TITLE – Impact of circadian time of dosing on cardiac-autonomous effects of glucocorticoids

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Abstract

Bioenergetic capacity is critical to adapt the high energy demand of the heart to circadian oscillations and diseased states. Glucocorticoids regulate the circadian cycle of energy metabolism, but little is known about how circadian timing of exogenous glucocorticoid dosing directly regulates cardiac bioenergetics through the primary receptor of these drugs, the glucocorticoid receptor (GR). While chronic once-daily intake of glucocorticoids promotes metabolic stress and heart failure, we recently discovered that intermittent once-weekly dosing of exogenous glucocorticoids promoted muscle metabolism and heart function in dystrophic mice. However, the effects of glucocorticoid intermittence on heart failure beyond muscular dystrophy remain unknown. Here we investigated the extent to which circadian time of dosing regulates the cardiac-autonomous effects of the glucocorticoid prednisone in conditions of single pulse or chronic intermittent dosing. In WT mice, we found that prednisone improved cardiac content of NAD^+ and ATP with light-phase dosing (ZT0), while the effects were blocked by dark-phase dosing (ZT12). The effects on mitochondrial function were cardiomyocyte-autonomous, as shown by inducible cardiomyocyte-restricted GR ablation, and depended on an intact activating clock complex, as shown by hearts from BMAL1-KO mice. Conjugating time-of-dosing with chronic intermittence, we found that once-weekly light-phase prednisone improved metabolism and function in heart after myocardial injury. Our study identifies cardiac-autonomous mechanisms through which circadian time and chronic intermittence reconvert glucocorticoid drugs to bioenergetic boosters for the heart.

INTRODUCTION

Bioenergetic capacity is critical to adapt the high energy demand of the heart to circadian oscillations and/or diseased states. Endogenous glucocorticoids are conserved pleiotropic hormones regulating the circadian cycle of energy utilization and storage in virtually all our organs [1]. However, little is known about the extent to which circadian timing of exogenous glucocorticoid dosing impacts function of the primary receptor of these drugs, the glucocorticoid receptor (GR). The question of time-restricted pharmacology is critical for the heart, which displays circadian oscillations in nutrient metabolism [2]. Indeed, genetic ablation of BMAL1, core component of the activating clock complex in every tissue, disrupts heart metabolism and function [3]. However, mechanisms and relevance of interplay between circadian time of glucocorticoid dosing and heart metabolism remain unaddressed.

The GR regulates and is in turn regulated by core components of the circadian clock [1, 4]. The autonomous circadian clock relies on the permutation between the activating complex, e.g. CLOCK/BMAL factors active during the light-phase, and the repressing complex, e.g. PER/CRY factors active during the dark-phase [5]. In mice, endogenous glucocorticoids entrain the circadian rhythm of GR activation by peaking during the dark phase and dipping during the light-phase [6]. Indeed, GR is known to regulate nocturnal factors like PER2 [1]. In turn, the clock factors Cry1/2 and CLOCK inhibit GR activity during the dark-phase [7] and during the late light-phase [8], respectively. This leads to a critical time window in the early light-phase (corticosterone trough [6]), where the GR is not repressed and not occupied by endogenous steroids, thus highly responsive to exogenous glucocorticoids [7, 9]. However, these molecular regulations were found in non-cardiac cells and therefore the effects of light-phase-restricted synthetic glucocorticoids (anti-physiologic to endogenous glucocorticoids) remain unknown for heart function and metabolism.

The glucocorticoid receptor (GR) is important for normal heart function and contractility [10, 11]. Indeed, GR activation by exogenous glucocorticoids improves calcium handling in cardiomyocytes [10, 12]. However, the cardiac-autonomous effects of GR pharmacology on heart metabolism are still unknown. This question is particularly important as the heart-autonomous versus body-wide effects of chronic glucocorticoid intake on cardiac health remain unresolved. Synthetic glucocorticoids are used chronically by >2.5M people in the US [13], but their regular once-daily intake promotes metabolic syndrome and cardiovascular diseases in a dose-dependent manner [14]. Opposite to once-daily dosing, we discovered that intermittent dosing (once-weekly) of glucocorticoids improves function and mitochondrial capacity in dystrophic striated muscle [15, 16]. Dosing intermittence could therefore reverse the adverse benefits/risks ratio of chronic steroid

regimens for the heart. However, the cardiac effects of intermittent glucocorticoid regimens must still be investigated in conditions of hearts failure beyond the context of muscular dystrophy.

Here we investigated the extent to which circadian time of dosing regulates the cardiac effects of the exogenous glucocorticoid prednisone in conditions of single pulse or chronic intermittent dosing. In WT mice, we found that prednisone improved NAD+ and ATP levels when dosed at the start of the light-phase, while these effects were blocked with dosing at the start of the dark-phase. The effects on mitochondrial function depended on cardiomyocyte-specific GR, as shown by inducible heart-restricted GR ablation, and on an intact activating clock complex, as shown by hearts from BMAL1-KO mice. Conjugating time-of-dosing with chronic intermittence, we found that once-weekly light-phase prednisone stimulated a transcriptional program relevant for circadian metabolism and promoted function in the failing heart after myocardial infarction. Our study identifies cardiac-autonomous mechanisms through which circadian time and chronic intermittence reconvert glucocorticoid drugs to bioenergetic boosters for the heart.

RESULTS

Light-phase-specific effects of prednisone on cardiac metabolites and gene expression

We sought to investigate the acute effects of exogenous glucocorticoids on heart according to circadian time of dosing. To this end, we treated WT mice (C57BL/6 background; 8 weeks of age) with a single injection of i.p. 1mg/kg prednisone at either ZT0 (light-phase) or ZT12 (dark-phase), assaying metabolites and gene expression in myocardial tissue every 4 hours starting at 1-hour post-pulse and during one circadian cycle post-pulse. The in vivo glucocorticoid dose mirrors what we used in prior studies in dystrophic mice [15].

For metabolite quantitation, we focused our analyses on myocardial NAD^+ and ATP content, quantitating these metabolites through targeted assays as metabolic biomarkers of mitochondrial function. NAD^+ is a fundamental determinant and biomarker of mitochondrial health in a variety of cardiovascular conditions [17]. ATP is produced by oxidative phosphorylation in mitochondria and the ATP synthase is directly regulated by the NAD^+ redox balance in heart [18]. As parallel analyses of gene expression, we monitored the effects of time-specific prednisone on myocardial expression of *Nampt* and *Ppargc1a*. *Nampt* encodes the homonymous rate-limiting enzyme for

NAD^+ biogenesis [19]. *Ppargc1a* encodes the mitochondrial regulator PGC1α, which promotes ATP production in heart through mitochondrial biogenesis and function [20].

Compared to vehicle, the light-phase prednisone pulse increased the levels of NAD^+ and ATP content **(Fig. 1A)**, as well as *Nampt* and *Ppargc1a* expression levels **(Fig. 1B)** over the course of the post-pulse circadian cycle. However, these effects were blunted or absent after dark-phase prednisone pulse **(Fig. 1A-B)**.

Thus, circadian time of intake regulated the acute effects of a single pulse of the GC prednisone in the WT heart

Acute effects of light-phase prednisone in heart require cardiac-specific GR

Considering the effects produced by a light-phase prednisone pulse, we asked the extent to which the glucocorticoid receptor (GR, encoded by the *Nr3c1* gene) mediates these effects. To this end, we generated inducible heart-specific GR-KO mice crossing *Myh6-CreERT*$^{+/-}$ [21] with *Nr3c1*$^{fl/fl}$ [22] transgenic lines on the C57BL/6 background. At 8 weeks of age, we induced GR ablation in heart through a sequence of i.p. tamoxifen injections (20mg/kg/day for 5 days) followed by 14 days on tamoxifen-containing chow (40mg/kg) and 2 days of regular chow for tamoxifen washout. Such induction paradigm ablated ~85% of GR in heart without immediate changes in plasma corticosterone or myocardial tissue OCR at baseline **(Fig. 2)**. We compared GR-WT (*Nr3c1*$^{fl/fl}$*; Myh6-CreERT*$^{-/-}$) versus GR-KO (*Nr3c1*$^{fl/fl}$*; Myh6-CreERT*$^{+/-}$) littermates for the effects of a light-phase prednisone pulse in vivo immediately after tamoxifen exposure and washout, i.e. at ~12 weeks of age. We injected GR-WT and GR-KO mice with a single i.p. 1mg/kg prednisone dose at ZT0 (light-phase) and monitored the effects in heart at 24-hours post-dose.

We quantitated the effects on mitochondrial function in the myocardium through parallel Seahorse respirometry analyses in myocardial tissue and isolated mitochondria. We quantitated basal tissue OCR adapting conditions previously reported for muscle tissue [23] and controlling the baseline OCR levels with rotenone/antimycin. To confirm that the gain in oxygen consumption correlated with actual mitochondrial respiration, we isolated mitochondria from the same left ventricle tissue samples and quantitated their respiratory control ratio (RCR). RCR estimates the capacity of isolated mitochondria to oxidize substrate to phosphorylate ADP and is indeed calculated as ratio of state III (ADP) over state IV_o (oligomycin) OCR values [24]. While in GR-WT mice the drug pulse increased both basal myocardial OCR and mitochondrial RCR as compared to vehicle, these effects were blocked in GR-KO hearts **(Fig. 3A)**. This correlated with analogous

trends in NAD+ and ATP content in the left ventricle myocardial tissue **(Fig. 3B)**. Intriguingly, inducible GR ablation blocked the transcriptional effects of the drug pulse on *Nampt* and *Ppargc1a* in heart **(Fig. 3C)**.

Thus, the cardiomyocyte-specific GR is required for the transcriptional and metabolic effects of light-phase prednisone in heart.

Light-phase intermittent prednisone activates a transcriptional program involving circadian-metabolic pathways in heart and improves function and metabolism after myocardial infarction.

We asked whether the effects of light-phase prednisone were significant in the context of myocardial infarction (MI), where mitochondrial capacity is a key target to improve overall cardiac function [25]. To that goal, we conjugated the circadian time of dosing with chronic glucocorticoid dosing. Regarding chronic steroid dosing, we used the once-weekly 1mg/kg prednisone intermittent dosing that we successfully tested with positive cardiac outcomes in dystrophic *mdx* and *Dysf-null* mice [15]. Intermittent prednisone dosing avoids the typical wasting and metabolic stress effects induced by chronic daily dosing [15]. We treated sham- or MI-operated WT mice from 2-weeks post-surgery to avoid early changes in pathophysiologic remodeling and provide a comparable control/dysfunction baseline before onset of treatments. MI or sham operations were conducted at 8 weeks of age after baseline echocardiography, and treatments (once-weekly i.p. 1mg/kg prednisone or vehicle at ZT0) started after confirming decreases in fractional shortening and stroke volume at 2-weeks post-MI. Treatment was administered for 6 weeks and analyses were conducted in both sham- and MI-operated mice.

Compared to vehicle at endpoint, light-phase intermittent prednisone treatment significantly increased fractional shortening and stroke volume after MI to levels close to control sham hearts. The trends were not significant in sham-operated hearts **(Fig. 4A)**.

At endpoint, we analyzed sham-vehicle, sham-treated, MI-vehicle and MI-treated hearts through RNA-seq. Principal component analysis of whole transcriptomes clustered samples according to surgery and treatment **(Fig. 4B)**. We then performed pathway analysis in the genes that were differentially expressed (DE) by treatment in each setting using the arbitrary thresholds of >2-fold change and >10CPM abundance (FDR<0.05). Intriguingly, we found that treatment increased the number of DE genes from 82 in sham hearts to 669 in MI hearts **(Suppl. Table 1)**. Also, the overall trend in treatment-driven regulation in DE genes shifted from 87% downregulated in sham to 96% upregulated in MI hearts **(Fig. 4C)**. Strikingly though, the gene ontology (GO)

analysis revealed a high degree of overlap in pathways (89% overlap in GO terms) between DE genes in sham versus MI settings **(Suppl. Table 2)**. Among these overlapping GO terms, we noticed several inflammation-related pathways, as well as circadian rhythm pathways **(Fig. 4C)**. We therefore analyzed concordant and discordant genes for genes contained in the circadian rhythm GO pathways. We defined "concordant genes" as upregulated by treatment in both sham and MI hearts, while "discordant genes" were upregulated by treatment in MI hearts but downregulated in sham hearts (no arbitrary thresholds). Intriguingly, we found that activating clock complex genes (*Arntl*, *Clock*, *Rora*), as well as metabolic regulators *Nampt*, *Ppargc1a* and *Ppara* (PGC1α co-factor) were among concordant genes, i.e. upregulated by treatment in both sham and MI hearts. Conversely, repressive clock complex genes (*Nr1d1*, *Per1-3*) were among discordant genes, i.e. upregulated by treatment in MI but not in sham hearts **(Fig. 4D)**.

Considering the concordant trends in genes encoding agonists of mitochondrial biogenesis and function, we quantitated mitochondrial respiration and abundance in the left ventricle myocardium of sham and MI hearts at endpoint. Treatment increased tissue OCR and mitochondrial RCR in sham and MI hearts, rescuing the levels in MI hearts to levels like in control sham hearts **(Fig. 4E)**. We quantitated mitochondrial abundance through two parallel assays, mitochondrial DNA/nuclear DNA ratio (mtDNA/nDNA; [26]) and unbiased Mitotracker Green FM fluorometry in left ventricle myocardial tissue. Mitotracker Green FM signal is independent from mitochondrial membrane potential [27]. Both assays showed gain of mitochondrial density with treatment in sham and MI hearts **(Fig. 4F)**, confirming the treatment effects on overall mitochondrial abundance in infarcted myocardium.

Thus, intermittent dosing of light-phase prednisone improved systolic function after MI, correlating with a pro-metabolic transcriptional program and increased levels of mitochondrial function and abundance in the myocardium.

Acute effects of light-phase prednisone in heart require an intact circadian clock

We were intrigued by the concordant trends induced by light-phase intermittent prednisone on cardiac expression of genes encoding factors of the activating clock complex. We thus instructed a proof-of-principle experiment to ask whether an intact activating clock complex was required for the acute cardiac effects of light-phase prednisone. Indeed, the activating clock complex, which encompasses BMAL1, is normally active during the light-phase in mice [5]. Importantly, BMAL1 (gene name, *Arntl*) promotes mitochondrial function and NAD^+ biogenesis [28] and plays a key

role in cardiomyocyte metabolism and function [3]. We therefore asked whether genetic deletion of BMAL1 abrogated the effects of light-phase prednisone in heart.

We analyzed hearts from BMAL1-WT (*Arntl*$^{wt/wt}$) versus BMAL1-KO (*Arntl*$^{null/null}$) littermate mice [29] at 24-hours after a pulse of light-phase prednisone versus vehicle at ZT0. We analyzed hearts at 8 weeks of age before onset of prominent dysfunction and wasting at ~6 months of age in this strain [30]. Compared to the drug effects in BMAL1-WT hearts, BMAL1 deletion abrogated or significantly blunted the light-phase prednisone effects on mitochondrial respiration **(Fig. 5A)**, NAD^+ and ATP levels **(Fig 5B)** and *Nampt-Ppargc1a* upregulation **(Fig. 5C)**, confirming that the cardiac clock is required for the acute effects of light-phase prednisone on gene expression and metabolism in heart.

Thus, an intact activating clock complex was required for the acute effects of light-phase prednisone in heart.

DISCUSSION

Despite their wide usage, the cardiomyocyte-autonomous effects of glucocorticoids on heart metabolism and function remain largely unknown. Moreover, endogenous glucocorticoids are intrinsically linked to the circadian rhythm, yet little is known about time-of-intake effects on exogenous glucocorticoid action. Here we report that the exogenous glucocorticoid prednisone regulated NAD^+, *Nampt*, ATP and *Ppargc1a* levels in heart dependent on time of dosing. Specifically, prednisone dosing at the start of the light-phase (ZT0) promoted those metabolites and gene levels in the murine heart, while dark-phase dosing (ZT12) did not. The effects were cardiac-autonomous and specific to the drug-GR axis in cardiomyocytes, as inducible ablation of cardiomyocyte-specific GR blocked the effects. Our findings are consistent with the reported cardiac dysfunction developed by mice with constitutive cardiac GR deletion [11]. In that regard, glucocorticoids are already known to improve calcium handling and contractility in cardiomyocytes [10]. The bioenergetic effects we are reporting here represent a complementary pathway of GR action in the heart. Importantly, in our inducible model, pathology was not a confounder as the acute experiment was performed immediately after ablation. Indeed, constitutive cardiac GR-knockout mice develop cardiomyopathy from 3-6 months of age [31].

In this study we used prednisone as exogenous glucocorticoid. Compared to other synthetic glucocorticoids, prednisone was effective in discriminating differences in time-of-intake likely due to short half-life and rapid uptake in striated muscles [32]. Moreover, the present data in WT, inducible GR-KO and BMAL1-KO hearts are consistent with our previous findings with pulsatile and intermittent dosing in dystrophic mice [15, 16, 33]. Indeed, in those prior studies injections were performed at 7AM (ZT1), a time-of-dose close to the ZT0 used here.

Here we did not address the role of endogenous corticosterone in mechanistically mediating or antagonizing the light-phase prednisone effects in heart. However, we did assess plasma corticosterone in our inducible cardiac GR-KO mice and did not find differences immediately after ablation, i.e. when the GR-WT and GR-KO hearts presented a differential response to light-phase prednisone. However, we recognize that the role of endogenous glucocorticoids as independent variable in exogenous glucocorticoid effects on heart metabolism in vivo remains unaddressed and should be investigated through specific studies.

Our study reports on the convergence between time of prednisone administration and peripheral clock on cardiac bioenergetics. Specifically, here the convergence relied on the light-phase-restricted time of prednisone dosing. Indeed, the light-phase is the circadian phase where the activating clock complex, including BMAL1, is active in all cells including cardiac cells. Consistent

with that notion of convergence, an intact clock was required for the light-phase prednisone effects, at least acutely in isolated hearts, as shown by hearts from BMAL1-KO mice. Our data are consistent with the protective role of BMAL1 in heart, as shown by prior studies in heart function [3] and heart rate [34]. As both GR and BMAL1 are epigenetic factors regulating gene expression, dedicated studies on the effects of time-specific drug dosing on the circadian transcriptome of cardiomyocytes will be informative in dissecting transient versus adaptive changes of chronic regimens.

With regards to the overall perspective of "chrono-pharmacology", we have shown here biomarkers and mechanisms of glucocorticoid action on cardiac bioenergetics, opening the perspective of circadian time as possible determinant of cardiac pharmacology outcomes. In that regard, it is important to note that circadian studies in mice are not immediately translatable to humans because of the mismatch in circadian cycles and the complexity of feeding/activity/sleep behavior in humans [35]. Nonetheless, the molecular oscillations governing heart metabolism and function are conserved [2]. Moreover, disruption of the circadian clock in the heart disrupts cardiac function in mice [3], aligning with the known cardiovascular and cardiometabolic insults induced by circadian disruption in humans [36, 37]. Thus, elucidation of molecular mediators of time-restricted pharmacology in murine models is still a cogent strategy to discern translatable mechanisms and biomarkers for the emerging clinical question of "when should you take your medicines?"[38].

Our study found notable effects on both NAD^+ and mitochondrial respiration/abundance in myocardial tissue, suggesting a comprehensive positive effect of light-phase prednisone on aerobic energy production in heart. Moreover, the effects of light-phase prednisone on *Nampt* upregulation and NAD^+ biogenesis in heart are consistent with the positive effects of NAD^+ replenishment in heart failure [18, 39-41]. However, our study was performed in male mice and additional studies are required to address whether effect size and mechanisms are replicable in female mice. Furthermore, the bioenergetic effects of chronic intermittent dosing in vivo correlated with functional improvements in models of MI and diabetic cardiomyopathy. This is remarkable as our findings with intermittent (once-weekly) prednisone oppose the known correlations between once-daily regimens and heart dysfunction. Indeed, chronic once-daily glucocorticoid intake in the human population increases the chance of heart failure events in a dose-dependent manner [14]. It is therefore possible that the combination of circadian time-of-intake and dosing intermittence, at least in our experimental conditions, increased the bioenergetic effects in heart without inducing metabolic stress, a notion strongly supported by our prior studies in dystrophic mice and patients

[15]. Importantly, our study did not address the extent to which circadian time-of-intake determines steroid effects on immune system and fibrosis. In liver, glucocorticoids induce circadian-independent effects on the immune system [60], but this still needs to be investigated in the context of heart remodeling.

In summary, this work reports cardiomyocyte-autonomous mechanisms elicited by pulsatile administration of exogenous glucocorticoids in the light-phase. These findings pave the way to more refined chrono-pharmacology studies in heart in vivo, as well as studies in non-myocyte components of the heart. The long-term potential of these studies is to rebalance pro-energetic versus off-target effects of glucocorticoid steroids in the context of cardiac-tailored therapies.

MATERIALS and METHODS

Mice

Mice were housed in a pathogen-free facility in accordance with the American Veterinary Medical Association (AVMA) and under protocols fully approved by the Institutional Animal Care and Use Committee (IACUC) at Cincinnati Children's Hospital Medical Center (#2020-0008). Consistent with the ethical approvals, all efforts were made to minimize suffering. Euthanasia was performed through carbon dioxide inhalation followed by cervical dislocation and heart removal.

Mice were maintained on a 12h/12h light/dark cycle. Mice were interbred on the C57BL/6 background from the following lines received from Jackson Laboratories (Bar Harbor, ME): WT C57BL/6 mice #000664; BMAL1-KO mice #009100; Myh6-CreERT mice #005657; GR-floxed mice #021021.

Gene ablation was induced with tamoxifen right before start of drug treatments using a combination of i.p. (20mg/kg/day for 5 days; Sigma #T5648) and chow-mediated (40 mg/kg until 48 hours prior to start; Harlan #TD.130860) administration [42]. Analyses were performed after 2 days of tamoxifen washout.

Prednisone administration in vivo

Single dose prednisone treatment consisted of i.p. injection of 1 mg/kg prednisone (#P6254; Sigma-Aldrich; St. Louis, MO) [15] and weekly prednisone treatment consisted of once-weekly i.p. injection of 1mg/kg prednisone. The injectable solution was diluted from a 5mg/ml stock in DMSO (#D2650; Sigma-Aldrich; St. Louis, MO) in 50μl volume. Injections were conducted either

at the beginning of the light-phase (ZT0; lights-on) or at the beginning of the dark-phase (ZT12; lights-off). Tissues were harvested 24 hours after single pulse or last injection in chronic treatment. A vehicle injection of PBS (Sigma #D5652-50L) and DMSO (Fisher #BP231-100) was administered to another mouse in parallel to each prednisone injected mouse.

Myocardial Infarction

Mice were anesthetized with briefly inhaled 2% isoflurane (Akorn 59399-106-01), intubated through the mouth, and ventilated throughout the procedure. A left lateral thoracotomy was performed and left coronary artery was identified and ligated using 8-0 prolene suture, just below the left atrium [3]. After surgery and before being placed in a recovery cage in an incubator, mice were given a single postoperative dosage of buprenorphrine-sustained release formula (3.25mg/kg body weight) by s.c. injection at 0.1 mg/kg. A sham surgical procedure was identical, but without ligature placement.

Echocardiography

Echocardiography was performed before the MI/Sham surgery (0 week), before treatment (2 weeks) and after treatment (8 weeks). Echocardiography was performed in mice under 2% isoflurane inhalation anesthesia and the measurements were performed using a Vevo 2100 instrument with 18–38 MHz transducer (VisualSonics) as previously described [42-44], using M- and B-mode recordings for fractional shortening and stroke volume quantitation. Cardiac output was calculated as heart rate (bpm) x stroke volume (ml). Echocardiographic measurements were performed in blind.

Mitochondrial density and NAD+-ATP targeted assays

The mtDNA/nDNA assay was performed on genomic DNA isolated using the Gbiosciences Omniprep kit (Gbiosciences #786-136). The ratio was obtained from qPCR values (absolute expression normalized to internal standard Rn45s and DNA concentration) of ND1 (mtDNA locus; primers: CTAGCAGAAACAAACCGGGC, CCGGCTGCGTATTCTACGTT) versus HK2 (nDNA locus; primers: GCCAGCCTCTCCTGATTTTAGTGT, GGGAACACAAAAGACCTCTTCTGG) [26]. For the Mitotracker assay, Mitotracker Green FM powder (Invitrogen #M7514) is resuspended in 373μL of DMSO (Fisher #BP231-100) to obtain a 200μM concentration. One microliter of this

resuspension is added to 1mL of Mammalian Ringer's Solution (Electron Microscopy Sciences #11763-10) containing isolated tissue from the ventricular myocardium. The solution containing myocardial tissue (~20mg cryopowder) and Mitotracker is then pipetted into a 96 well plate (Corning #9017) in increments of 200μL. This plate is then read at the plate reader for fluorescence with excitation set to 490nm and emission set to 516nm. Values are then normalized to protein content, assayed in each well after the Mitotracker assay through homogenization and Bradford assay. NAD+-ATP targeted assays were performed on ~20mg of cryopulverized myocardial tissue (per assay) using dedicated assays: a colorimetric assay for NAD (Cayman Chemical #600480) and a luminometric assay for ATP (Cayman Chemical #700410), both then performed using a Biotek Synergy H1 Microplate Reader. All mitochondrial and metabolite dosing assays were conducted blinded to treatment groups.

Respirometry on tissues and isolated mitochondria

Basal tissue OCR values were obtained from basal rates of oxygen consumption of myocardial biopsies at the Seahorse XF96 Extracellular Flux Analyzer platform (Agilent, Santa Clara, CA) using previously detailed conditions [15]. Basal OCR was calculated as baseline value (average of 3 consecutive reads) minus value after rotenone/antimycin addition (average of 3 consecutive reads). Basal OCR values were normalized to total protein content, assayed in each well after the Seahorse run through homogenization and Bradford assay. Nutrients: 1mM palmitate-BSA (#G7021, #P0500; Millipore-Sigma, St Louis, MO); inhibitors: 0.5μM rotenone + 0.5μM antimycin A (Agilent).

Respiratory control ratio (RCR) values were obtained from isolated mitochondria from myocardial tissue. Tissues form left ventricle are harvested from the mouse and cut up into very fine pieces. The minced tissue is placed in a 15mL conical tube (USA Scientific #188261) and 5mL of MS-EGTA buffer with 1mg Trypsin (Sigma #T1426-50MG) is added to the tube. The tube is quickly vortexed, and the tissue is left submerged in the solution. After 2 minutes, 5mL of MS-EGTA buffer with 0.2% BSA (Goldbio #A-421-250) is added to the tube to stop the trypsin reaction. MS-EGTA buffer: Mannitol- ChemProducts #M0214-45, Sucrose- Millipore #100892, HEPES- Gibco #15630-080, EGTA- RPI #E14100-50.0. The tube is inverted several times to mix then set to rest. Once the tissue has mostly settled to the bottom of the tube, 3mL of buffer is aspirated and the remaining solution and tissue is transferred to a 10mL glass tissue homogenizer (Avantor # 89026-382). Once sufficiently homogenized the solution is transferred back into the 15mL conical tube and spun in the centrifuge at 1,000g for 5 minutes at 4 degrees Celsius. After

spinning, the supernatant is transferred to a new 15mL conical tube. The supernatant in the new tube is then centrifuged at 12,000g for 10 minutes at 4 degrees Celsius to pellet the mitochondria. The supernatant is discarded from the pellet and the pellet is then resuspended in 7mL of MS-EGTA buffer and centrifuged again at 12,000g for 10 minutes at 4 degrees Celsius. After spinning, the supernatant is discarded, and the mitochondria are resuspended in 1mL of Seahorse medium (Agilent #103335-100) with supplemented 10μL of 5mM pyruvate (Sigma #P2256-100G) and 10μL of 5mM malate (Cayman Chemical #20765). After protein quantitation using a Bradford assay (Bio-Rad #5000001), 2.5μg mitochondria are dispensed per well in 180μl total volumes and let to equilibrate for 1 hour at 37 degrees C. 20μL of 5mM ADP (Sigma #01905), 50μM Oligomycin (Milipore #495455-10MG), 100μM Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (TCI #C3463), and 5μM Rotenone (Milipore #557368-1GM)/Antimycin A (Sigma #A674-50MG) are added to drug ports A, B, C, and D respectively to yield final concentrations of 0.5mM, 50μM, 10μM, and 0.5μM. At baseline and after each drug injection, samples are read for three consecutive times. RCR was calculated as the ratio between state III (OCR after ADP addition) and uncoupled state IV (OCR after oligomycin addition). All Seahorse measurements were conducted blinded to treatment groups.

Western Blot

Protein analysis was performed on ~50μg total lysates from heart homogenized in PBS supplemented with 1mM CaCl2, 1mM MgCl2 (#C1016, #M8266, Sigma-Aldrich; St. Louis, MO) and protease and phosphatase inhibitors (#04693232001, #04906837001, Roche, Basel, Switzerland). Blocking and stripping solutions: StartingBlock and RestorePLUS buffers (#37543, #46430, Thermo Fisher Scientific, Waltham, MA). Primary antibodies (all diluted 1:1000 for O/N incubation at +4C): rabbit anti-GR (#A2164, ABclonal, Woburn, MA). Secondary antibody (diluted 1:5000 for 1-hour incubation at room temperature): donkey anti-rabbit (#sc-2313, Santa Cruz Biotech, Dallas, TX). Counterstain for loading control was performed with ponceau (#P7170, Sigma-Aldrich; St. Louis, MO). Blots were developed with SuperSignal Pico (cat#34579; Thermo Scientific, Waltham, MA) using the iBrightCL1000 developer system (cat #A32749; Thermo Scientific, Waltham, MA) with automatic exposure settings. WB gels and membranes were run/transferred in parallel and/or stripped for multiple antibody-based staining for densitometry analyses. Protein density was analyzed using the Gel Analysis tool in ImageJ software [45] and expressed as fold changes to control samples. All protein analyses were conducted blinded to treatment groups.

RNA extraction, cDNA preparation and quantitative PCR:

For RT-qPCR assays, total RNA was extracted from cryo-pulverized myocardial tissue with Trizol (#15596026; Thermo Fisher Scientific, Waltham, MA) and 1mg RNA was reverse-transcribed using 1X qScript Supermix (#95048; QuantaBio, Beverly, MA). RT-qPCRs were conducted in three replicates using 1X Sybr Green Fast qPCR mix (#RK21200, ABclonal, Woburn, MA) and 100nM primers at a CFX96 qPCR machine (Bio-Rad, Hercules, CA; thermal profile: 95C, 15sec; 60C, 30sec; 40X; melting curve). The 2-ΔΔCT method was used to calculate relative gene expression. was used to calculate the relative gene expression (Schmittegen and Livak, 2008). GAPDH was used as the internal control. Primers were selected among validated primer sets from the MGH PrimerBank; IDs: Fkbp5 (56753884a1), Tsc22d3 (11907580a1), Nampt (10946948a1), Ppargc1a (6679433a1), Ppara (31543500a1), Rora (1869971a1), Clock (21950741a1), Arntl (4586560a1), Gapdh (6679937a1).

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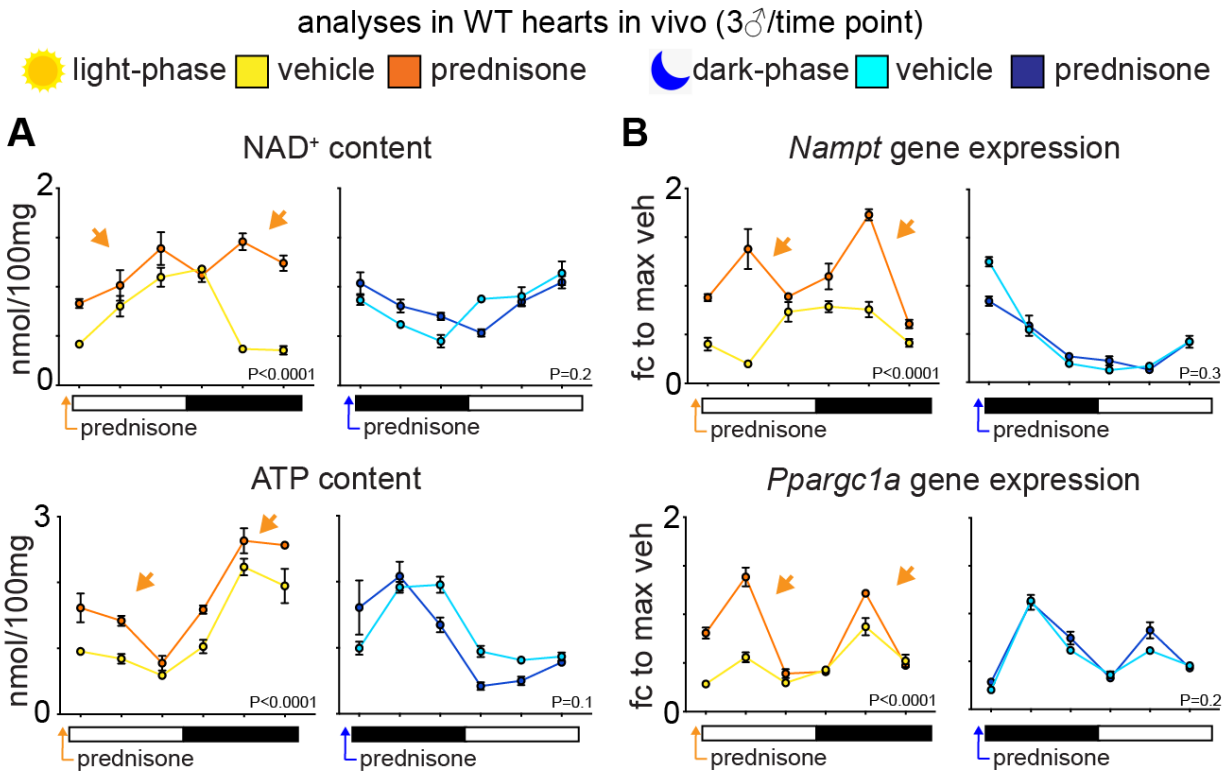

Figure 1. In vivo light-phase injection of one prednisone dose increases myocardial bioenergetic biomarkers during one circadian cycle. Mice were injected with one dose of 1mg/kg i.p. prednisone in vivo and sampled every 4 hours from 1-hour post-injection. Light-phase prednisone (pulse at ZT0) increased NAD+ and ATP content **(A),** as well as *Nampt* and *Ppargc1a* expression **(B)** in hearts in vivo. These effects were blocked with a dark-phase drug pulse (ZT12) Test: 2w ANOVA.

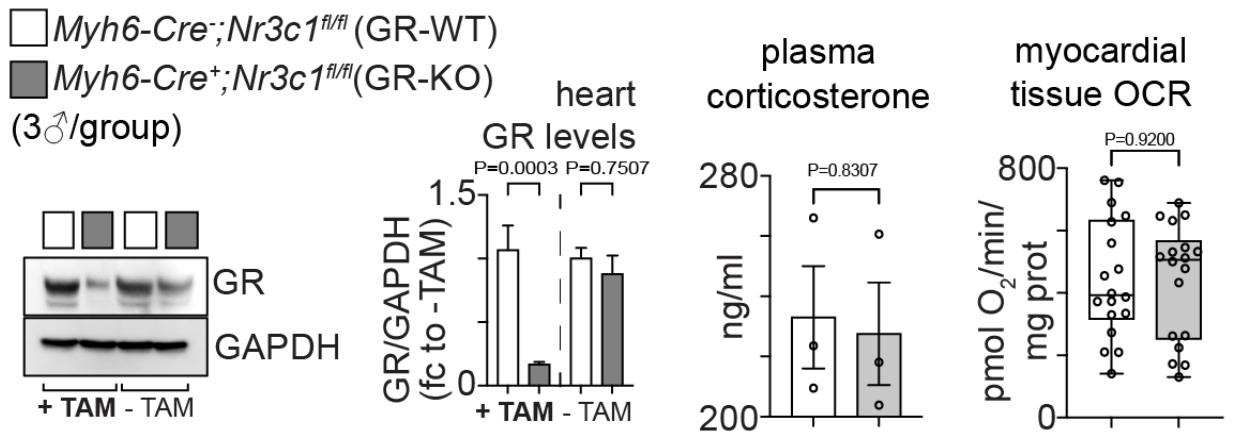

Figure 2. Inducible ablation of GR in post-natal hearts. GR was ablated for ~85% of its WT levels after tamoxifen treatment and washout at 8 weeks of age. Immediately after cardiac-restricted ablation and washout, GR-WT and GR-KO mice did not show significant changes in plasma corticosterone or myocardial mitochondrial respiration at baseline. Tests: 1w ANOVA + Sidak for GR levels, Welch's t-test for corticosterone and OCR.

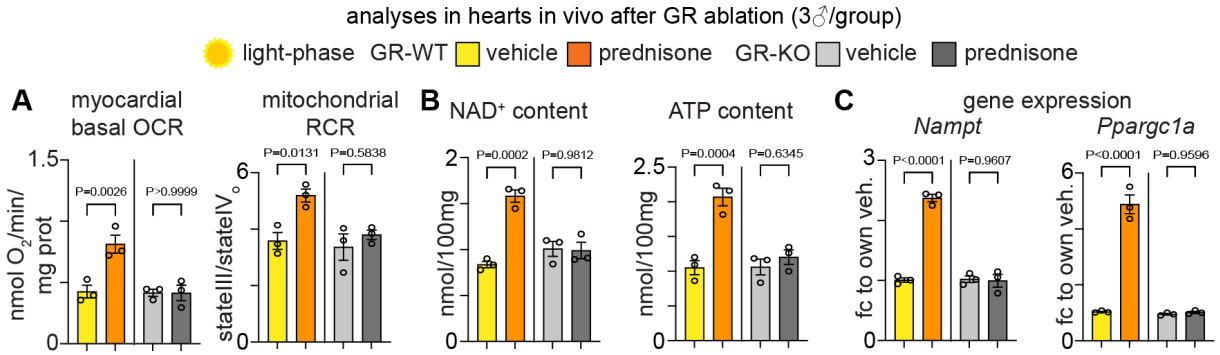

Figure 3. Inducible ablation of GR in the post-natal heart blocks the bioenergetic effects of light-phase prednisone in vivo. A) At 24-hours post-injection, light-phase prednisone increased mitochondrial respiration in GR-WT but not GR-KO hearts, as compared to respective vehicle controls. **B-C)** Analogous trends were found in myocardial content of NAD^+ and ATP, as well as expression levels of *Nampt* and *Ppargc1a*. Test: 1w ANOVA + Sidak.

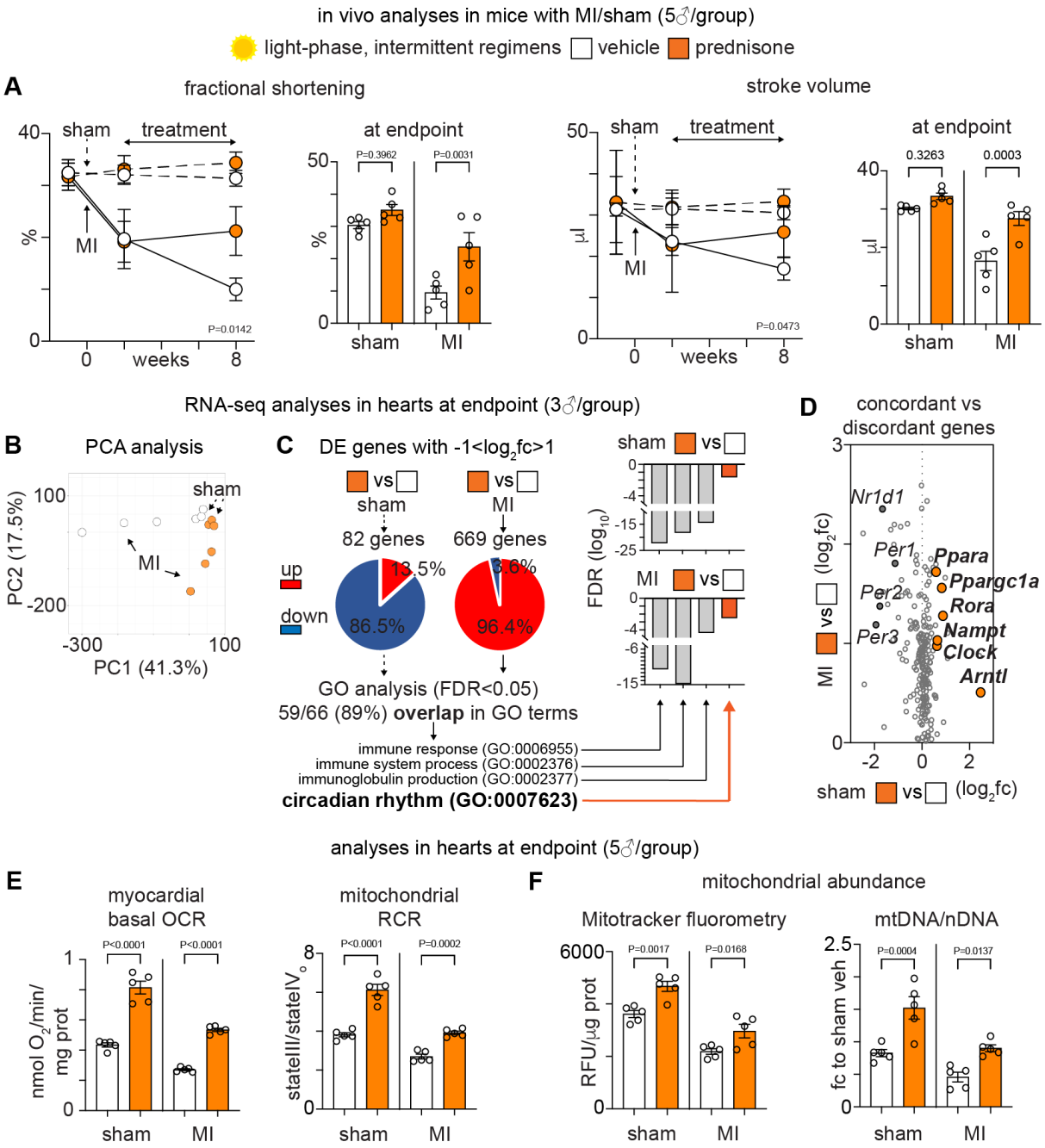

Figure 4. Chronic treatment with light-phase intermittent prednisone improves heart function and metabolism after myocardial infarction. A) WT mice were randomized into sham or myocardial infarction (MI) cohorts and treated with once-weekly prednisone/vehicle through light-phase 1mg/kg doses for 6 weeks from 2-weeks post-MI. Treatment improved fractional shortening and stroke volume in mice with MI. **B)** PCA analysis of RNA-seq profiles showed treatment-associated clustering in both sham and MI hearts. **C)** Differentially expressed (DE) genes in treated versus control hearts showed 89% overlap in GO analysis between sham and MI conditions. Among overlapping GO terms were inflammation and circadian rhythm pathways. **D)** Chart presenting genes with concordant versus discordant regulation by treatment in sham versus MI conditions. Concordant genes (right side) were upregulated by treatment in both sham and MI hearts. Activating clock complex genes (*Arntl*, *Clock*, *Rora*), as well as metabolic regulators *Nampt*,

Ppargc1a and *Ppara* are among concordant genes. Discordant genes (left side) were upregulated by treatment in MI hearts but downregulated in sham hearts. Repressive clock complex genes (*Nr1d1*, *Per1-3*) were among discordant genes. **E-F)** Treatment increased mitochondrial respiration (tissue OCR and mitochondrial RCR) and mitochondrial abundance (Mitotracker fluorometry in myocardial tissue and mtDNA quantitation) in both sham and MI hearts. Tests: 1w ANOVA + Sidak for histograms, 2w ANOVA + Tukey for curves, Benjamini-Hochberg for GO term FDR.

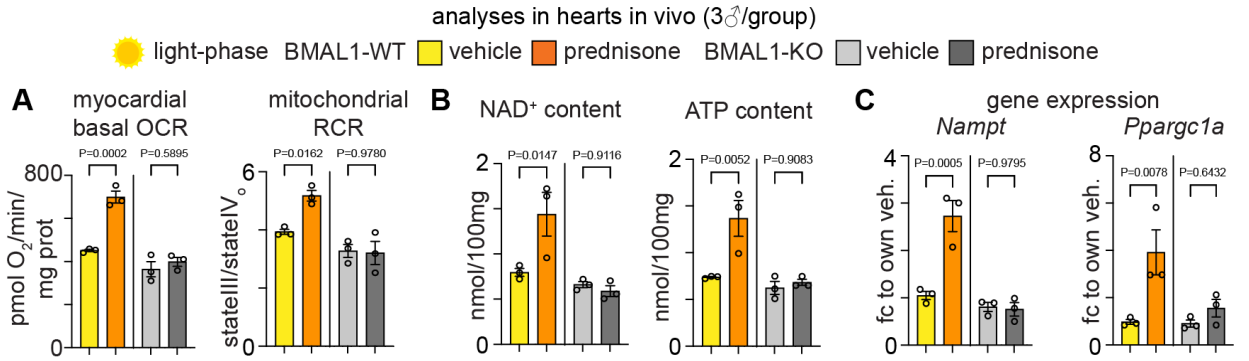

Figure 5. Light-phase prednisone fails to induce acute bioenergetic effects in hearts from BMAL1-KO mice. At 24-hours post-injection, compared to the drug effect in BMAL1-WT hearts, genetic deletion of BMAL1 blocked the effects of light-phase prednisone on mitochondrial respiration **(A)**, bioenergetic biomarkers **(B)** and related gene expression **(C)**. Test: 1w ANOVA + Sidak.

Supplementary Table 1 – List of genes with >2 fold-change variation and >10CPM abundance found with treatment versus vehicle comparisons in sham and MI hearts.

Supplementary Table 2 – List of GO terms enriched from DE genes in sham and MI heart comparisons.

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