

# The Circadian Clock within the Cardiomyocyte Is Essential for Responsiveness of the Heart to Fatty Acids<sup>\*[5]</sup>

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David J. Durgan<sup>‡1</sup>, Nowice A. Trexler<sup>‡1</sup>, Oluwaseun Egbejimi<sup>‡</sup>, Tracy A. McElfresh<sup>§</sup>, Hee Yun Suk<sup>¶</sup>, Lauren E. Petterson<sup>‡</sup>, Chad A. Shaw<sup>||</sup>, Paul E. Hardin<sup>\*\*</sup>, Molly S. Bray<sup>‡</sup>, Margaret P. Chandler<sup>§</sup>, Chi-Wing Chow<sup>¶</sup>, and Martin E. Young<sup>‡2</sup>

From the <sup>‡</sup>United States Department of Agriculture/Agricultural Research Service Children's Nutrition Research Center, Department of Pediatrics, Baylor College of Medicine, Houston, Texas 77030, the <sup>§</sup>Department of Physiology and Biophysics, Case Western Reserve University, Cleveland, Ohio 44106, the <sup>¶</sup>Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, New York 10461, the <sup>||</sup>Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, Texas 77030, and the <sup>\*\*</sup>Department of Biology, Texas A & M University, College Station, Texas 77204

Cells/organs must respond both rapidly and appropriately to increased fatty acid availability; failure to do so is associated with the development of skeletal muscle and hepatic insulin resistance, pancreatic  $\beta$ -cell dysfunction, and myocardial contractile dysfunction. Here we tested the hypothesis that the intrinsic circadian clock within the cardiomyocytes of the heart allows rapid and appropriate adaptation of this organ to fatty acids by investigating the following: 1) whether circadian rhythms in fatty acid responsiveness persist in isolated adult rat cardiomyocytes, and 2) whether manipulation of the circadian clock within the heart, either through light/dark (L/D) cycle or genetic disruptions, impairs responsiveness of the heart to fasting *in vivo*. We report that both the intramyocellular circadian clock and diurnal variations in fatty acid responsiveness observed in the intact rat heart *in vivo* persist in adult rat cardiomyocytes. Reversal of the 12-h/12-h L/D cycle was associated with a re-entrainment of the circadian clock within the rat heart, which required 5–8 days for completion. Fasting rats resulted in the induction of fatty acid-responsive genes, an effect that was dramatically attenuated 2 days after L/D cycle reversal. Similarly, a targeted disruption of the circadian clock within the heart, through overexpression of a dominant negative CLOCK mutant, severely attenuated induction of myocardial fatty acid-responsive genes during fasting. These studies expose a causal relationship between the circadian clock within the cardiomyocyte with responsiveness of the heart to fatty acids and myocardial triglyceride metabolism.

It has long been appreciated that both experimental animals and humans exhibit diurnal variations in multiple cardiovascular parameters, including blood pressure, peripheral vascular

resistance, heart rate, electrical properties of the heart (e.g. Q–T interval duration), and cardiac output (1–7). These rhythms have been ascribed primarily to fluctuations in neurohumoral influence during the day, which in turn are because of both environmental factors and the central circadian clock within the suprachiasmatic nucleus (8–10). Diurnal variations in physiological cardiovascular parameters have been attributed to the same factors (e.g. sympathetic activity) responsible for diurnal variations in fatal cardiovascular events, which have an increased incidence in the early hours of the morning (11–13). However, few studies have investigated whether the intrinsic properties of the heart fluctuate during the day or whether a loss of synchronization between the presence of a stimulus (e.g. increased sympathetic activity in the early hours of the morning) and responsiveness of the heart plays a role in the instigation of contractile dysfunction.

One extracellular influence that the heart must adapt to rapidly is a change in circulating fatty acid levels. Fatty acids are the primary fuel source for the normal myocardium, which generates ~70% of the ATP required for contraction from fatty acid  $\beta$ -oxidation (14). However, fatty acids are more than just a fuel for cardiomyocytes because they act as both structural and signaling precursors (15). As such, a balance between fatty acid availability and rates of fatty acid  $\beta$ -oxidation must be maintained. If the latter fails, detrimental fatty acid derivatives accumulate within the myocardium, which has been linked to the pathogenesis of various cardiomyopathies, including those observed in obese and diabetic subjects (15–17). A major way in which the heart prevents accumulation of intramyocellular fatty acids during periods of increased availability is through induction of fatty acid-responsive genes that promote  $\beta$ -oxidation (15, 18).

Increasing evidence exists in support of the hypothesis that the circadian clock intrinsic to the cardiomyocyte plays a critical role in synchronizing cardiac metabolism to the environment (19–23). Circadian clocks are intracellular transcriptional mechanisms composed of positive and negative feedback loops, with a free running period of ~24 h (24). This molecular mechanism confers the selective advantage of anticipation, permitting the cell to respond rapidly to a given stimulus at the appropriate time of the day (24). Circadian clocks have been identified in a vast array of mammalian cell types, including components of the cardiovascular system (19, 20, 25, 26).

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<sup>[5]</sup> The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. 1–6.

<sup>1</sup> Both authors contributed equally to this work.

<sup>2</sup> To whom correspondence should be addressed: United States Department of Agriculture/Agricultural Research Service Children's Nutrition Research Center, Baylor College of Medicine, Dept. of Pediatrics, 1100 Bates St., Houston, TX 77030. Tel.: 713-798-7567; Fax: 713-798-7101; E-mail: meyoung@bcm.edu.

Recently we characterized the circadian clock within the cardiomyocytes of the rat heart (19). Isolated adult rat cardiomyocytes (ARCs),<sup>3</sup> when cultured in the presence of serum, exhibit oscillations in mRNAs encoding for known circadian clock components (e.g. *bmal1* and *rev-erbaα*). These oscillations exhibit a periodicity of between 20 and 24 h, with temporal patterns similar to those observed in the intact heart *in vivo*, consistent with the operation of a circadian clock within the cardiomyocyte (19).

Circulating nonesterified fatty acid (NEFA) levels exhibit a robust diurnal variation, with increased levels occurring during the resting phase for mammals. As such, we previously hypothesized that the circadian clock within the heart would synchronize fatty acid availability (i.e. circulating NEFA levels) with the capacity of the myocardium to oxidize this substrate (21). Somewhat surprisingly, a dyssynchrony between these two parameters is observed in the rat; despite increased circulating NEFA levels during the light phase, the rat heart exhibits not only increased expression of genes promoting fatty acid oxidation during the dark phase but also increased responsiveness to fatty acids (21). The purpose of this study was to test the hypothesis that the circadian clock within the cardiomyocyte mediates diurnal variations in the responsiveness of the heart to fatty acids. Through the use of both *in vitro* and

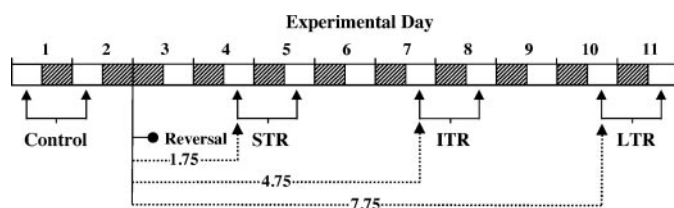
*in vivo* models, we report conclusive evidence in support of this hypothesis. The following two strategies were employed: 1) investigation of responsiveness of cardiomyocytes to fatty acids *ex vivo*; and 2) disruption of the circadian clock within the heart *in vivo*. Like the circadian clock, diurnal variations in the responsiveness of cardiomyocytes to fatty acids persist in culture. Furthermore, disruption of the circadian clock within the cardiomyocyte severely impairs responsiveness of the heart to fatty acids *in vivo*. These studies are the first to identify a function for the circadian clock within the cardiomyocytes of the heart.

## EXPERIMENTAL PROCEDURES

**Manipulation of the Light/Dark Cycle**—Male Wistar rats (175–200 g; Harlan) were initially housed under controlled conditions (23 ± 1 °C; 12-h light/12-h dark cycle) and received standard laboratory chow and water *ad libitum*. The light/dark (L/D) cycle was controlled by means of a timer. Zeitgeber time 0 (ZT0) corresponds to the time at which the lights turn on (normally 7 a.m.).

Reversal of the L/D cycle is known to induce a rapid re-entrainment of the central circadian clock within the suprachiasmatic nucleus, followed by re-entrainment of peripheral circadian clocks, at tissue-specific rates (27). To determine whether manipulation of the L/D cycle affected the circadian clock within the heart and/or responsiveness of this organ to fatty acids, the following experimental strategy was utilized, as depicted in Fig. 1. Briefly, rats were divided into two main groups on day 1 as follows: 1) 102 control rats maintained on the normal L/D cycle (lights on at 7 a.m.; ZT0); and 2) 306 rats subjected to a reverse in the L/D cycle (lights on at 7 p.m.; new ZT0). Within each group, half the rats were fed *ad libitum*, whereas the other half were fasted; in the latter case, food was withdrawn in the middle of the light phase (i.e. ZT6) on the day of the experiment. Fasting was performed such that the responsiveness of the heart to increased circulating fatty acid levels could be investigated. Hearts were isolated every 3 h from the control group, starting at ZT6 on day 1. Rats in group 2 were subdivided into short term recovery (STR), intermediate term recovery (ITR), and long term recovery (LTR) groups. Subdivision into recovery length duration groups was performed to determine the time course of re-entrainment of the circadian clock within the heart. Hearts were isolated every 3 h starting

<sup>3</sup> The abbreviations used are: ARCs, adult rat cardiomyocytes; FCS, fetal calf serum; L/D, light/dark; NEFA, nonesterified fatty acid; STR, short term recovery; LTR, long term recovery; PPARs, peroxisome proliferator-activated receptors; ITR, intermediate term recovery; CCM, cardiomyocyte CLOCK mutant; BHK, baby hamster kidney; dnCLOCK, dominant negative CLOCK; ZT, Zeitgeber time.



**FIGURE 1. L/D cycle manipulation protocol.** Rats were divided into four main groups as follows: control, STR, ITR, and LTR (see under “Experimental Procedures” and “Results”). These groups were subdivided into fed *versus* fasted rats; in the latter case, food was removed in the middle of the light phase (i.e. ZT6) on the day of the experiment. All rats were maintained in a 12-h/12-h L/D cycle prior to the start of experimental day 3, at which time the L/D cycle was reversed. Isolation of hearts from control, STR, ITR, and LTR rats was initiated at ZT6 on experimental days 1, 4, 7, and 10, respectively. Hearts were isolated from fed and fasted rats every 3 h, for the subsequent 24 h.

**TABLE 1**  
Primer and probe sequences used in real time quantitative reverse transcription-PCR

Gene	Primer/probe	Sequence
<i>adpn</i>	Forward	5'-CTATCAAGGGTACCTGGACGC-3'
	Reverse	5'-AAGTTTGCCATTTTCCAAGCA-3'
	Probe	5'-FAM-TGTGGCCCGTTACAGATGCCATTCT-TAMRA-3'
<i>atgl</i>	Forward	5'-GGTCTCCGAGAGATGTGC-3'
	Reverse	5'-TGGTTCAGTAGGCCATTCTC-3'
	Probe	5'-FAM-CAGGGCTACAGAGATGGACTTCGATTCTT-TAMRA-3'
<i>clock</i>	Forward	5'-AAAGACGGCGAGAAGTGG-3'
	Reverse	5'-GGAGGCAGAGAGGTTGGG-3'
	Probe	5'-FAM-CTGAGACAGCTGCTGACAAAAGCCAAG-TAMRA-3'
<i>dgat2</i>	Forward	5'-GGCTGGCATTGACTGGAA-3'
	Reverse	5'-TGGTCAGCAGGTTGTGTCT-3'
	Probe	5'-FAM-AAGAAAGGTGGCAGGAGATCGCAGTG-TAMRA-3'
<i>e4bp4</i>	Forward	5'-GGTGCAGGTGACGAACATTC-3'
	Reverse	5'-AGTTTTCGCGCCAGTTCT-3'
	Probe	5'-FAM-AGATTGGTCCCTCAGATCGGAACACTG-TAMRA-3'

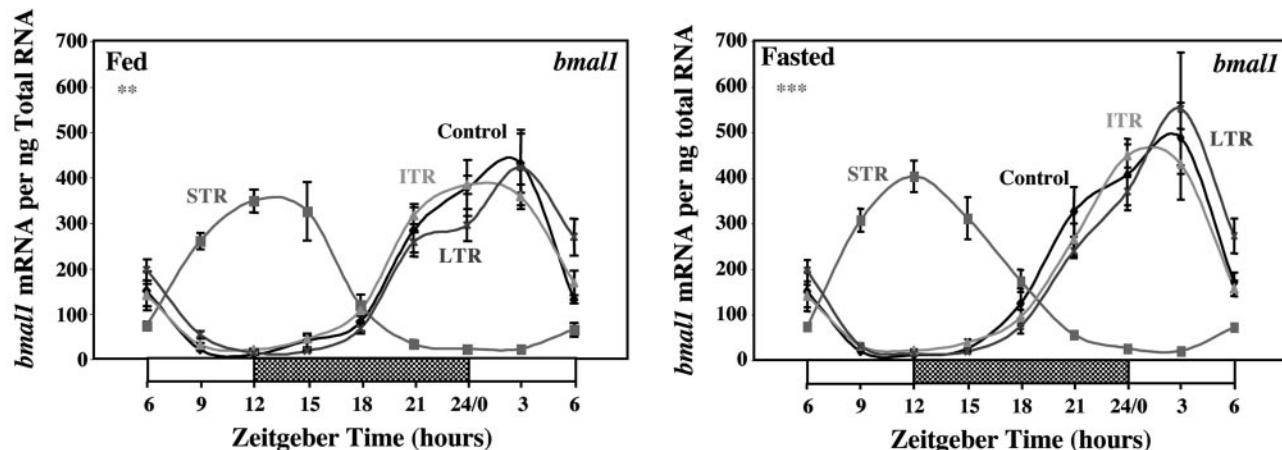
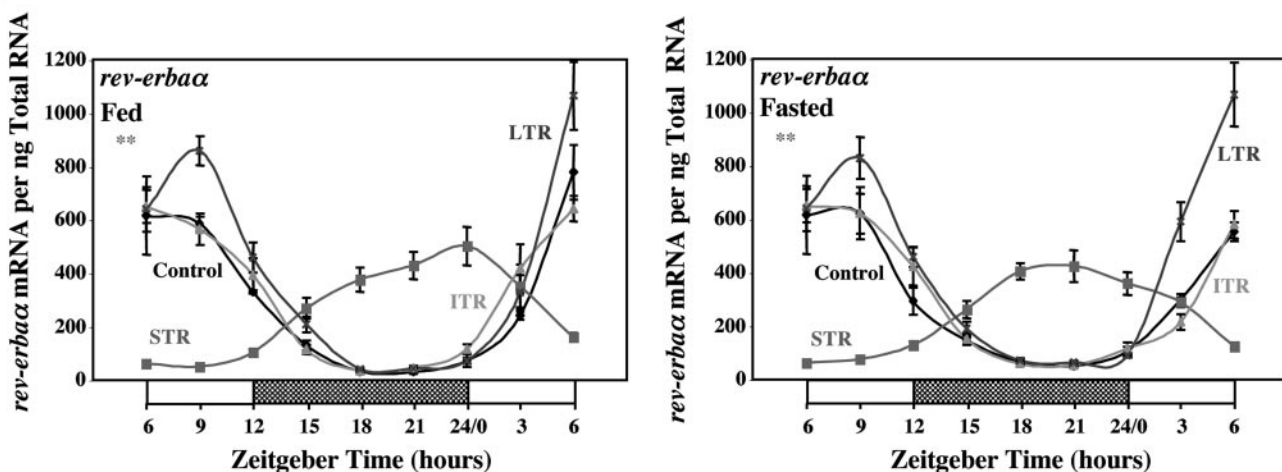
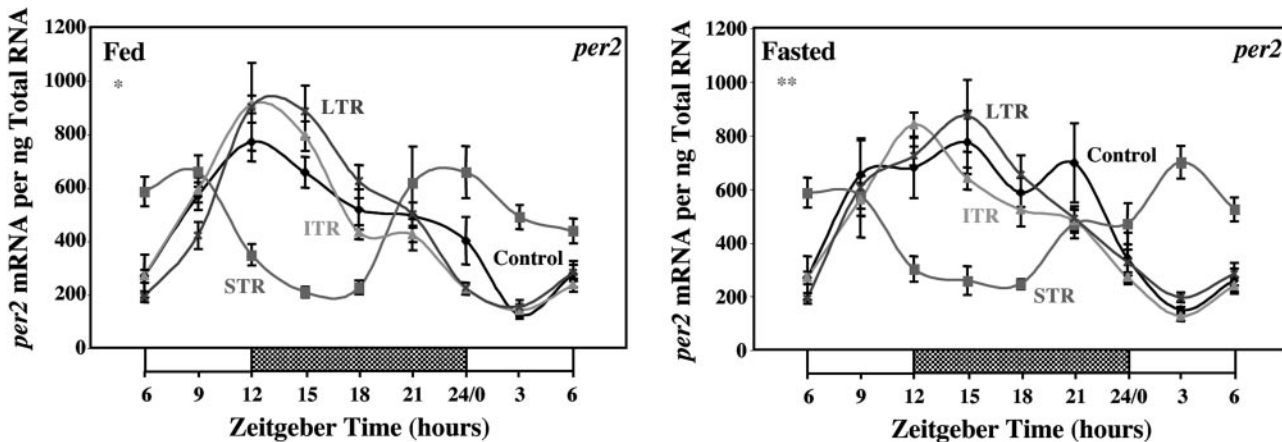
A) *bmal1* mRNAB) *rev-erba* mRNAC) *per2* mRNA

FIGURE 2. Effects of L/D cycle manipulation on myocardial gene expression (A–F) and plasma NEFA levels (G) in fed versus fasted male Wistar rats. The L/D cycle was manipulated as described under “Experimental Procedures” and “Results” and as depicted in Fig. 1. Myocardial genes investigated were *bmal1* (A), *rev-erba* (B), *per2* (C), *dbp* (D), *pdk4* (E), and *ucp3* (F). Each panel contains four groups: control (◆), STR (■), ITR (▲), and LTR (✕). Values are shown as the mean  $\pm$  S.E. for six separate observations at each time point. Gene expression data are represented as number of mRNA molecules (per ng of total RNA). Plasma NEFA levels are represented as mM. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; and \*\*\*,  $p < 0.001$  STR versus control hearts (phase); \$,  $p < 0.05$ ; \$\$,  $p < 0.01$ ; and \$\$\$,  $p < 0.001$  ITR versus control hearts (phase); #,  $p < 0.05$ ; ##,  $p < 0.01$ ; and ###,  $p < 0.001$  LTR versus control hearts (phase).

1.75, 4.75, and 7.75 days following reversal of the L/D cycle, for STR, ITR, and LTR, respectively. All hearts were stored at  $-80^{\circ}\text{C}$  prior to RNA extraction.

**Adult Rat Cardiomyocyte Isolation and Culture**—ARCs were isolated from male Wistar rats (175–200 g; Harlan) and cultured on laminin-coated plates, as described previously (19).

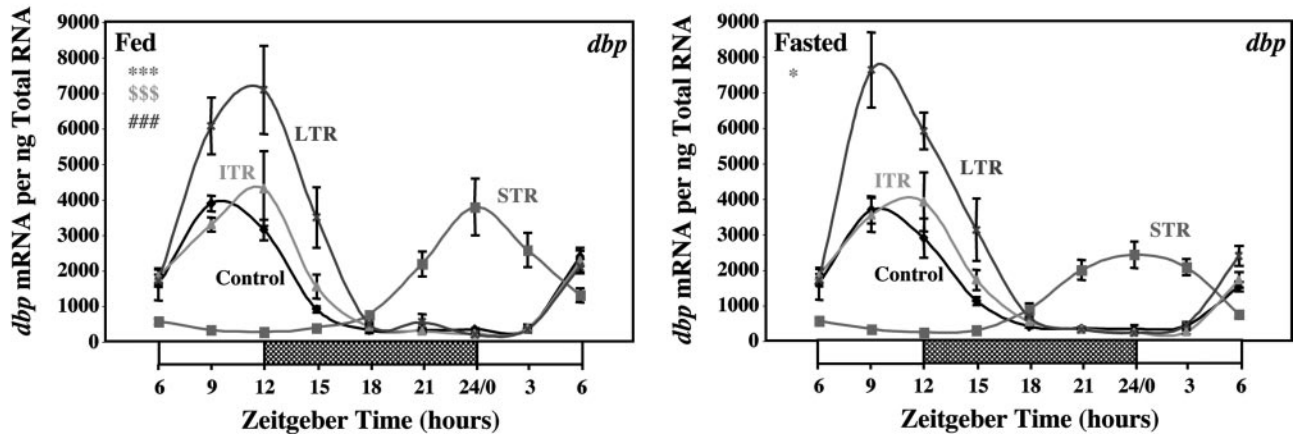
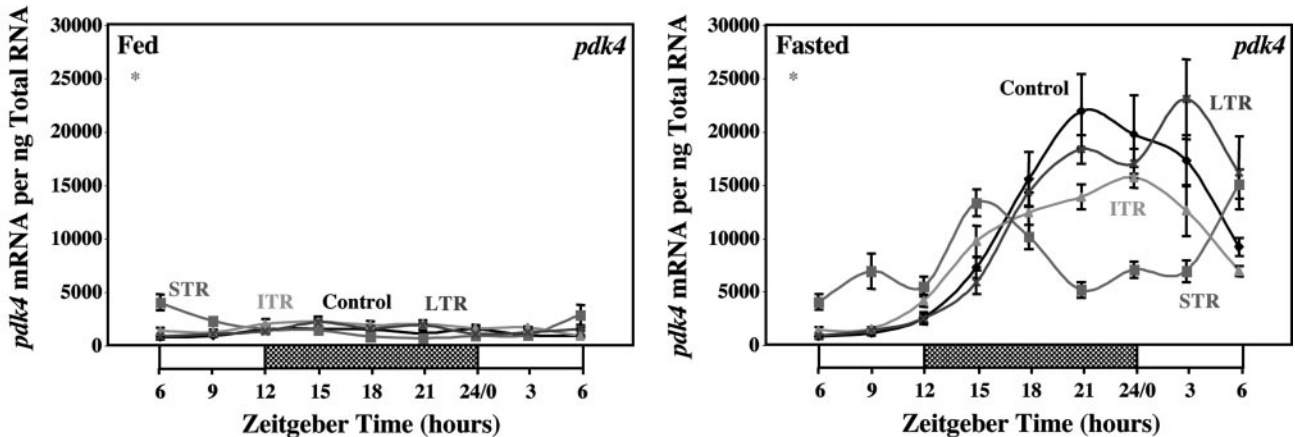
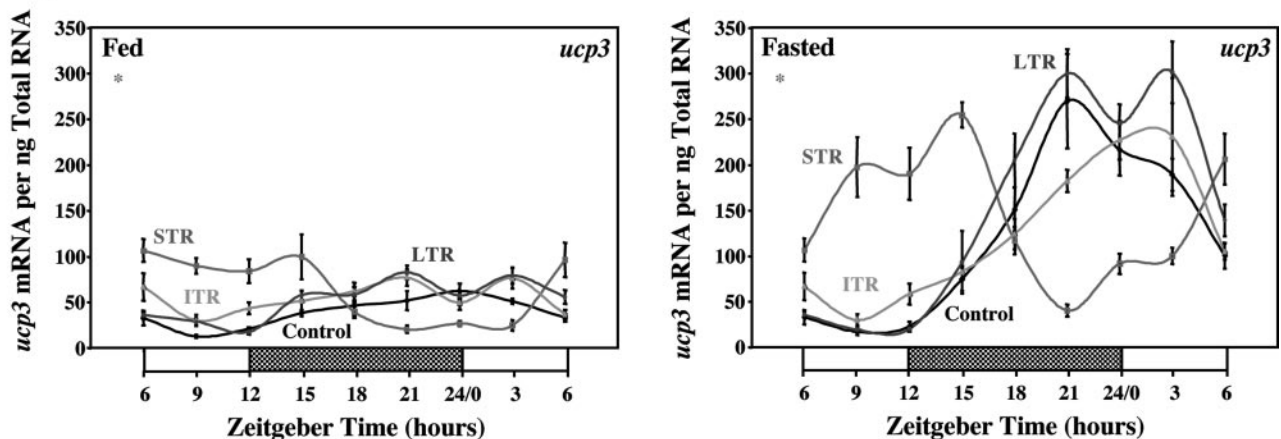
D) *dbp* mRNAE) *pdk4* mRNAF) *ucp3* mRNA

FIGURE 2—continued

Cardiomyocytes were cultured in low glucose (5 mM), glutamine-free Dulbecco's modified Eagle's medium (Sigma) supplemented with 1 mM pyruvate, 4 mM NaHCO<sub>3</sub>, 8.6 mM HEPES (pH 7.3), 5 mM creatine, 2 mM L-carnitine, 5 mM taurine, 10  $\mu$ M cytosine  $\beta$ -D-arabinofuranoside, 1% bovine serum albumin, 100 IU/ml penicillin, and 100 mg/ml streptomycin. The culture medium was also supplemented with a defined concentration of fetal calf serum (FCS). Following plating of cells to laminin

for 2 h (5% FCS-supplemented plating medium), cells were cultured overnight (equilibration medium with or without 2.5% FCS; as specified). At time 0 (11 a.m. the following morning; ~24 h post-isolation), cardiomyocytes were challenged with fresh medium (challenge medium with or without 50% FCS; as specified). At time 2 h, cells were washed twice with wash medium (with or without 2.5% FCS; as specified) and subsequently cultured in post-challenge medium (with or without

## G) Plasma NEFA

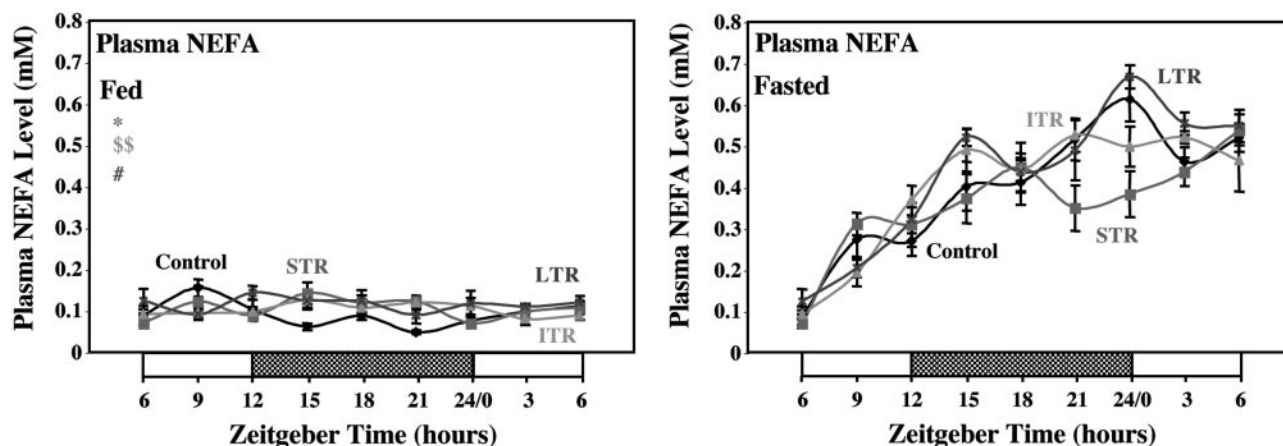


FIGURE 2—continued

2.5% FCS; as specified). For a sub-set of cells, 0.4 mM oleate was added to challenge, wash, and post-challenge media. All media were pre-equilibrated to 37 °C in an atmosphere of 5% CO<sub>2</sub> for at least 2 h prior to utilization (use of 25-cm<sup>2</sup> phenolic style cap cell culture flasks; Corning Glass). Furthermore, all media changes were performed on a 37 °C pre-equilibrated heat pad within a sterile tissue culture cabinet, thereby minimizing temperature fluctuations. Cells were terminated at multiple time points and stored at –80 °C prior to RNA extraction.

**Generation of Transgenic Mice**—Mice with a cardiomyocyte-selective expression of a dominant negative CLOCK (dnCLOCK) mutant protein (*i.e.* cardiomyocyte CLOCK mutant (CCM) mice) were generated, in which a truncated *clock* gene (which corresponds to the first 541 amino acids of the wild-type CLOCK protein, and therefore lacks a functional transactivation domain) was constructed, whose expression was driven by the  $\alpha$ -myosin heavy chain promoter. The  $\alpha$ -myosin heavy chain promoter targets expression to cardiomyocytes but not other cell types in the heart. Use of a dominant negative strategy was performed, as opposed to a knock-out strategy, because of the high level of redundancy of the mammalian circadian clock mechanism (*e.g.* NPAS2, a CLOCK homologue, is as highly expressed in the heart as CLOCK and may therefore compensate if CLOCK is limited) (20). Transfection studies were performed in BHK fibroblasts using standard techniques, to ensure the CLOCK mutant protein exhibited a dominant negative effect over wild-type CLOCK and BMAL1 (both endogenous and exogenous). Human CLOCK, BMAL1, and (per2)<sub>3</sub>-luciferase constructs utilized for *in vitro* characterization of the dnCLOCK mutant were the kind gifts from Dr. Steven McKnight (University of Texas Southwestern). Genotyping of transgenic mice was performed by PCR.

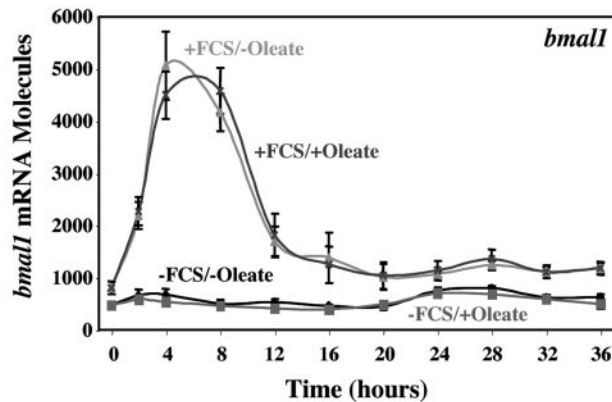
**RNA Extraction and Quantitative Reverse Transcription-PCR**—RNA extraction and quantitative reverse transcription-PCR were performed using methods described previously (28–30). Specific quantitative assays were designed from rat and mouse sequences available in GenBank<sup>TM</sup> for four core circadian clock genes (circadian locomotor output cycles kaput (*clock*), brain and muscle arnt-like protein 1 (*bmal1*), *rev-erba*, and period 2 (*per2*)), two circadian clock-regulated genes (albu-

min d-element binding protein (*dbp*) and *e4bp4*), two fatty acid-responsive genes (pyruvate dehydrogenase kinase 4 (*pdk4*), and uncoupling protein 3 (*ucp3*), three triglyceride metabolism genes (diacylglycerol acyltransferase 2 (*dgat2*), adipose triglyceride lipase (*atgl*), and adiponutrin (*adpn*)), and one housekeeping gene (cyclophilin); primer and probe sequences for these assays have been published previously, with the exception of *clock*, *e4bp4*, *dgat2*, *atgl*, and *adpn*, which are presented in Table 1 (31, 32). The *clock* assay was designed to recognize mRNAs encoding for both wild-type *clock* and *dnclock*. For the ARC studies, gene expression data are represented as mRNA molecules per approximate 20 ng of total RNA; because of the lower yield of total RNA for isolated cardiomyocytes *versus* intact hearts, accuracy in spectrophotometric determination of RNA concentration is reduced (typically 20 ng of total RNA is used for each cardiomyocyte gene expression measurement). For intact rodent heart studies, gene expression data are represented as mRNA molecules per ng of total RNA. For transgenic mouse studies investigating nonmyocardial tissues, gene expression data are normalized to the housekeeping gene cyclophilin, to allow for sample-to-sample variability in total RNA (see Supplemental Material). The latter representation is because of the lower sample sizes (*i.e.*  $n = 3$ ) for these studies, which dictate a stricter normalization method, as compared with normalization to total RNA levels.

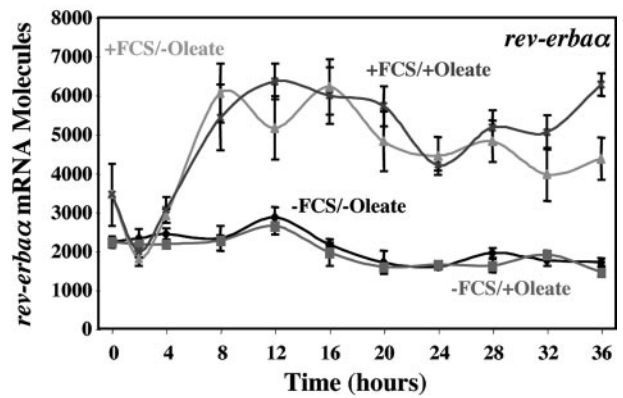
**Metabolite Measurements**—Immediately prior to heart isolation, 1 ml of blood was withdrawn from all rodents. The sample was placed on ice prior to centrifugation for 10 min at full speed using a desktop microcentrifuge. The plasma was retained and stored at –80 °C until NEFA levels were measured spectrophotometrically with a commercially available kit (Wako Chemicals). Specimen blanks were prepared for all samples to allow for possible hemolysis. Myocardial triglyceride content was measured from homogenate extracts using enzymatic spectrophotometric methods as described previously (33).

**Western Blotting**—Native CLOCK and dnCLOCK proteins were detected in heart homogenates, using standard Western blotting techniques. Myocardial proteins were separated by electrophoresis on a 10% gel, transferred to nitrocellulose

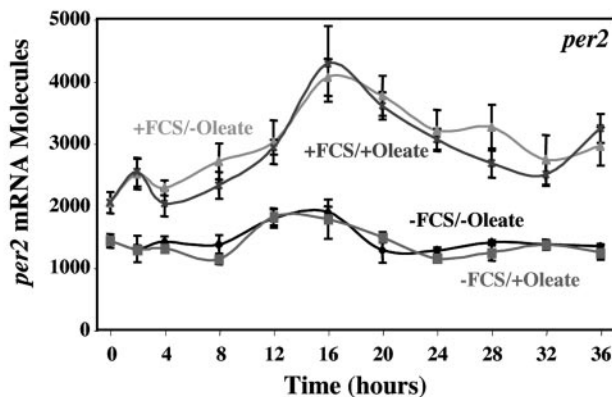
A) *bmal1* mRNA



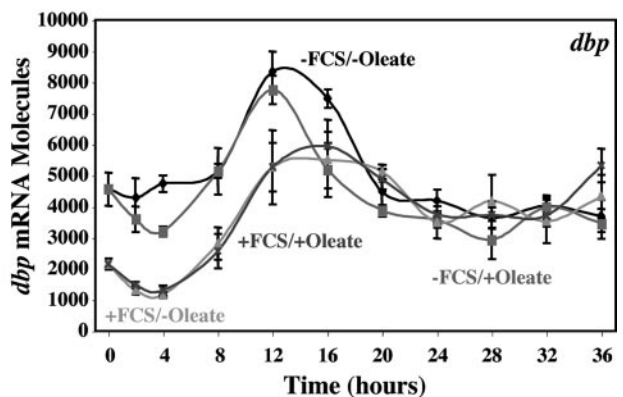
B) *rev-erba* mRNA



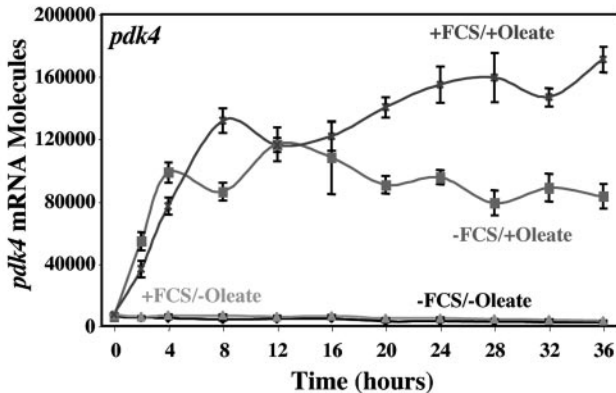
C) *per2* mRNA



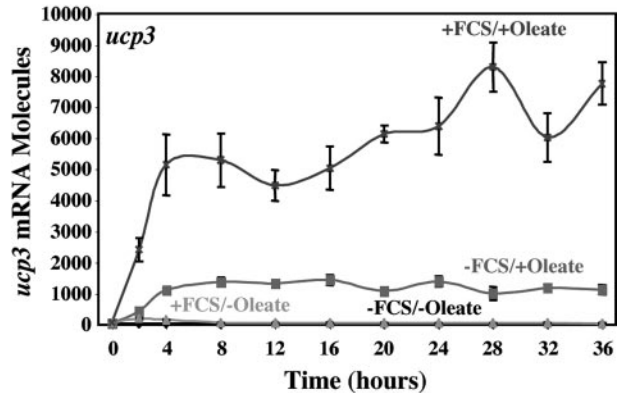
D) *dbp* mRNA



E) *pdk4* mRNA



F) *ucp3* mRNA



**FIGURE 3. Gene expression of *bmal1* (A), *rev-erba* (B), *per2* (C), *dbp* (D), *pdk4* (E), and *ucp3* (F) for ARCs challenged with FCS and/or oleate.** When challenged with FCS, 2.5% FCS was present in equilibration, wash, and post-challenge media, whereas 50% FCS was present in the challenge medium; FCS was omitted from all media for ARCs not challenged with FCS. When challenged with oleate, bovine serum albumin (1%) and oleate (0.4 mM) were present in challenge, wash, and post-challenge media; only 1% bovine serum albumin was present in these media for control cells. The four groups are as follows: 1) control (no FCS, no oleate;  $\diamond$ ); 2) FCS (FCS-challenged, no oleate;  $\blacksquare$ ); 3) oleate (no FCS, 0.4 mM oleate;  $\blacktriangle$ ); and 4) FCS plus oleate (FCS-challenged, 0.4 mM oleate;  $\blacklozenge$ ). Values are shown as the mean  $\pm$  S.E. for five separate observations at each time point. Data are represented as number of mRNA molecules (per approximate 20 ng of total RNA). Significant ( $p < 0.01$ ) oscillations are observed in ARCs cultured in the presence of FCS for *bmal1*, *rev-erba*, *per2*, and *dbp*; significant ( $p < 0.01$ ) oscillations are observed in control cells for *dbp* only.

membranes, and probed with a goat anti-CLOCK antibody that recognizes the N terminus of both the native CLOCK and dnCLOCK proteins (Santa Cruz Biotechnology). Secondary probing was achieved using a rabbit anti-goat antibody, followed by detection through enhanced chemiluminescence.

**Statistical Analysis**—For data involving oscillations, periodicity analysis was performed in R stat analysis environment using non-

linear least squares estimation as supported by the method nls. Prior to fitting, all gene expression data were normalized to remove linear time trends and to remove global shifts. For all experimental versus control data, a simple wave form was fit:  $Y(t) = A \times \cos(t/T \times 2\pi + P)$ . A likelihood ratio test was performed to determine the significance of each fit for each gene in each experiment compared with the null hypothesis of a constant

time pattern (a straight line). We call a periodic fit significant if the likelihood ratio test  $p$  value was less than 0.05.

For nonoscillatory data, two-way analysis of variance was conducted to investigate the main effects of group (tissue or experimental condition) and time. The general linear model procedure in SAS software, version 8.2, was used for this analysis (SAS Institute, Inc). A full model, including second-order interactions, was conducted for each experiment. Significant differences were determined using type III sums of squares. The null hypothesis of no model effects was rejected at  $p < 0.05$ . Repeated measures analysis was not utilized because of the fact that samples in each group were from different animals.

## RESULTS

**Light/Dark Cycle Reversal Impairs Responsiveness of the Heart to Fasting**—We investigated whether the cardiomyocyte-specific circadian clock modulates responsiveness of the heart to circulating fatty acids, by disrupting the cardiomyocyte circadian clock *in vivo* through reversal of the L/D cycle. Fig. 2, A–D, depicts myocardial circadian gene expression data for key clock components in fed and fasted rats. Clock genes exhibit marked circadian oscillations in expression in the intact rat heart (Fig. 2, A–D; *control group*). Comparison of control hearts with STR hearts shows that following reversal of the L/D cycle, the circadian clock within the heart remains antiphase to its environment  $\sim 2$  days post-reversal; a 9–12-h phase difference exists between control and STR hearts for the four clock genes examined (Fig. 2, A–D). Comparison of control hearts with ITR hearts shows an almost complete re-entrainment of the circadian clock within the heart by  $\sim 5$  days post-reversal of the L/D cycle; a 3-h phase difference remains for all clock genes examined (Fig. 2, A–D). Re-entrainment of the circadian clock genes is complete in LTR hearts (Fig. 2, A–D). The rate of re-entrainment of the circadian clock components was relatively unaffected by acute fasting (Fig. 2, A–D).

Fig. 2, E and F, depicts myocardial circadian gene expression for key fatty acid-responsive genes in fed *versus* fasted rats. As anticipated, fasting caused a marked induction in both *pd4* and *ucp3* in control hearts, whose expression peaked at ZT21 (15 h of fasting; Fig. 2, E and F), and declined thereafter. STR hearts initially exhibited a more rapid induction of *pd4* and *ucp3* following food withdrawal, compared with control hearts, peaking at ZT15 (9 h of fasting; Fig. 2, E and F). Thereafter, *pd4* and *ucp3* expression profiles in STR hearts were antiphase to control hearts, exhibiting a trough in expression at ZT21 (Fig. 2, E and F). Fasting-mediated induction of *pd4* and *ucp3* in ITR hearts was similar to that observed in control hearts, although an approximate 3-h phase difference and diminished zenith persisted (Fig. 2, E and F). Profiles of these fatty acid-responsive genes were normalized in LTR hearts (Fig. 2, E and F). Thus, the rate of re-entrainment of the circadian clock within the heart following reversal of the L/D cycle was identical to the rate of normalization of the responsiveness of the heart to acute fasting.

To ensure that the level of the stimulus (*i.e.* fatty acids) for control, STR, ITR, and LTR rats was not mediating differences in the induction of fatty acid-responsive genes during fasting, plasma NEFA levels were measured. Withdrawal of food at ZT6

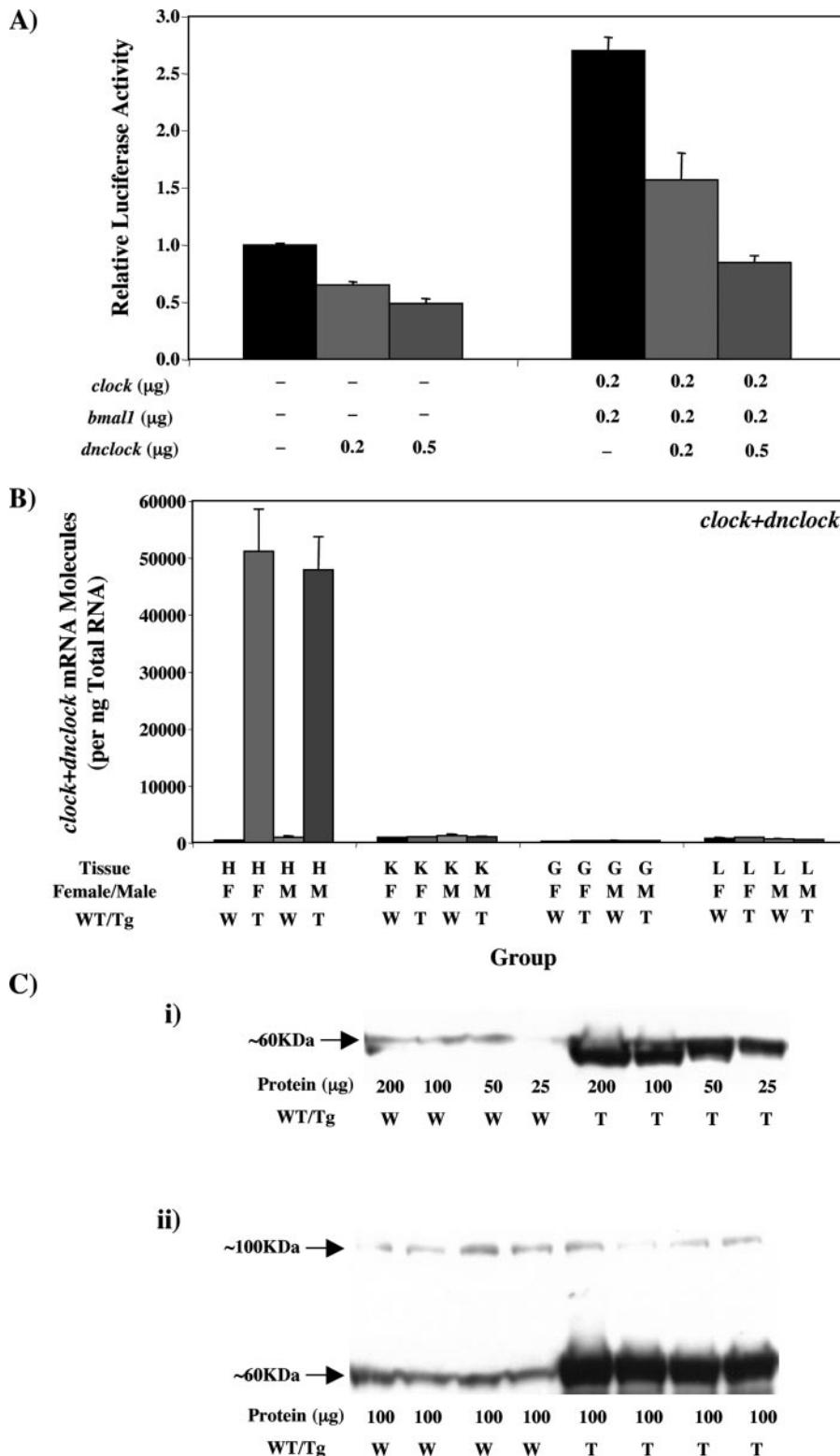
was associated with a rapid increase in plasma NEFA levels in all four groups investigated (an average of 2.8-fold within 3 h; Fig. 2G). The temporal patterns of plasma NEFA levels were similar between the four groups investigated following food withdrawal (Fig. 2G).

**Diurnal Variations in the Responsiveness of Cardiomyocytes to Fatty Acids Persist in Culture**—Because reversal of the L/D cycle disrupts all circadian clocks within the organism, as well as normal rhythms in multiple neurohumoral factors, two strategies were next performed to dissect the influence of the intra-cardiomyocyte circadian clock *versus* neurohumoral factors, as mediators of diurnal variations in the responsiveness of the heart to fatty acids. These were as follows: 1) use of isolated adult rat cardiomyocytes (ARCs) in culture; and 2) generation of a cardiomyocyte-specific CLOCK mutant (CCM) mouse.

Challenging ARCs with 50% FCS for 2 h (termed serum shock) has been shown to establish circadian clock gene oscillations in isolated ARCs (19). As such, challenging isolated ARCs with serum shock induced circadian oscillations in the expression of the four clock genes investigated (*bmal1*, *rev-erba*, *per2*, and *dbp*; see Fig. 3, A–D). In contrast, ARCs cultured in the absence of serum exhibited significant oscillations only in *dbp* (Fig. 3D). Furthermore, oscillations in *dbp* for ARCs cultured in the absence of serum were noticeably lower in amplitude (*i.e.* nadir-to-zenith) as compared with those cardiomyocytes subjected to serum shock (Fig. 3D). Thus, circadian clock oscillations persist in ARCs cultured in the presence of serum.

Oscillations in circadian clock genes were unaffected by the presence of the fatty acid oleate (0.4 mM) in the medium (Fig. 3, A–D). However, challenging cardiomyocytes with oleate caused a rapid induction of *pd4* and *ucp3*, in both the absence and presence of FCS (*e.g.* 6- and 12-fold induction of *pd4* and *ucp3*, respectively, within 2 h for cardiomyocytes challenged with serum shock; Fig. 3, E and F). For cardiomyocytes cultured in the absence of FCS, induction of *pd4* and *ucp3* reached a peak after 4 and 8 h, respectively; thereafter, expression of these fatty acid-responsive genes remained constant (Fig. 3, E and F). In contrast, for cardiomyocytes challenged with serum shock, a rapid induction of *pd4* and *ucp3* in response to oleate was followed by a slight repression (trough at time 12 h; Fig. 3, E and F). Next, a second induction phase occurred, peaking at time 28 h (Fig. 3, E and F); in the case of *ucp3*, the level of induction of this fatty acid-responsive gene was as great during the second induction phase (12–28 h) as observed for the first induction phase (0–12 h; Fig. 3F). A second repression phase occurred after time 28 h (Fig. 3, E and F). These data show that diurnal variations in the responsiveness of cardiomyocytes to fatty acids persists *in vitro*, under conditions in which the circadian clock is fully operational (*i.e.* in the presence of FCS).

**Characterization of CCM Mouse Hearts**—Transfection studies were performed in BHK fibroblasts to ensure the CLOCK mutant protein exhibited a dominant negative effect over wild-type CLOCK and BMAL1 (both endogenous and exogenous). As such, BHK fibroblasts were transfected with the luciferase gene downstream of a CLOCK/BMAL1-responsive promoter ((*per2*)<sub>3</sub>-



**FIGURE 4. Characterization of the dnCLOCK mutant.** A, effects of *dnClock* gene transfection into BHK fibroblasts on *clock*- and *bmal1*-mediated transcription *in vitro* ( $n = 3$ ). B, gene expression of *clock* and *dnClock* in hearts (H), kidneys (K), gastrocnemius muscles (G), and livers (L) in female (F) and male (M) wild-type (W) and CCM transgenic (T) mice ( $n = 10-17$ ). C, protein expression of CLOCK (100 kDa) and dnCLOCK (60 kDa) in male wild-type (W) and CCM transgenic (T) mice.

luciferase). Co-transfection of the *dnClock* gene into BHK fibroblasts resulted in a dose-dependent repression of luciferase activity (Fig. 4A). Co-transfection of wild-type *clock* and *bmal1*

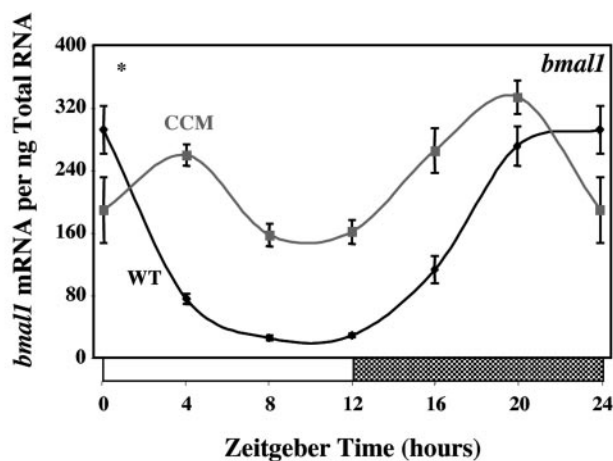
genes into BHK fibroblasts caused an increase in luciferase activity (Fig. 4A). The latter was dose-dependently repressed by co-transfection of the *dnClock* gene (Fig. 4A). Similar observations were made in CV-1, L6, and COS-1 cells (data not shown). Thus, the dnCLOCK mutant is functional *in vitro*.

Gene expression of both *clock* and *dnClock* was next measured in hearts, kidneys, gastrocnemius muscles, and livers isolated from age-matched (2 months old) wild-type and CCM mice. Fig. 4B shows a specific expression of the *dnClock* gene within the heart; expression of the *dnClock* gene is ~80-fold higher than native *clock*, in both female and male CCM mice. This high level of *dnClock* expression relative to *clock* expression was also observed at the protein level; expression of the dnCLOCK protein (60 kDa) was markedly greater than native CLOCK (100 kDa) (Fig. 4C). A low intensity nonspecific band at ~60 kDa was also observed in wild-type hearts (Fig. 4C).

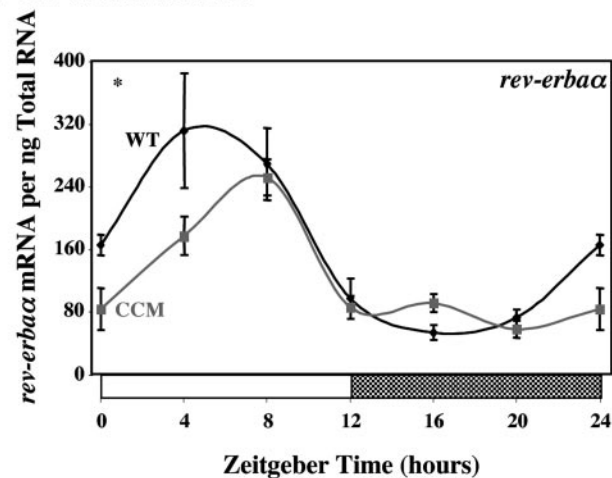
Whether forced expression of the dnCLOCK protein within the cardiomyocytes of the heart was associated with impairment of the circadian clock was next investigated by measuring circadian expression patterns of known clock components, as well as fatty acid-responsive genes, in CCM mice. Female wild-type hearts exhibited oscillations in the expression of all clock and fatty acid-responsive genes investigated (Fig. 5, A-H). Hearts isolated from CCM mice exhibited higher levels of *bmal1* expression (Fig. 5A), consistent with the loss of negative loops of the circadian clock. In contrast, oscillations in *rev-erba* and *per2* expression tended to be decreased in CCM hearts (Fig. 5, B and C). Oscillations in the clock output gene *dbp* were markedly attenuated in CCM versus wild-type hearts (Fig. 5D).

In contrast, expression of *e4bp4* (which antagonizes DBP transcriptional activity) was significantly increased in CCM hearts (Fig. 5E). As such, oscillations in the *dbp/e4bp4* ratio (an indirect marker of circadian

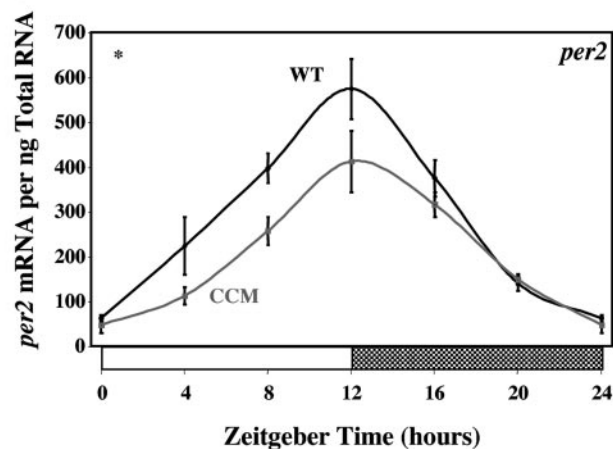
A) *bmal1* mRNA



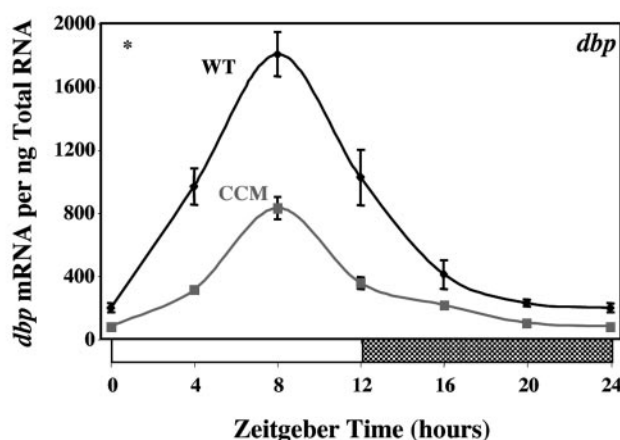
B) *rev-erbaα* mRNA



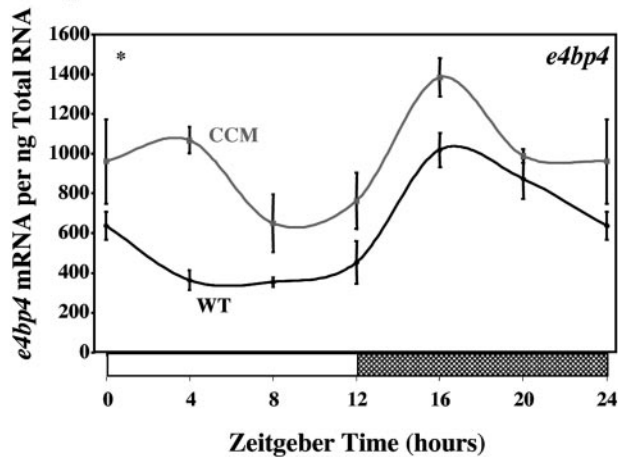
C) *per2* mRNA



D) *dbp* mRNA



E) *e4bp4* mRNA



F) *dbp* mRNA/*e4bp4* mRNA Ratio

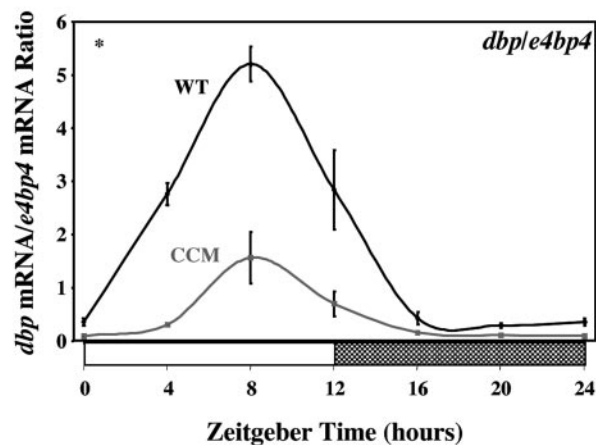


FIGURE 5. Circadian expression of *bmal1* (A), *rev-erbaα* (B), *per2* (C), *dbp* (D), *e4bp4* (E), *dbp/e4bp4* ratio (F), *pdk4* (G), and *ucp3* (H) in hearts isolated from female wild-type (WT; ♦) and CCM mice (■). Values are shown as the mean  $\pm$  S.E. for 3–9 separate observations at each time point. Gene expression data are represented as number of mRNA molecules (per ng of total RNA). \*,  $p < 0.05$  CCM versus wild-type hearts (amplitude).

clock output) were dramatically attenuated in CCM hearts, as were oscillations in *pdk4* and *ucp3* (Fig. 5, F–H). Expression of the circadian clock genes *bmal1*, *rev-erbaα*, *per2*, and *dbp* was essentially identical between wild-type and CCM

mice for kidneys, gastrocnemius muscles, and livers (see Supplemental Material).

To ensure that the transgene did not influence the circadian clock in a gender-specific manner, expression of clock genes

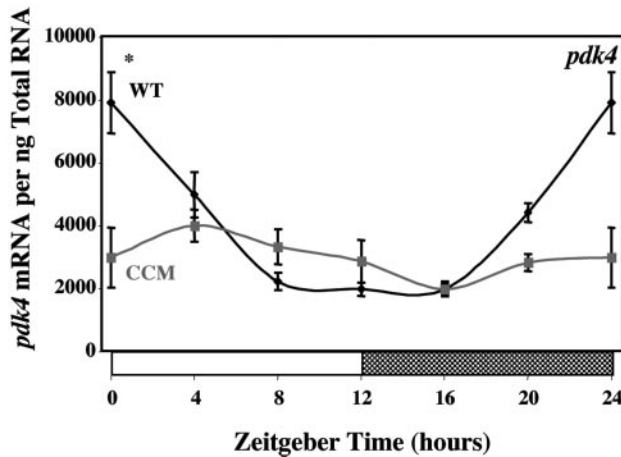
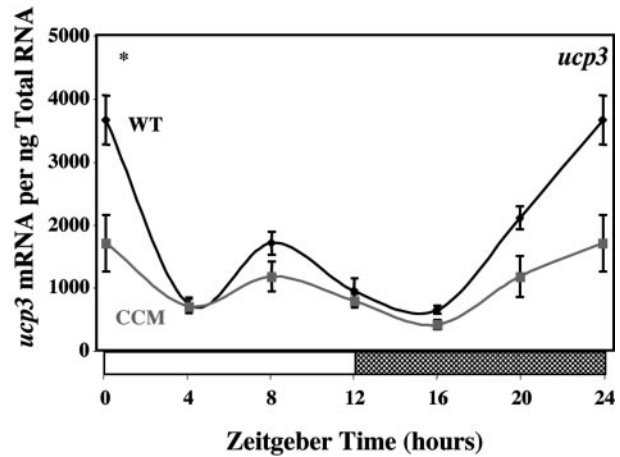
G) *pdk4* mRNAH) *ucp3* mRNA

FIGURE 5—continued

was next investigated in male wild-type and CCM hearts, at both ZT8 and ZT20. For all clock genes investigated, identical patterns of expression were observed in male CCM mice (Fig. 6, A–F), as observed previously in female mice (Fig. 5, A–F). Furthermore, identical data were obtained in male mice for the fatty acid-responsive genes *pdk4* and *ucp3* (Fig. 6, G and H). Taken together, these data show that forced expression of a dominant negative CLOCK mutant protein in the cardiomyocytes of the mouse heart results in impairment of the circadian clock within the heart, without affecting extra-cardiac tissues, in a gender-independent manner. Persistence of dampened circadian clock gene oscillations within CCM hearts likely represents functional circadian clocks within noncardiomyocytes of the heart (e.g. vascular smooth muscle cells, endothelial cells, and fibroblasts) that constitute ~30% of the cells within the heart.

**Altered Responsiveness of CCM Hearts to Fasting**—The potential role for the circadian clock within the cardiomyocyte as a modulator of the responsiveness of the heart to fatty acids was investigated further through utilization of the CCM mouse. As male rats were utilized in the L/D cycle manipulation studies, and that the circadian clock was impaired equally in both male and female CCM mice, male mice were utilized in the following study. Wild-type and CCM mice were fasted for either 12 or 24 h (food withdrawal at ZT8); *ad libitum* fed mice served as controls. Fasting wild-type mice resulted in a significant induction of both myocardial *pdk4* and *ucp3* within 12 h. Consistent with the data presented in Fig. 2, E and F, for the rat heart, prolongation of fasting into the light phase results in a lesser induction of *pdk4* and *ucp3* (i.e. wild-type mice following 12 h versus 24 h of fasting). Hearts isolated from 12-h fasted CCM mice exhibit a marked attenuation in the induction of both *pdk4* and *ucp3* (Fig. 7A). Prolongation of the fast to 24 h resulted in a greater induction of *pdk4* and *ucp3* in CCM hearts versus wild-type hearts (Fig. 7A). In marked contrast, fasting wild-type and CCM mice elicited similar responses in the induction of *pdk4* and *ucp3* in gastrocnemius muscles (Fig. 7B), as well as similar increases in plasma NEFA levels (Fig. 7C). Taken together, these data show that a functional circadian

clock within the cardiomyocytes of the heart mediates diurnal variations in the responsiveness of the heart to fatty acids.

To investigate further the responsiveness of CCM hearts to fasting, the difference in myocardial triglyceride content was measured at ZT24 between fed versus fasted wild-type and CCM mice. We hypothesized that impaired induction of  $\beta$ -oxidation promoting genes in CCM hearts during fasting would result in accumulation of myocardial triglyceride. Fasting significantly increased triglyceride content in wild-type hearts by 63% (compared with fed controls; Fig. 7D). However, fasting had no significant effects on myocardial triglyceride content in CCM mice (Fig. 7D).

Gene expression of key lipogenesis (*dgat2*) and lipolysis (*atgl*, *adpn*) enzymes were next investigated, in an attempt to explain the lack of triglyceride accumulation in CCM hearts during fasting (34–36). Messenger RNAs encoding for *dgat2* and *adpn* (but not *atgl*) exhibited significant circadian oscillations in female wild-type hearts, with peak levels of expression at ZT16 and ZT20, respectively (Fig. 8A). Oscillations in *dgat2* and *adpn* were markedly attenuated in CCM hearts (Fig. 8A). Furthermore, *dgat2* expression was constitutively repressed in CCM hearts (–48% compared with wild-type hearts;  $p < 0.05$ ), whereas *adpn* expression was constitutively induced (159%;  $p < 0.05$ ). Expression of *atgl* was not significantly different between wild-type and CCM hearts (Fig. 8A). Similar observations were made in male wild-type versus CCM hearts (Fig. 8B). Fasting male wild-type mice resulted in a time-dependent induction of *dgat2* and *atgl*, as well as a repression of *adpn* (Fig. 8C). Fasting-induced alterations in the expression of these tri-glyceride metabolism genes were blunted in CCM hearts (Fig. 8C). These observations not only identify triglyceride metabolism genes as being directly regulated by the circadian clock within the cardiomyocyte, but they also suggest that the lack of triglyceride accumulation in fasted CCM hearts is because of increased lipolytic capacity concomitant to decreased lipogenic capacity.

## DISCUSSION

This study is the first to ascribe a functional role for the circadian clock within the cardiomyocytes of the heart *in vivo*. We have

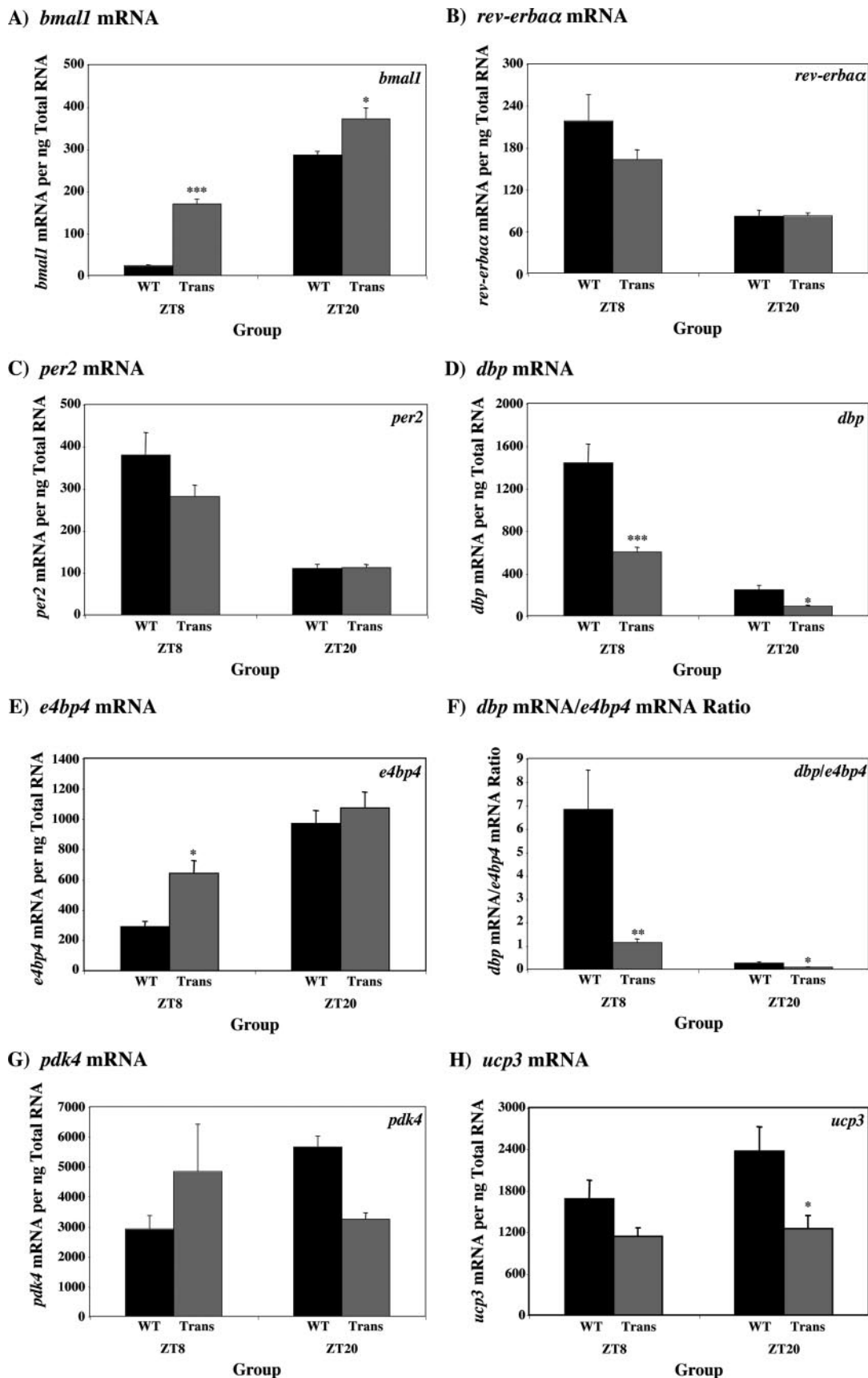
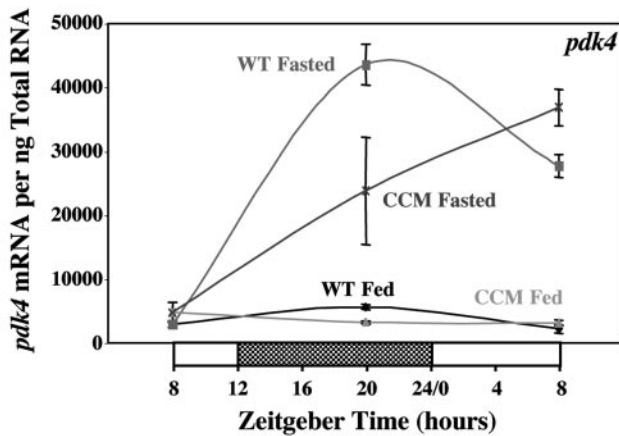


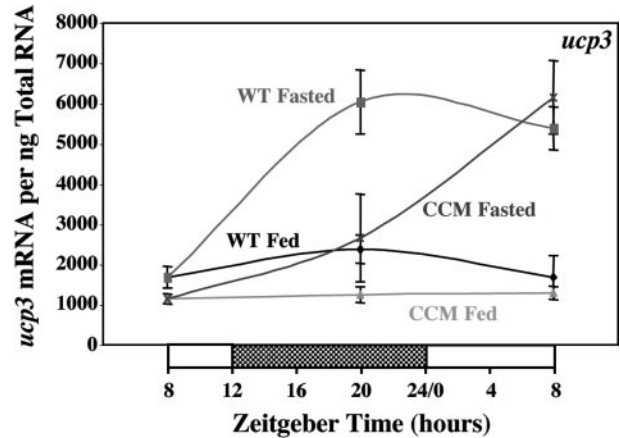
FIGURE 6. Gene expression of *bmal1* (A), *rev-erba* (B), *per2* (C), *dbp* (D), *e4bp4* (E), *dbp/e4bp4* ratio (F), *pdk4* (G), and *ucp3* (H) in hearts isolated from male wild-type (WT) and CCM mice at ZT 8 and ZT20. Values are shown as the mean  $\pm$  S.E. for 6–13 separate observations at each time point. Gene expression data are represented as number of mRNA molecules (per ng of total RNA). \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; and \*\*\*,  $p < 0.001$  CCM versus wild-type hearts at the same time point.

A) Heart

i) *pdk4* mRNA

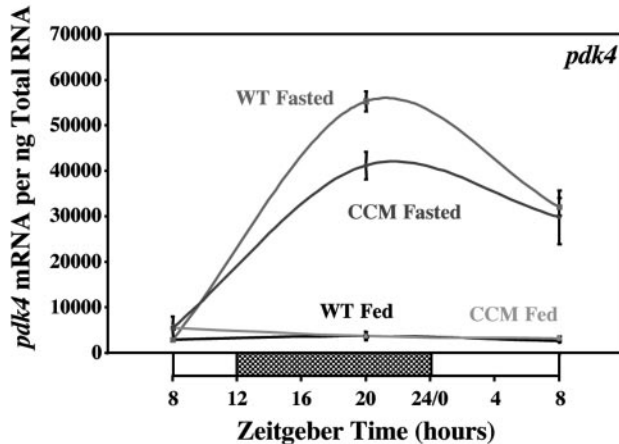


ii) *ucp3* mRNA

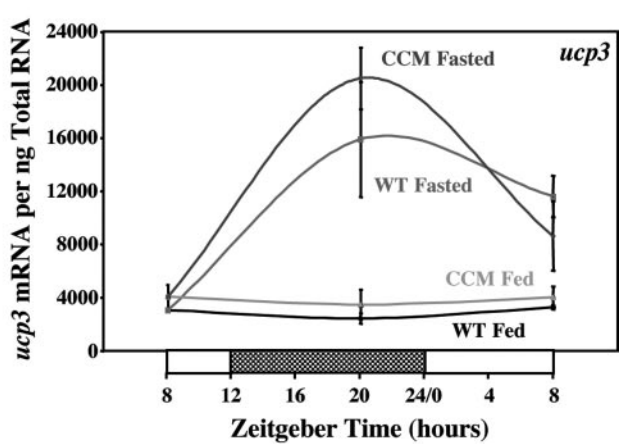


B) Gastrocnemius Muscle

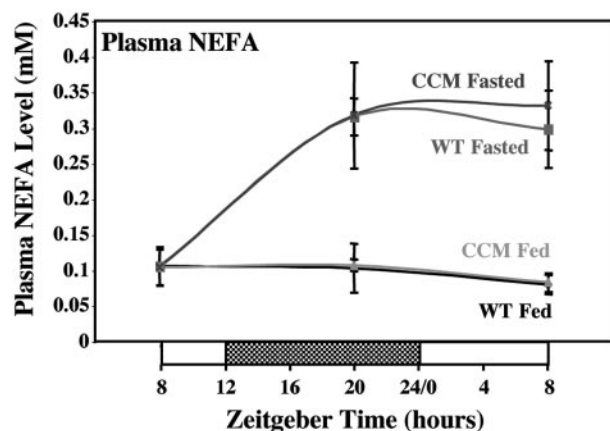
i) *pdk4* mRNA



ii) *ucp3* mRNA



C) Plasma NEFA



D) Myocardial Triglyceride

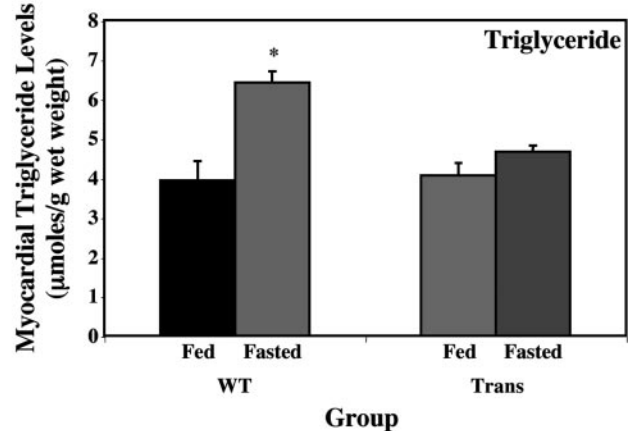


FIGURE 7. Gene expression (A and B), plasma NEFA levels (C), and myocardial triglyceride (TAG) levels (D) in fed versus fasted male wild-type (WT) and CCM mice. Each figure panel contains four groups as follows: wild-type fed (◆), wild-type fasted (■), CCM fed (▲), and CCM fasted (✕) mice. Values are shown as the mean  $\pm$  S.E. for 3–8 separate observations at each time point. Gene expression data are represented as number of mRNA molecules (per ng of total RNA). Plasma NEFA levels are represented as mM. Myocardial TAG levels were measured in hearts isolated at ZT24; data are represented as  $\mu$ mol/g wet weight. \*,  $p < 0.05$  fed versus fasted hearts.

found the following. 1) Re-entrainment of the circadian clock within the heart and normalization of the responsiveness of the heart to fasting exhibit essentially identical temporal patterns following reversal of the L/D cycle. 2) Diurnal variations in the

responsiveness of the heart to fatty acids persist *in vitro* only when the circadian clock is fully functional. 3) Specific impairment of the circadian clock within the cardiomyocyte (through genetic manipulation) dramatically attenuates responsive-

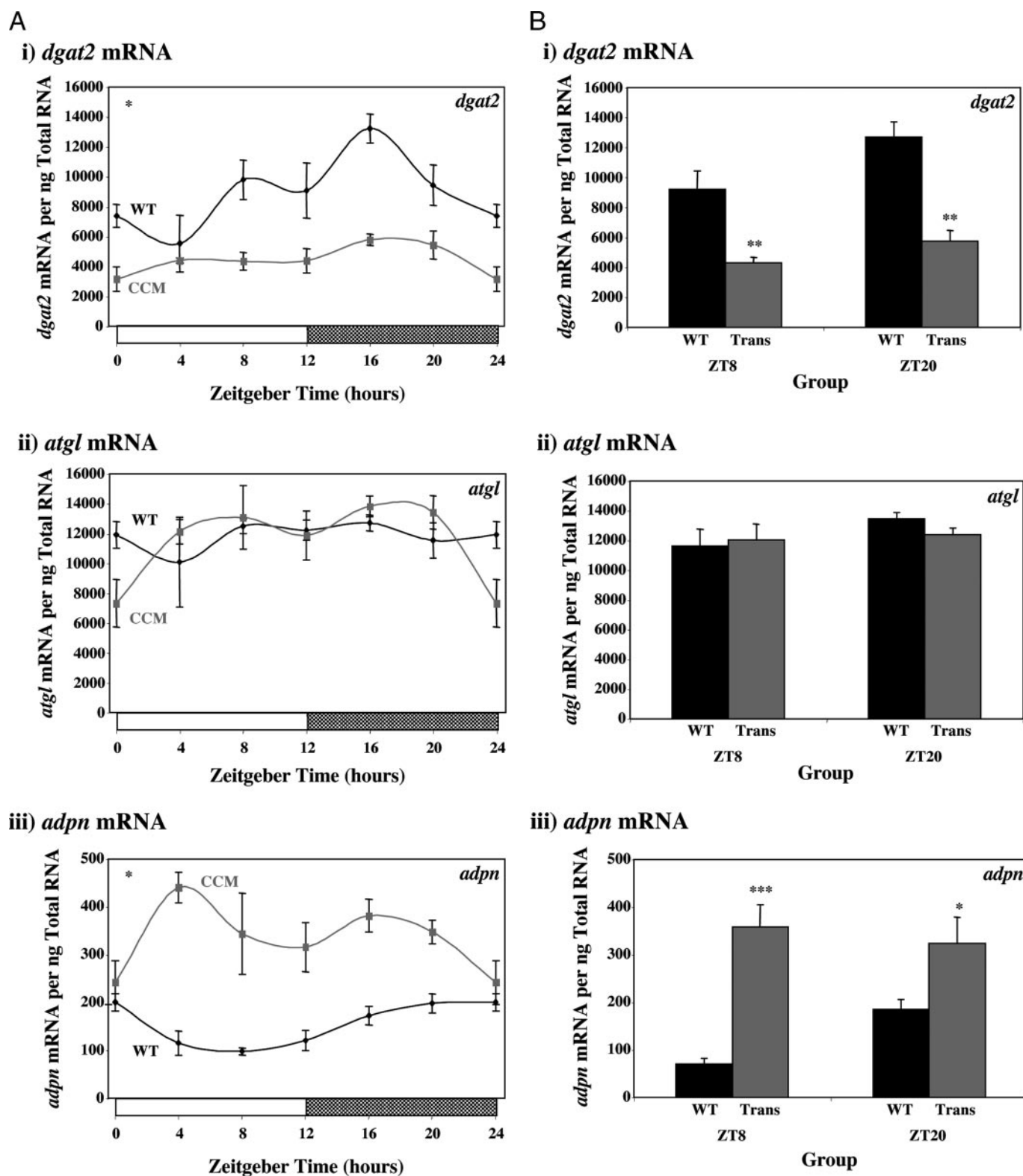


FIGURE 8. Myocardial expression of triglyceride metabolism genes in female wild-type (WT) (♦) and CCM (■) mice (A), male wild-type versus CCM mice (B), and male fed versus fasted wild-type and CCM mice (C). Each panel in C contains four groups as follows: wild-type fed (♦), wild-type fasted (■), CCM fed (▲), and CCM fasted (✱) mice. Values are shown as the mean  $\pm$  S.E. for 3–13 separate observations in each group. Gene expression data are represented as number of mRNA molecules (per ng of total RNA). \*,  $p < 0.05$  CCM versus wild-type hearts (amplitude; A). \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; and \*\*\*,  $p < 0.001$  CCM versus wild-type hearts at the same time point (B).

ness of the heart to fasting. Taken together, these observations provide direct evidence that the circadian clock within the cardiomyocyte regulates responsiveness of the heart to

fatty acids. In addition to identifying a novel mechanism that regulates myocardial metabolism, these observations have significant clinical implications regarding the pathogenesis

C

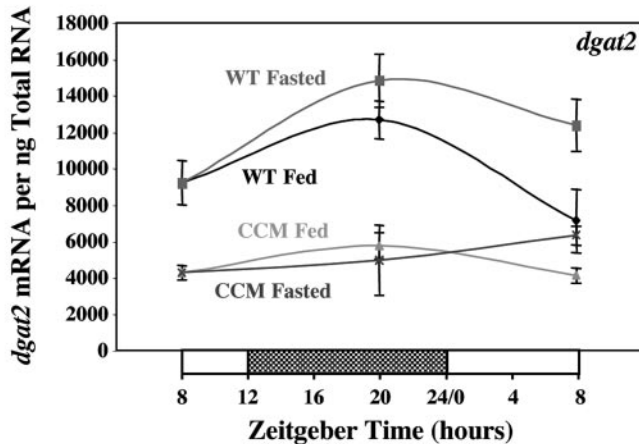
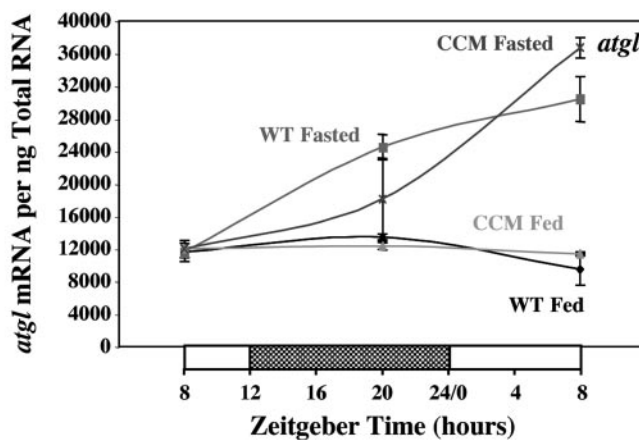
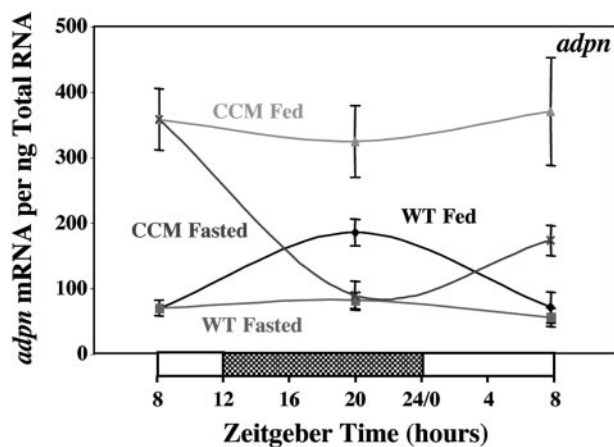
i) *dgat2* mRNAii) *atgl* mRNAiii) *adpn* mRNA

FIGURE 8—continued

of cardiovascular disease observed during hypertension, obesity, diabetes, and prolonged shift work.

**The Circadian Clock within the Heart as a Novel Regulator of Myocardial Metabolism**—We have recently established a link between the circadian clock within the cardiomyocyte and myocardial metabolism. Through the use of ARCs in culture,

we reported persistence in oscillations of mRNAs encoding for both *pd4* and *ucp3* (19). Oscillations in these metabolic genes were observed *in vitro* only under conditions in which the circadian clock was operational. Furthermore, metabolic gene oscillations exhibited the same temporal pattern as observed in the intact heart *in vivo*, when compared with circadian clock genes (19). Consistent with these observations, the present study reports severe attenuation of *pd4* and *ucp3* mRNA circadian oscillations in hearts isolated from CCM mice (Fig. 5, *D* and *E*). In addition, we show that circadian oscillations in expression of the triglyceride metabolism enzymes *dgat2* and *adpn* are markedly attenuated in CCM hearts (Fig. 8*A*). Taken together, these observations show that the circadian clock within the cardiomyocyte modulates myocardial metabolic genes.

We have previously reported diurnal variations in the responsiveness of the heart to fatty acids, with increased sensitivity during the dark phase for the rat (21). Consistent with these observations, Fig. 2 shows the greatest level of *pd4* and *ucp3* induction, following food withdrawal, for control hearts during the dark phase (zenith at ZT21). To dissect whether diurnal variations in the responsiveness of the heart to fatty acids are because of diurnal variations in neurohumoral factors and/or the intramyocellular circadian clock, two strategies were employed as follows: 1) investigation of responsiveness of cardiomyocytes to fatty acids *ex vivo*; and 2) disruption of the circadian clock within the heart *in vivo*. By using oscillations of circadian clock genes as a marker of the subjective time perceived by the cardiomyocytes, experiments presented in Fig. 3 show that diurnal variations in the responsiveness of cardiomyocytes to fatty acids persist *ex vivo*. For example, *bmal1* expression peaks at ZT0 in the intact rat heart *in vivo*, and peaks 4 h post-serum shock for ARCs *in vitro* (Figs. 2*A* and 3*A*, respectively). As such, a trough in the oleate-mediated induction of fatty acid-responsive genes 12 h post-serum shock in ARCs is equivalent to ZT8 in the intact rat heart; ZT9 is the time at which the lowest responsiveness of the heart to fatty acids is observed *in vivo* (control hearts in Fig. 2, *D* and *E*). Similarly, a peak in the oleate-mediated induction of fatty acid-responsive genes 28 h post-serum shock is equivalent to ZT24 in the intact rat heart; ZT21 is the time when the greatest responsiveness of the heart to fatty acids is observed *in vivo* (control hearts in Fig. 2, *D* and *E*). With the exception of *dbp*, oscillations in both the circadian clock genes and the induction of fatty acid-responsive genes are not observed in ARCs in the absence of serum shock (Fig. 3). Thus, diurnal variations in the responsiveness of the heart to fatty acids are preserved *in vitro* but only when the circadian clock within the cardiomyocyte is fully functional. Furthermore, disruption of the circadian clock within the heart, either through manipulation of the L/D cycle (Fig. 2) or through use of genetically modified mice (Fig. 7), severely impairs responsiveness of the heart to fasting. These data expose a causal relationship between the circadian clock within the heart and responsiveness of this organ to fatty acids.

Myocardial fatty acid oxidation capacity (at the level of gene expression) is greatest during the dark phase for the rat. Additionally, responsiveness of the rat heart to fatty acids is greatest during the dark phase. Both of these phenomena are under the

control of the circadian clock within the cardiomyocyte. Given that circadian clocks allow anticipation of environmental stimuli, the question arises as to why a dyssynchrony exists between diurnal variations in fatty acid availability (highest during the light phase) and responsiveness of the myocardium to fatty acids. This led us to hypothesize that diurnal variations in fatty acid responsiveness may be the result of anticipation of prolongation of the resting phase fast, if the animal in the wild is initially unsuccessful in its forage for food upon awakening; a rapid induction of fatty acid oxidation enzymes at this time would allow effective utilization of readily abundant fatty acids, during continued foraging (20–22). Consistent with this hypothesis, we find that responsiveness of the heart to fasting is severely attenuated when the intramyocardial circadian clock is impaired.

The precise mechanism linking the intramyocellular circadian clock to responsiveness of the heart to fatty acids has not been elucidated in this study. Fatty acids rapidly induce gene expression through direct activation of a family of nuclear receptors known as peroxisome proliferator-activated receptors (PPARs) (37). All three PPAR family members ( $\alpha$ ,  $\beta/\delta$ , and  $\gamma$ ) identified to date are expressed within cardiomyocytes to varying extents, with expression of PPAR $\alpha$  and PPAR $\beta/\delta$  predominating over PPAR $\gamma$  (18, 38, 39). Upon heterodimerization with retinoid X receptor family members, the PPAR/retinoid X receptor dimer binds to fatty acid-response elements located within the promoter of various target genes (40). Known PPAR $\alpha$  (and PPAR $\beta/\delta$ ) target genes include those promoting fatty acid oxidation (e.g. *ucp3*) and repressing carbohydrate oxidation (e.g. *pd4*) (32, 41). Similar to diurnal variations in the responsiveness of the heart to fatty acids, the heart is more sensitive to the PPAR $\alpha$ -specific agonist WY-14,643 during the dark phase, suggesting that sensitivity of the PPAR $\alpha$  system exhibits circadian rhythmicity (21). Furthermore, we found a coordinated induction of transcriptional activators of the PPAR $\alpha$  system (*ppara*, *rxra*, *pgc1*, and *p300*) in the rat heart during the night (21). Kassam *et al.* (42) have shown that the circadian clock component REV-ERBA $\alpha$  antagonizes PPAR $\alpha$ , through binding of overlapping sequences in the promoter of target genes. Consistent with its antagonistic function, oscillations in *rev-erba $\alpha$*  expression are antiphase to those of *ppara* (21). However, the observed decreased responsiveness of CCM hearts to 12 h of fasting, despite decreased *rev-erba $\alpha$*  expression, suggests that *rev-erba $\alpha$* -independent mechanisms may link the circadian clock within the cardiomyocyte to fatty acid responsiveness.

**Synchronization of the Circadian Clock in the Heart with the Environment**—An additional observation reported in this study that warrants further discussion relates to the rate of re-entrainment of the circadian clock within the heart following reversal of the L/D cycle. Fig. 2 shows that the circadian clock within the heart takes between 5 and 8 days to fully resynchronize following reversal of the L/D cycle. In contrast, alterations in the L/D cycle and/or restricted feeding induces rapid phase shifting of blood pressure, heart rate, and behavioral activity circadian rhythms in rats, which are completely resynchronized within 1–2 days (5). This means that the circadian clock within the heart of rodents is dyssynchronous with the environment between 3 and 7 days following reversal of the L/D cycle. In humans, shift workers show an essentially complete re-entrain-

ment of blood pressure rhythms within the first 24 h of the shift rotation (43, 44). Assuming that the rate of re-entrainment for the circadian clock within the human heart mirrors that of rodents, one would hypothesize that the repetitive reversal of the L/D by shift workers would prevent synchrony between the circadian clock within the heart and its environment. As such, hearts of shift workers would lack the selective advantage of anticipation, thereby attenuating normal adaptation to diurnal variations in environmental stimuli (*i.e.* neurohumoral factors and blood pressure). It is tempting to speculate that such an impairment in this anticipatory mechanism may contribute toward increased risk of cardiovascular disease observed in these subjects (45–47). As with L/D cycle alterations, both pressure overload-induced hypertrophy and diabetes mellitus result in alterations in the circadian clock within the heart; both of these stresses are cardiovascular disease risk factors (31, 48). Furthermore, additional cardiovascular disease risk factors, such as obesity and sleep apnea, would be anticipated to affect the circadian clock within the heart (although no formal reports have been made to date).

The concept of impairment of peripheral circadian clocks as contributors toward the pathogenesis of disease states extends beyond cardiovascular disease. This study clearly demonstrates that impairment of the circadian clock within the heart attenuates responsiveness of the myocardium to fatty acids. A mismatch between fatty acid availability and fatty acid oxidation rates results in accumulation of detrimental intracellular fatty acid derivatives. One way in which a cell prevents accumulation of detrimental fatty acid derivatives, in addition to  $\beta$ -oxidation, is through storage as triglyceride; certain cells, including cardiomyocytes, possess the ability to export triglyceride in the form of lipoproteins. We report that impairment of the circadian clock within the cardiomyocyte not only attenuates induction of  $\beta$ -oxidation promoting enzymes but also markedly attenuates triglyceride synthesis during fasting. Accumulation of detrimental fatty acid derivatives is associated not only with development of myocardial contractile dysfunction but also with hallmarks of type 2 diabetes mellitus and the metabolic syndrome, such as adiposity, insulin resistance, and insulin insufficiency (15–17, 49, 50). If the circadian clock within adipocytes, skeletal myocytes, hepatocytes, and pancreatic  $\beta$ -cells also regulates responsiveness of these cell types to fatty acids, then impairment of this intracellular molecular mechanism would accelerate accumulation of detrimental lipid derivatives. Indeed, we find that, as for the heart, re-entrainment of the circadian clock within skeletal muscle exhibits an identical temporal pattern as normalization of responsiveness to fatty acids.<sup>4</sup> In addition, it is likely that circadian clocks allow peripheral tissues to anticipate a plethora of environmental stimuli, in addition to fatty acids. These may include diurnal variations in sympathetic activity, nutrients (e.g. glucose), hormones (e.g. insulin, thyroid hormone), as well as blood pressure. An inability to respond appropriately to one or more of these environmental stimuli would be expected to contribute to the development of multiple disease states, such as obesity, type 2 diabetes mellitus, and cardiovascular disease.

<sup>4</sup> D. J. Durgan and M. E. Young, unpublished observations.

**Summary**—This study reports the persistence of diurnal variations in the responsiveness of the cardiomyocyte to fatty acids when in culture. Manipulation of the L/D cycle is associated with alterations in the responsiveness of the heart to fatty acids and a concomitant loss of synchronization between the circadian clock within the heart and the environment. The rate of re-entrainment of the circadian clock within the heart was identical to the rate of normalization in the responsiveness of the heart to fatty acids. Additionally, responsiveness of the heart to fasting was markedly attenuated following specific impairment of the circadian clock within cardiomyocytes of the heart (*i.e.* CCM mice). Taken together, these data expose a causal link between the responsiveness of the heart to fatty acids and the circadian clock within the cardiomyocyte. Whether alterations in the circadian clock within the cardiomyocyte contribute toward cardiovascular disease progression requires further investigation.

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