, 31, 37). However, few studies have defined the time course of these events. Here, we report that metabolically-relevant fatty acid responsive genes (*Pdk4*, *Ucp3*, *Mcd*, *Dgat2*, and *Cte1*) increased in hearts of fasted mice, with greatest induction (i.e., greatest rate of change within a 4-hr period) observed during the dark phase (Figure 3A). Interestingly, with the exception of *Cte1*, the expression of these genes decreased at the onset of the light phase (i.e., change in expression from ZT20 to ZT4), despite prolongation of the fast (Figure 3A). Next we investigated whether these fluctuations in gene expression translated to alterations in protein levels. Given that *Pdk4* mRNA was markedly induced by fasting during the active period, and that this kinase directly regulates myocardial glucose oxidation, we focused on PDK4 protein. In hearts of fed mice, PDK4 did not exhibit a significant time-of-day-dependent variation (Figure 3B). Fasting did not significantly induce PDK4 protein levels until ZT20 (i.e., 16-hr fast; Figure 3B). Furthermore, the rate of PDK4 induction appeared to be greatest between ZT16 and ZT24 (Figure 3B). Next, myocardial oxidative metabolism was interrogated following short-term (4-hr fasting, from ZT4 to ZT8) and prolonged (16-hr fasting, from ZT4 to ZT20) food withdrawal (matching times at which PDK4 was either not altered [short-term] or increased

[prolonged]). Neither glucose nor oleate oxidation were significantly affected by the 4-hr fast (Figure 3C). In contrast, hearts isolated from 16-hr fasted mice exhibited decreased glucose oxidation, concomitant with a trend ($p=0.055$) for elevated fatty acid oxidation, relative to hearts isolated from *ad libitum* fed mice at this time (Figure 3C). It is interesting that, consistent with previously published studies (8), hearts isolated from fed mice at ZT20 exhibit increased rates of glucose oxidation concomitant with decreased rates of oleate oxidation, compared to hearts isolated at ZT8 (Figure 3C). No significant differences were observed between the experimental groups for contractile function parameters (e.g., cardiac power, rate pressure product; data not shown). Collectively, these data demonstrate a delayed adaptation of the murine heart to fasting, at the level of glucose and fatty acid metabolism, which peaks towards the middle/end of the dark/active phase.

Rapid alterations in protein synthesis and autophagy markers in the heart during fasting

During completion of the fasting time course studies, biventricular weight (BVW) was assessed. In *ad libitum* fed mice, BVW tends to increase towards the end of the dark phase; this oscillation is absent in hearts of fasted mice (Figure 4Ai). Interestingly, the effects of time-of-day and fasting on BVW do not appear to be secondary to fluctuations in glycogen levels, as: 1) myocardial glycogen content decreases at the end of the dark phase in *ad libitum* fed mice (when BVW increases); and 2) at the end of the dark phase, hearts from fasting mice have increased glycogen levels (relative to *ad libitum* fed hearts), yet BVW tends to be lower (Figure 4Ai–ii). These observations prompted us to investigate protein turnover parameters. We initially investigated mTOR, a nutrient sensitive kinase known to promote protein synthesis and inhibit autophagy (18). In *ad libitum* fed mice, p-mTOR^{Ser2448} did not exhibit a significant time-of-day-dependent oscillation (although a trend for increased levels during the dark phase was observed; Figure 4B). As predicted, p-mTOR^{Ser2448} decreased during fasting, reaching a trough after 12-hrs (i.e., ZT16; Figure 4B). Somewhat surprising, p-mTOR^{Ser2448} increased thereafter, reaching normal levels after 24-hrs (i.e., ZT4; Figure 4B). Measurement of p-S6^{Ser240/244}, a known mTOR target, revealed a similar pattern to that seen for p-mTOR^{Ser2448} (Figure 4C). Myocardial protein synthesis rates were next investigated *in vivo*, both 12-hr (ZT16) and 24-hr (ZT4) after initiation of the fast, matching times at which p-mTOR was either repressed or not altered, respectively. Myocardial protein synthesis rates were significantly decreased after the 12-hr fast (Figure 4D). In contrast, the 24-hr hour fast did not significantly influence myocardial protein synthesis (Figure 4D). The autophagy markers LC3II (lipidated form of LC3) and p62 were next investigated. *Ad libitum* fed mouse hearts exhibited elevated LC3II levels at ZT4 (Figure 4E). Fasting maintained LC3II at high levels during the light phase (Figure 4E). Interestingly, this autophagy marker decreased somewhat during the dark period despite continued fasting (Figure 4E). LC3II levels appeared to increase once again in fasted hearts during the light phase (i.e., ZT0 to ZT4; Figure 4E). A time-of-day-dependent oscillation in p62 was also observed in hearts of *ad libitum* fed mice, being antiphase to oscillations in LC3II (i.e., peaking at ZT16; Figure 4F). Fasting initially attenuated the rise of p62 during the dark phase, while prolongation of the fast into the light phase led to a robust elevation of p62 levels (i.e., ZT0 to ZT4; Figure 4F). Collectively, these data reveal that fasting induces rapid alterations in myocardial protein synthesis and autophagy markers.

Effects of time-of-day on responsiveness of the heart to fasting

The fasting studies described above have two variables: duration of fasting and time-of-day. To investigate the contribution of time-of-day on responsiveness of the heart to fasting, hearts collected from a previously published study were utilized in which mice underwent a daily 12-hr fast either during the dark or light phase (3). Given the marked responses of p-mTOR^{Ser2448} and LC3II to fasting, these two parameters were investigated. Light phase (LP) fasted mice exhibited an approximate 2-fold oscillation in cardiac p-mTOR^{Ser2448} levels, which was absent in dark phase (DP) fasted mice (Figure 5A). Hearts isolated from LP fasted mice exhibit an approximate 5-fold oscillation in LC3II levels, peaking at the end of the fasting period (i.e., ZT12; Figure 5B). In contrast, the LC3II peak at the end of the 12-hr fasting period in DP fasted mice (i.e., ZT0/24) is markedly attenuated (Figure 5B). Collectively, these findings suggest that the heart exhibits a greater response to fasting during the light/sleep phase, in terms of p-mTOR attenuation and LC3II augmentation.

DISCUSSION

Numerous studies suggest that restriction of caloric intake to the active period is beneficial for a number of cardiometabolic parameters, including cardiac function. However, little is known regarding the temporal response of the heart to fasting, which hinders mechanistic understanding of sleep phase fasting benefits. In this study we have defined the integrated temporal adaptation of the mouse heart to fasting, with an important focus on time-of-day influences. Consistent with previously published reports, which have shown that fasting induced fatty acid responsive genes in the heart, in association with a decrease in glucose oxidation. However, this adaptation appeared to be time-of-day-dependent, being augmented during the dark/active period. Similarly, fasting-induced attenuation of myocardial protein synthesis appeared to be greatest during the dark/active period. In marked contrast, autophagic markers (e.g., LC3 lipidation) were most responsive to fasting during the light/sleep phase. Collectively, these data reveal that metabolic adaptation of the murine heart to fasting is temporally partitioned.

Previous studies have shown that fasting induces profound transcriptional alterations, particularly for genes encoding metabolic enzymes. For example, β -oxidation enzymes are increased in the heart during fasting, likely functioning in a feed-forward manner, thereby facilitating fatty acid oxidation in the face of increased circulating non-esterified fatty acid (consequent to increased adipose tissue lipolysis) (11). Fasting-induced transcriptional changes have also been reported that would be anticipated to diminish myocardial glucose utilization (such as increased *Pdk4* expression) (38). Alterations in myocardial gene expression during fasting are mediated by various transcription factors, including multiple isoforms of SREBP, FOXO, and PPAR (11, 14, 24). In the latter case, direct binding of fatty acids to PPAR α results in induction of target genes, such as *Pdk4*, *Ucp3*, *Mcd*, and *Cte1* (7, 30, 38–40).

Consistent with these prior studies, here we report that fasting induces PPAR α target genes in the heart, associated with increased myocardial fatty acid oxidation (and a concomitant repression of glucose oxidation; Figure 3). However, although whole body metabolic adaptation to fasting appeared to be rapid (e.g., RER decreases within 1-hr of food

withdrawal, indicative of diminished glucose reliance concomitant with increased fatty acid oxidation; Figure 2Aiv), adaptation of myocardial substrate selection occurs at a slower rate. Indeed, no significant effects were observed for PPAR α target genes, PDK4 protein levels, nor for myocardial glucose and fatty acid oxidation rates (measured *ex vivo*) 4-hr after food withdrawal (Figure 3). In contrast, dramatic fluctuations in these parameters were observed 16-hr after food withdrawal. Interestingly, continuation of the fast beyond 16-hr was associated with a return of multiple mRNA species towards fed state levels (e.g., *Pdk4*, *Ucp3*, *Mcd*, *Dgat2*). This phenomenon has been reported previously in the rat heart, but not in mice, and may be secondary to diminished responsiveness of PPAR α to fatty acids during the light phase (31). Consistent with time-of-day, as opposed to duration, of the fast playing an important role in transcriptional responses, we have previously reported that a 12-hr fast during the dark/active phase induces cardiac *Pdk4* to a greater extent than a 12-hr fast during the light/sleep phase (6.0-fold versus 2.4-fold, respectively) (3). Collectively, these observations suggest that fasting during the dark/active phase leads to greater effects on parameters such as induction of fatty acid responsive genes and substrate switching (between glucose and fatty acids), relative to light/sleep phase fasting.

Recent studies indicate that the mass of specific organs may exhibit time-of-day-dependent oscillations. For example, Sinturel *et al* reported that liver weight is highest at the dark-to-light phase transition (i.e., ZT0), whereas heart weight (normalized to body weight) did not exhibit a time of day dependent oscillation (28). In contrast, we recently reported that biventricular weight (normalized to tibia length) tended ($p=0.07$) to oscillate over the course of the day, being higher at ZT0 (21). The current study revealed a similar pattern in biventricular weight, and suggests that these rhythms may be dependent on the feeding-fasting cycle. More specifically, a decrease in the biventricular weight of fasting mice was only observed 16-hr after food withdrawal (i.e., ZT20; $p<0.05$ by t-test); biventricles from fed and fasted mice had identical weights when fasting was continued to 24-hrs (i.e., ZT4 on day 2; Figure 4Ai). Rhythms in biventricular weight did not seem to be secondary to myocardial glycogen levels, which were either decreased (8-hr fast; ZT12), increased (20-hr fast; ZT24/0; $p<0.05$ by t-test), or not influenced (all other time points) by fasting (relative to fed mice; Figure 4Aii). This led us to hypothesize that protein turnover contributes towards rhythms in biventricular weight. Consistent with this concept, we have recently reported increased protein synthesis in the heart at the dark-to-light phase transition (i.e., ZT0) (21). Investigation of p-mTOR (a kinase that influences both protein synthesis and autophagy (10, 18, 27)) revealed a slight oscillation in the heart of *ad libitum* fed mice, peaking at the beginning of the dark phase (consistent with increased circulating insulin levels at this time; Figure 4B). As predicted, fasting decreased p-mTOR levels, with a maximal effect at ZT16 (i.e., 12-hr fast). Somewhat surprisingly, p-mTOR increased thereafter, returning to normal levels at ZT4, despite continuation of the fast; similar results were observed for pS6 (Figures 4B–C). Consistent with these observations, fasting-induced attenuation of myocardial protein synthesis was greater after a 12-hr fast, relative to a 24-hr fast (Figure 4D). Given this striking observation, we next investigated whether the time-of-day (as opposed to fasting duration) played an important role in the responsiveness of p-mTOR to food withdrawal. Unlike light phase fasting, a 12-hr fast during the dark phase was unable to decrease p-mTOR levels (Figure 5A). Similarly, a greater induction of the autophagy marker LC3II

was observed during light phase fasting (Figure 4E); fasting during the dark phase had no significant effect on this parameter (Figure 5B). Collectively, these studies suggest a greater impact of fasting on protein turnover (i.e., simultaneous synthesis and degradation) in the mouse heart when performed during the light (sleep) phase, with a relative resistance to fasting-induced alterations in these parameters during the dark (awake) phase.

It is important to acknowledge both strengths and shortcomings of the current study. With regards to strengths, this study has revealed temporal adaption of the murine heart to fasting, highlighting that fasting depresses both glucose oxidation and protein synthesis to the greatest extent during the active/dark phase. In contrast, both protein synthesis and autophagy markers are elevated in the heart when fasting occurs during the sleep/light phase. The latter suggests that turnover of protein is augmented by sleep phase fasting, which would promote maintenance of cellular function through replacement of damaged proteins (and potentially other cellular constituents). Another strength of the current study includes its potential utility as a guide for future fasting studies; the results clearly indicate that both the duration and time-of-day of a fast markedly influence various outcomes (e.g., cardiac glycogen and p-mTOR levels), which, in turn, impacts conclusions drawn. Shortcomings of ours studies are as follows. Although we investigated a vast array of potential mediators, it should be noted that no mechanistic links were established. Another limitation relates to assessment of autophagy. We investigated only markers of autophagy, but not autophagic flux. Unfortunately attempts to measure autophagic flux during discrete time periods (i.e., 4 hour intervals, through the use of chloroquine) failed, potentially due to low absolute rates of autophagy in the heart. Finally, the possibility exists that increased protein turnover is detrimental during fasting, due to, for example, increasing energetic demand.

In summary, the current study reveals that both time-of-day and duration of a fast markedly influence metabolic adaptation of the heart (illustrated in Figure 6). We speculate that temporal partitioning of the fasting response likely plays an important role in maintenance of contractile function of the heart, as the animal in the wild continues its forage for food. Furthermore, we postulate that one mechanism by which sleep-phase fasting improves cardiac function is through facilitation of cellular constituent turnover, as a daily maintenance function. These findings also serve as reference for future studies designed to interrogate discrete metabolic parameters in the mouse heart in response to fasting.

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Figure 1.

Time-of-day-dependent variations in whole body energy balance parameters in mice. Non-invasive assessment of food intake (A), physical activity (beam breaks; B), energy expenditure (C), and RER (respiratory exchange ratio, an indication of carbohydrate versus fat oxidation; D) was performed by placement of mice in CLAMS cages for a 2-week period ($n=20$). All data are shown as mean $\pm$ SEM.

Figure 2.

Whole body adaptation to fasting in mice. For this fasting study, food was withdrawn at ZT4, and continued for up to 24-hrs; *ad libitum* fed mice are indicated by solid lines, while fasted mice are indicated by dashed lines. Non-invasive assessment of food intake (Ai), physical activity (beam breaks; Aii), energy expenditure (Aiii), and RER (respiratory exchange ratio, an indication of carbohydrate versus fat oxidation; Aiv) was performed by placement of mice in CLAMS cages. Body weight (Bi) and change in body weight from baseline (i.e., ZT4; Bii). Diurnal variations in plasma insulin (Ci), non-esterified fatty acids (NEFA; Cii), and glucose (Ciii), as well as blood glucose (Civ). All data are shown as mean

$\pm$ SEM, for 6 separate observations per experimental group. *, $p < 0.05$ for fed versus fasted within a ZT.

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Figure 3.

Myocardial metabolic adaptation to fasting in mice. For this fasting study, food was withdrawn at ZT4, and continued for up to 24-hrs; *ad libitum* fed mice are indicated by solid lines/bars, while fasted mice are indicated by dashed lines/hatched bars. Diurnal variations in *pdh* (Ai), *ucp3* (Aii), *mcd* (Aiii), *dgat2* (Aiv), and *cte1* (Av) mRNA levels were assessed by RT-PCR. Diurnal variations in PDK4 (B) protein levels were assessed by Western blotting. Light/dark differences in rates of glucose (Ci) and oleate (Cii) oxidation were assessed in *ex vivo* perfused hearts through use of radiolabeled tracers. All data are shown as mean $\pm$ SEM, for 6 separate observations per experimental group. *, $p < 0.05$ for fed versus fasted within a ZT.

Figure 4ad

Figure 4ef

Figure 4.

Influence of fasting on heart size and autophagy markers in mice. For this fasting study, food was withdrawn at ZT4, and continued for up to 24-hrs; *ad libitum* fed mice are indicated by solid lines, while fasted mice are indicated by dashed lines. Biventricular weight (BVW; Ai) and myocardial glycogen content (Aii) were assessed gravimetrically and spectrophotometrically, respectively. Diurnal variations in mTOR^{Ser2448} (B) and p-S6^{Ser240/244} (C) were assessed by Western blotting. Light/dark differences in rate of protein synthesis (D) were assessed *in vivo* through use of radiolabeled tracers. Diurnal variations in LC3II (E), and p62 (F) were assessed by Western blotting. All data are shown as mean $\pm$ SEM, for 6 separate observations per experimental group. *, $p < 0.05$ for fed versus fasted within a ZT.

Figure 5.

Time-of-day modulates responsiveness of autophagy markers to fasting in the murine heart. For this fasting study, food was withdrawn only during the 12-hr light phase (LP; ZT0 to ZT12) or 12-hr dark phase (DP; ZT12 to ZT24); LP fasted mice are indicated by solid lines, while DP fasted mice are indicated by dashed lines. Diurnal variations in mTOR^{Ser2448} (A) and LC3II (B) were assessed by Western blotting. All data are shown as mean $\pm$ SEM, for 6 separate observations per experimental group. *, $p < 0.05$ for fed versus fasted within a ZT; #, $p = 0.0515$ for fed versus fasted within a ZT.

Figure 6.

Summary of study findings. Data from the current study suggests that fasting increases LC3 lipidation to the greatest extent during the light phase, whereas this intervention has the greatest inhibitor effect on glucose oxidation and protein synthesis during the dark phase. These findings suggest that cellular constituent turnover (i.e., simultaneous high rates of synthesis and degradation) would be observed during light phase fasting.