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PRE-CLINICAL RESEARCH

The Primary Benefits of Angiotensin-Converting Enzyme Inhibition on Cardiac Remodeling Occur During Sleep Time in Murine Pressure Overload Hypertrophy

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Objectives Our objective was to test the hypothesis that there is a significant diurnal variation for the therapeutic benefit of angiotensin-converting enzyme (ACE) inhibitors on pressure-overload cardiovascular hypertrophy.

Background Physiological and molecular processes exhibit diurnal rhythms that may affect efficacy of disease treatment (chronotherapy). Evidence suggests that the heart primarily remodels during sleep. Although a growing body of clinical and epidemiological evidence suggests that the timing of therapy, such as ACE inhibition, alters diurnal blood pressure patterns in patients with hypertension, the benefits of chronotherapy on myocardial and vascular remodeling have not been studied.

Methods We examined the effects of the short-acting ACE inhibitor, captopril, on the structure and function of cardiovascular tissue subjected to pressure overload by transverse aortic constriction (TAC) in mice. Captopril (15 mg/kg intraperitoneally) or placebo was administered at either murine sleep time or wake time for 8 weeks starting 1 week after surgery.

Results TAC mice given captopril at sleep time had improved cardiac function and significantly decreased heart: body weight ratios, myocyte cross-sectional areas, intramyocardial vascular medial wall thickness, and perivascular collagen versus TAC mice given captopril or placebo during wake time. Captopril induced similar drops in blood pressure at sleep or wake time, suggesting that time-of-day differences were not attributable to blood pressure changes. These beneficial effects of captopril were correlated with diurnal changes in ACE mRNA expression in the heart.

Conclusions The ACE inhibitor captopril benefited cardiovascular remodeling only when administered during sleep; wake-time captopril ACE inhibition was identical to that of placebo. These studies support the hypothesis that the heart (and vessels) remodel during sleep time and also illustrate the importance of diurnal timing for some cardiovascular therapies. (J Am Coll Cardiol 2011;57:2020–8) © 2011 by the American College of Cardiology Foundation

Biological and physiological rhythms in mammals, including humans, play an important role in health and disease. Molecular clocks appear to exist in practically all cells and have been shown to affect fundamental biological functions (1–5). In peripheral tissues such as the heart, local function and the underlying mRNA expression patterns also display circadian rhythms (5,6). Recent studies have also linked biological clocks to heart disease. For example, myocardial infarction and sudden cardiac death peaks between 6 AM and 10 AM (7,8); also, night-shift workers who experience disrupted circadian rhythms have increased risks of heart disease (9).

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Biological rhythms also provide an opportunity to enhance therapeutic efficacy and perhaps minimize risk through appropriate timing of treatment. Timed-release strategies are being developed in diabetes, for example, to modulate the release of insulin based on biosensor assessment of blood glucose levels (10). Recent reports suggest that timing of chemotherapy can also increase drug efficacy and reduce toxicity in cancer patients (11,12). Consideration of timing of treatment might be particularly relevant in cardiovascular diseases, such as hypertension. In most people, blood pressure "dips" 10% to 20% during the night. A large percentage of hypertensives do not show normal reductions in blood pressure during the night (nondippers), and these patients are particularly susceptible to target organ damage (13–15). Antihypertensive therapies, including angiotensin-converting enzyme (ACE) inhibitors administered at night have been demonstrated to restore the diurnal blood pressure dipping pattern (16,17). However, there are no studies, animal or human, examining the actual benefit of this strategy on target organ damage.

In this study, we examine the diurnal efficacy of the ACE inhibitor captopril in the transverse aortic constriction (TAC) murine model of pressure overload. ACE inhibition has been shown to ameliorate target organ damage in this model (18,19); in addition, targeting the renin-angiotensin-aldosterone system (RAAS) is a clinical mainstay of our approach to patients with hypertension or heart failure or after myocardial infarction (20,21). We chose captopril as the drug of choice because it can be easily administered and has a short half-life, which allows it to be administered in a selectively diurnal manner (i.e., only at sleep time vs. only at wake time); this would allow us to determine whether there was any time dependence to the efficacy of ACE inhibition on target organ damage.

Methods

Animals. All animal work was conducted under the guidelines of the Canadian Council on Animal Care. Male mice (C57BL6J, Jackson Laboratories, Bar Harbor, Maine) were housed under a 12-h light, 12-h dark cycle, with lights on at 7 AM (zeitgeber time, ZT0) and off at 7 PM (ZT12). At 8 to 10 weeks of age, TAC was applied to the descending aorta by placing a 7-0 silk suture with the aid of a 27-gauge hypodermic needle. Sham mice underwent the same surgical procedures except the ligature was not tightened; surgical details have been published previously (22).

Captopril. To investigate the influence of timing of captopril treatment on efficacy in TAC mice, we used the experiment summarized in Figure 1. Mice were subjected to TAC for 9 weeks (1 week of recovery plus 8 weeks of treatment). Treatment consisted of either captopril (C4042, Sigma-Aldrich, Canada) at a dose of 15 mg/kg body weight or vehicle (sterile water), administered either in the morning (sleep time) or evening (wake time) intraperitoneally (0.1-ml bolus injection). See Figure 1 legend for details. All treatments were suspended 24 h before end point measurements to exclude possible acute effects of captopril on the cardiovascular system. Previous studies established that the effects of a single dose of captopril last about 2 to 6 h, and in humans with normal renal function, the half-life is <2 h (23,24).

Abbreviations and Acronyms

ACE = angiotensin-converting enzyme
BP = blood pressure
HW = heart weight
LVEDD = left ventricular end-diastolic dimension
LVESD = left ventricular end-systolic dimension
%FS = percent fractional shortening
RAAS = renin-angiotensin-aldosterone system
TAC = transverse aortic constriction
ZT = zeitgeber time

Echocardiography. Echocardiography was performed using a 13-MHz linear array probe (Sequoia, Acuson, California) on mice anesthetized with 0.75% isoflurane (maintaining dose). Left ventricular end-diastolic diameter (LVEDD) and left ventricular end-systolic diameter (LVESD), LV diastolic anterior and posterior wall thickness (AWT and PWT),

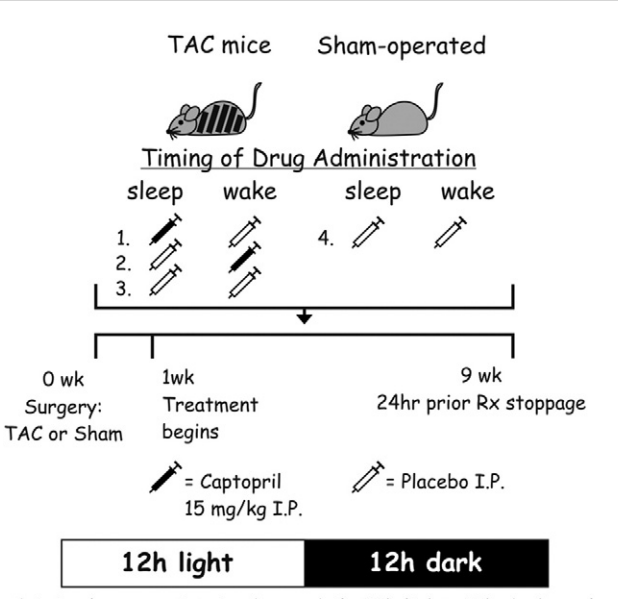

Figure 1 **Longer-Term Benefit of Captopril on Cardiac Remodeling, When the Drug Is Administered at Sleep Versus Wake Time, for 8 Weeks of Dosing**

Group 1, TAC mice: captopril at sleep time (1 h after lights on, zeitgeber time [ZT] 1), placebo at wake time (1 h before lights off, ZT11). Group 2, transverse aortic constriction (TAC) mice: placebo sleep time, captopril wake time. Group 3, TAC mice: placebo sleep and wake time. Group 4, sham mice: similar conditions. Treatments were terminated 24 h before sample collection to ensure that captopril with its half-life of 2 to 6 h (23) clears from the system before any experimental measures.

percent fractional shortening (%FS) calculated as: [(LVEDD − LVESD)/LVEDD × 100], and heart rate were measured. All studies were performed in a blinded manner at fixed times.

Radiotelemetry. PA-C10 murine telemetry transmitters (Data Sciences International, St. Paul, Minnesota) were used to monitor and collect blood pressure data from conscious, freely moving TAC and sham mice (n ≥3) for 7 days. Animals were administered captopril at either the onset of sleep or wake time, and blood pressure changes were monitored over 1 week. Data were analyzed using Dataquest A.R.T. (Data Sciences International), with samples taken every 5 min for 30 s and then averaged for each hour of the day.

Tissue collection for gravimetric and histological analyses. Animals were sacrificed at the same time of day (ZT02 to ZT04), under isoflurane anaesthetic, and KCl was injected into the ventricle to arrest the heart in end diastole. Hearts collected for pathology were weighed and then placed into 10% formalin. Multiple sections were evaluated at the level of the mitral valve. Masson Trichrome staining was used to evaluate myocyte cross-sectional area, intramyocardial vessel medial wall area, perivascular fibrosis, and lumen area with the aid of ImageJ (National Institutes of Health, Bethesda, Maryland).

mRNA measurements. A second identical set of littermates not subjected to heart function studies were used. Animals were sacrificed by cervical dislocation every 4 h for 1 complete diurnal cycle (n = 3/timepoint/group), beginning at 1 h before sleep time (ZT23). RNA was extracted using TRIzol reagent (Invitrogen, Canada), and quantity and quality were verified by A260/280-nm measurements (Agilent 2100 Biosystem, Mississauga, Ontario, Canada) and on a 1% agarose-formaldehyde

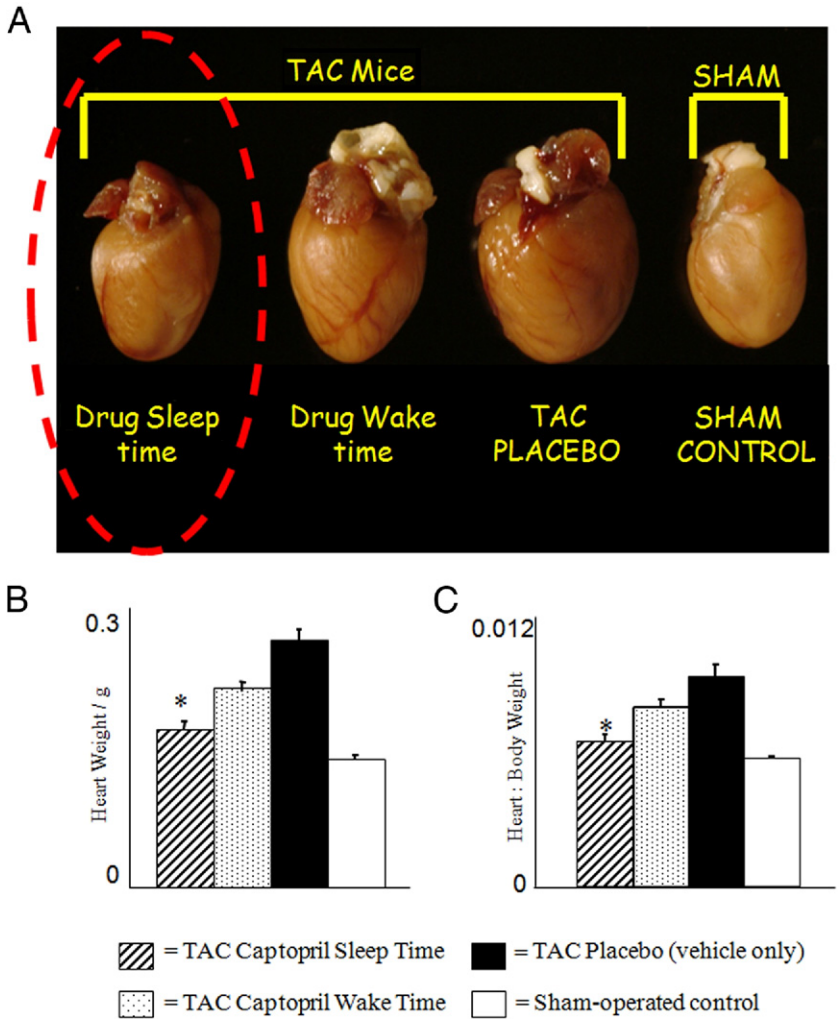

Figure 2 Cardiac Morphometry, Gross View of Heart

(A) Left to right: transverse aortic constriction (TAC), captopril sleep time; TAC, captopril wake time; TAC, placebo (no drug); sham control. TAC mice given captopril at sleep time exhibited decreased heart size versus other TAC groups (were most similar to sham control). In contrast, TAC mice given captopril at wake time had significantly enlarged hearts (most similar to TAC placebo). **(B)** Heart weight, **(C)** Heart/body weight ratio demonstrating that the most significant reduction in heart size was in TAC mice treated with captopril at sleep time, consistent with the other morphometric findings noted above. *$p < 0.005$, mean ± SEM, n = 10 mice/group.

denaturing gel. ACE cardiac gene expression and circadian gene rhythmicity (period, bmal1) were examined by real-time polymerase chain reaction (ABI7000 sequence detection system, Applied Biosystems, StreetsVille, Ontario, Canada) (Online Appendix).

Statistical analyses. Data are expressed as mean ± SEM. Statistical comparisons were made either by use of the independent student *t* test for comparing individual groups or by 1-way analysis of variance followed by Tukey multiple test for comparisons of more than 2 groups. Analysis is performed using SPSS statistical software (version 12.0.0, SPSS, Chicago, Illinois). Results of $p < 0.05$ are considered statistically significant.

Results

Hearts from TAC mice in the placebo group had clear evidence of cardiac hypertrophy compared with those in the sham group as indicated by increased heart size and elevated heart weight (HW) ($p < 0.005$) (Fig. 2). Captopril treatment during sleep time but not wake time reduced ($p < 0.005$) HW as well as HW-to-body weight ratios induced by TAC compared with the placebo-treated group.

To examine whether these time-dependent differences in HW-to-body weight ratios correlated with in vivo structural and functional differences, we performed echocardiography. Figure 3 and Table 1 show that, compared with TAC

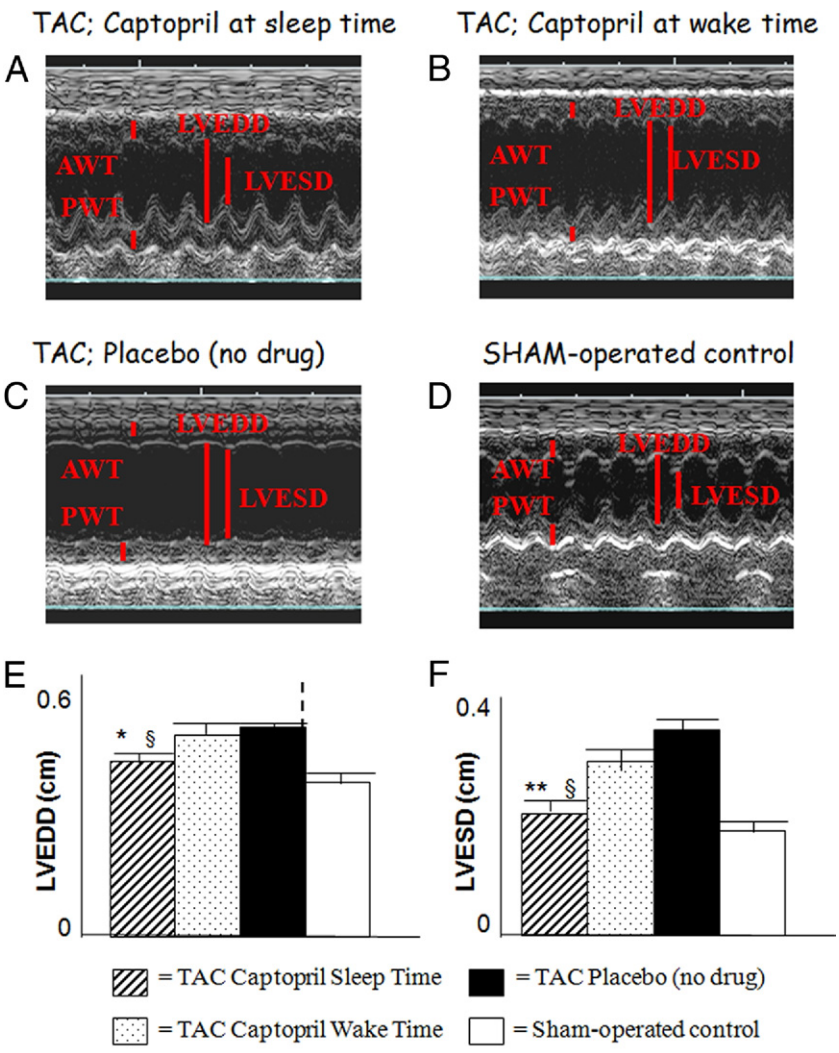

Figure 3 Cardiac Indexes Indices Using Representative Echocardiographic Images

(A) Transverse aortic constriction (TAC), captopril sleep time, **(B)** TAC, captopril wake time, **(C)** TAC, placebo, **(D)** sham control. Measurements shown are left ventricular end-systolic dimension (LVESD) and left ventricular end-diastolic dimension (LVEDD), anterior wall thickness (AWT) and posterior wall thickness (PWT). **(E)** LVEDD was reduced (*$p < 0.05$) in TAC mice given captopril at sleep time versus TAC placebo and versus TAC captopril wake time. **(F)** LVESD was reduced in TAC captopril mice treated at sleep time versus TAC placebo (**$p < 0.005$) and versus TAC captopril wake time (§$p < 0.05$). Mean ± SEM, n = 10 mice/group.

Table 1 Pathophysiology of Pressure-Overload TAC Heart in Mice Given Captopril Chronotherapy at Sleep Versus Wake Time

	TAC (Banded) Mice			
	Captopril Sleep Time (n = 10)	Captopril Wake Time (n = 10)	Placebo (n = 10)	Sham Mice, Placebo (n = 10)
HR (beats/min)	567.0 ± 16.8	582.9 ± 11.1	563.7 ± 7.6	545.2 ± 13.3
AWT (cm)	0.07 ± 0.00	0.08 ± 0.00	0.08 ± 0.00	0.07 ± 0.00
PWT (cm)	0.07 ± 0.00	0.07 ± 0.00	0.08 ± 0.00	0.06 ± 0.00
LVEDD (cm)	0.40 ± 0.01*	0.46 ± 0.02	0.48 ± 0.02	0.36 ± 0.01
LVESD (cm)	0.20 ± 0.01*	0.29 ± 0.02	0.34 ± 0.03	0.17 ± 0.01
FS (%)	50.0 ± 1.8*	39.2 ± 3.4	31.1 ± 3.6	51.6 ± 1.40

Values are mean ± SEM. *$p < 0.05$ TAC mice treated with captopril at sleep time versus TAC placebo group.
AWT = anterior wall thickness; %FS = percent fractional shortening; HR = heart rate; LVEDD = left ventricular end-diastolic dimension; LVESD = left ventricular end-systolic dimension; PWT = posterior wall thickness; TAC = transverse aortic constriction.

placebo, the TAC mice treated with captopril at sleep time had reduced ($p < 0.05$) LVESD (0.20 ± 0.01 cm vs. 0.34 ± 0.03 cm) and LVEDD (0.40 ± 0.01 cm vs. 0.48 ± 0.02 cm), accompanied by increased ($p < 0.05$) fractional shortening (50.0 ± 1.8% vs. 31.1 ± 3.6%). Consistent with heart size measurements, captopril treatment of TAC mice at wake time provided less ($p > 0.05$) benefit on LVESD, LVEDD, and %FS than TAC mice treated at sleep time.

To establish whether functional improvements were associated with reduced myocardial fibrosis and structural remodeling, histological analysis was performed. As expected, Figure 4 (4A to 4D and 4I) reveals that myocyte size was increased ($p < 0.005$) in TAC mice (207.6 ± 14.0 μm^2) compared with sham mice (110.0 ± 3.4 μm^2). TAC also increased ($p < 0.05$) wall-to-lumen area ratios (0.90 ± 0.13) of large arterial vessels compared with sham (0.64 ± 0.04) (Figs. 4E to 4H and 4J). More important, cardiac histology revealed decreased ($p < 0.005$) hypertrophy in TAC-captopril mice treated during sleep time (140 ± 10 μm^2) compared with TAC placebo (207.6 ± 14.0 μm^2). Reductions in myocyte size with captopril sleep-time treatment were correlated with thinner ($p < 0.05$) medial and adventitial areas, quantified by wall-to-lumen area ratios (0.90 ± 0.13 vs. 0.60 ± 0.14). As might be expected from the functional data, myocyte hypertrophy (195 ± 11 μm^2) and wall-to-lumen area ratio (0.80 ± 0.11) were not significantly reduced ($p > 0.05$) in TAC mice given captopril during waking hours.

Because differences between groups noted above could be related to the blood pressure (BP) responses for the mice to captopril, we next measured diurnal BP patterns. As expected, TAC mice showed significantly higher BP profiles compared with sham mice (Fig. 5A), and both groups reduced BP at night (i.e., dipper profile). Administration of captopril to TAC mice at sleep time resulted in reduced systolic pressures that followed a clear pattern, wherein the reduction was greatest within the first 2 h of drug injection and reductions declined thereafter, consistent with the short-acting nature of captopril (Figs. 5B and 5C, Online Appendix) (23,24). These results indicate that the sleep-time benefits for cardiac remodeling are not directly attributable to BP response alone.

Because a number of diseases show diurnal molecular influences and because organs themselves show daily circadian molecular rhythms, we next explored whether TAC may disrupt normal diurnal patterns of cardiac gene expression and whether captopril might influence gene rhythms depending on the time of administration. We found that the core circadian clock genes in the myocardium were not changed by either the TAC procedure or captopril, regardless of timing (Figs. 6A and 6B). Specifically, mRNA levels of key circadian oscillatory genes, *mper2* mRNA and *mbmal1*, were unaffected by TAC-induced mechanical overload, consistent with our previous results (22), or by captopril treatment. An alternative influence that might contribute to the time-dependent benefit of captopril is the diurnal rhythm in RAAS signaling pathways, as proposed. Consistent with a previous study (25), our microarray results (Fig. 6C) show that ACE myocardial mRNA expression exhibits diurnal fluctuations. In addition, ACE gene expression was increased in TAC mice as compared with sham mice (Fig. 6C). Importantly, ACE mRNA expression increased ($p < 0.05$) in TAC mice compared with sham controls immediately before entering the sleep time (ZT23/ZT03) period (TAC, 122.93 ± 28.76 vs. sham, 42.55 ± 14.74). Conversely, ACE myocardial mRNA expression did not differ ($p > 0.05$) in TAC mice as they entered the wake time (ZT11/ZT15) period (TAC, 143.38 ± 23.17 vs. sham, 100.53 ± 25.53). Taken together, these results show increased levels of ACE mRNA at sleep time, which is precisely when the ACE inhibition with captopril was found to be beneficial.

Discussion

In this study, the short-acting ACE inhibitor, captopril, was used to test the hypothesis that the efficacy of ACE inhibitors depends on the time of treatment. We found, in a murine model of pressure overload, that captopril administered to mice only at sleep time provides protection against cardiac remodeling, whereas captopril given at wake time had no measurable benefit and was identical to placebo. Our

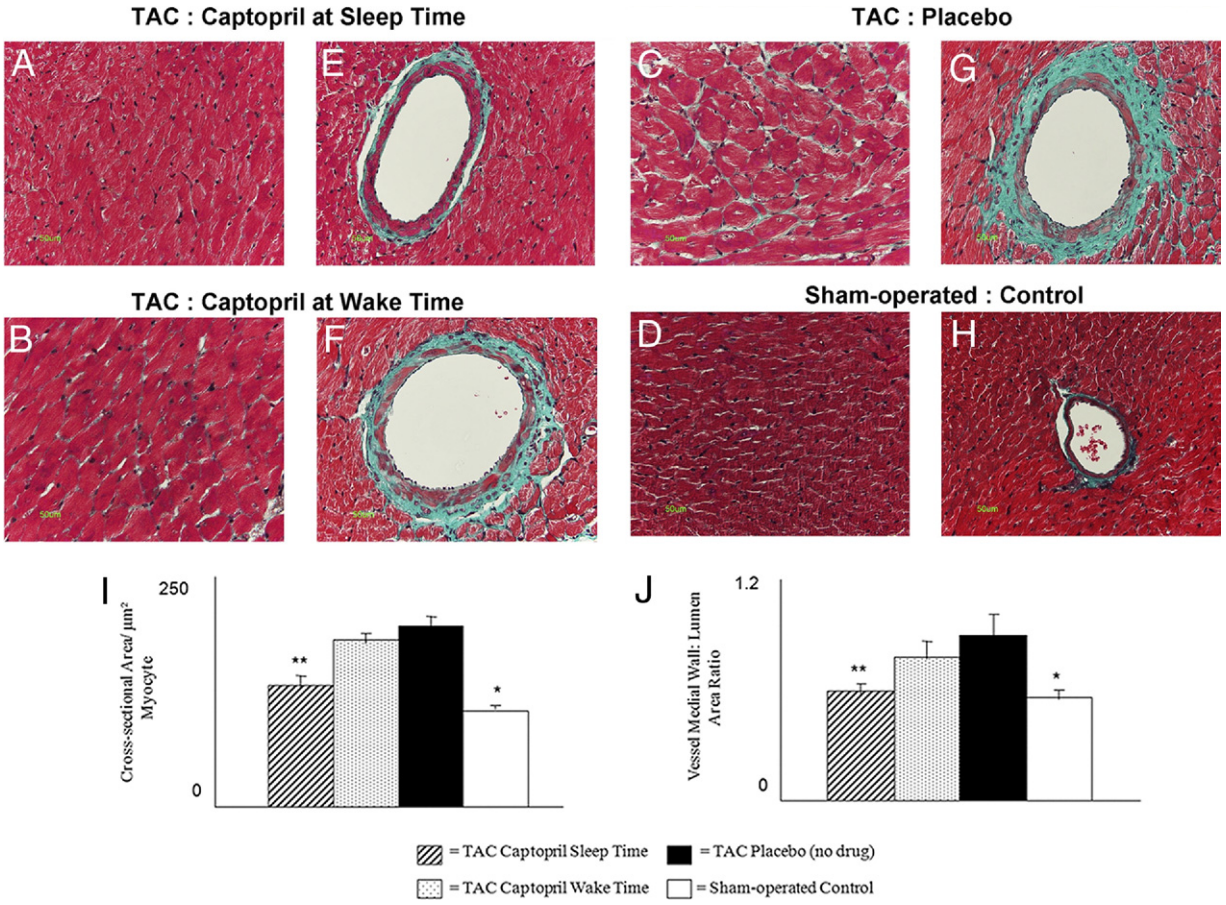

Figure 4 **Cardiovascular Histology Demonstrating Reduced (Beneficial) Remodeling in Hearts of TAC Mice Treated With Captopril at Sleep Time**

(A to D) Myocellular hypertrophy follows a similar pattern as with overall cardiac and left ventricular hypertrophy. That is, **(A)** transverse aortic constriction (TAC) captopril sleep time hearts show the least cellular hypertrophy, most similar to sham-operated controls. **(B)** In contrast, TAC captopril wake time or **(C)** placebo hearts exhibit the greatest levels of myocyte hypertrophy. **(D)** Sham. **(E to H)** Perivascular fibrosis in the myocardium followed the similar and consistent pattern. **(E)** TAC captopril sleep time, **(F)** TAC captopril wake time, **(G)** TAC placebo, and **(H)** sham. Magnification 20×, scaled at 50 μm, Masson's Trichrome stain. **(I)** Quantification of myocyte cross-sectional area. *p $<$ 0.005 TAC placebo versus sham. **p $<$ 0.005 TAC captopril sleep time versus TAC captopril wake time. **(J)** Intramyocardial vessel medial wall area to lumen area ratio. *p $<$ 0.05 TAC placebo versus sham. **p $<$ 0.05 TAC captopril sleep time versus TAC captopril wake time. Mean ± SEM, n = 5 mice/group.

results further show that the time dependence of the benefits of captopril coincided with an observed diurnal increase in the myocardial expression of the key RAAS gene, ACE. The beneficial effects of administering ACE inhibitors at sleep time may be mediated, at least in part, by interfering with the peaking actions of RAAS on cardiovascular remodeling. The inclusion of molecular markers of cardiac dysfunction (e.g., atrial natriuretic factor, myosin heavy chain beta) and diurnal cycling of tissue ACE activity may also aid in understanding patient benefit to chronotherapy. Moreover, these diurnal benefits may be particularly apparent for ACE inhibitors like captopril, which are short-acting agents with little or no bioavailability 12 h after administration (i.e., half-life 2 to 6 h) (23,24). In a corollary to our experiments with ACE inhibition, the cardiac response to a very short-term elevation of BP by intraperitoneal injections of angiotensin II in rats during sleep time was compared with the response to 24-h angiotensin infusion to the same peak blood pressure. In spite of the great differences in exposure to angiotensin II, both groups exhibited the same cardiac enlargement (26).

The benefit conferred by captopril when administered at sleep time could be mediated partially via enhanced changes in blood pressure. ACE inhibition is anticipated to drop blood pressure, and these changes are expected to be the greatest when ACE inhibitors are administered at the time when RAAS signaling peaks (i.e., at sleep time), thus inducing nocturnal dips in blood pressure. Previous studies have documented the impact of "dipper versus nondipper profiles" in human hypertensives on cardiovascular morbidity, with hypertensive nondippers experiencing greater target organ damage (13,14). We first postulated that dipping may be more pronounced at night time and that nondipping

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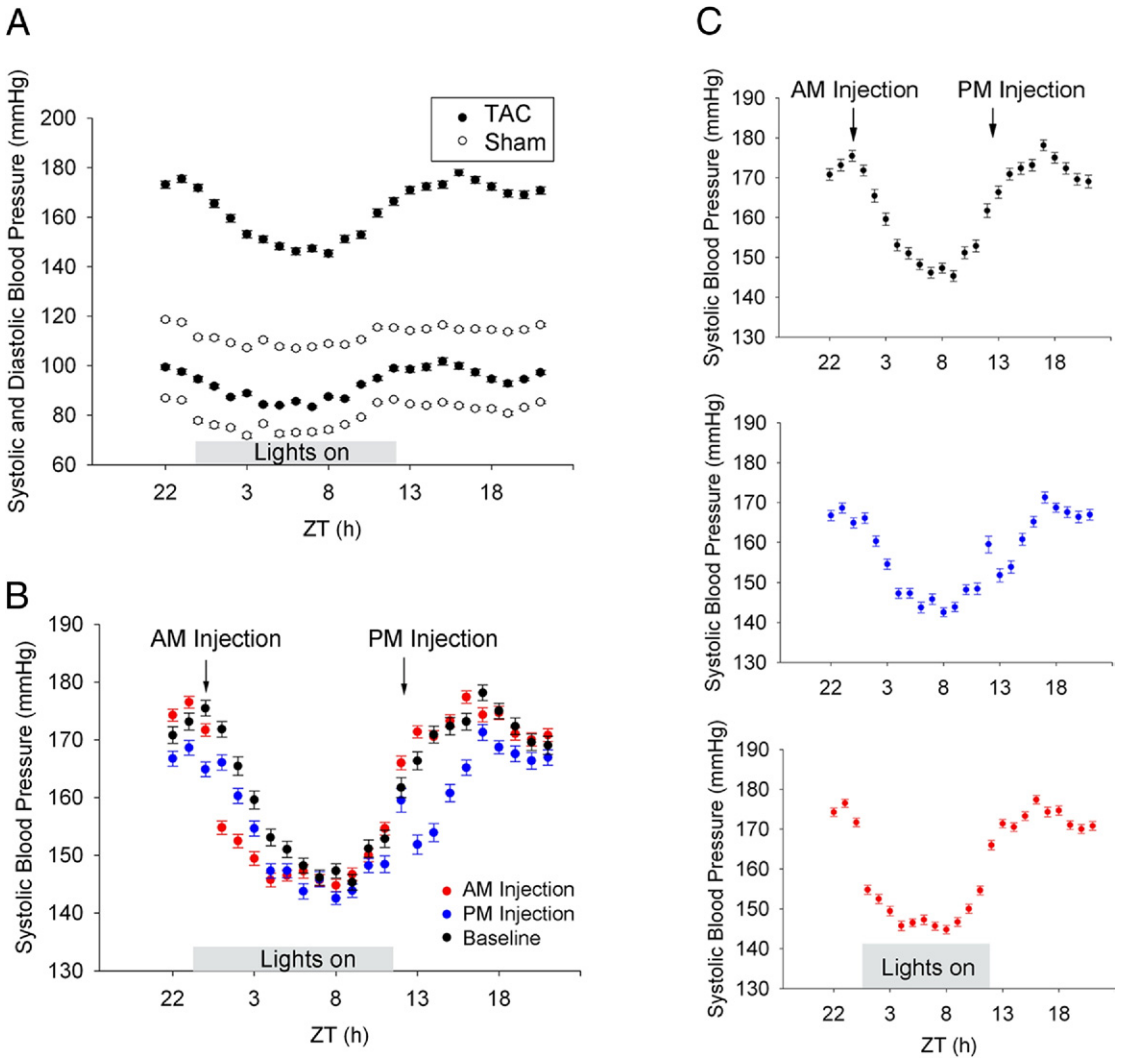

Figure 5 Diurnal Blood Pressure Profiles by Radiotelemetry

(A) Average systolic **(upper solid circles)** and diastolic **(lower solid circles)** blood pressure rhythms for transverse aortic constriction (TAC) and sham **(open circles)** plotted versus 24-h daily time. Each point is mean ± SEM of n ≥ 3 mice/group. **Bottom bar** denotes lights on (murine sleep time). TAC mice show significantly increased blood pressure versus sham; both groups maintain the dipper profile. **(B)** Average systolic blood pressure for TAC mice administered captopril at sleep time (AM injection) or wake time (PM injection). **(C)** Conserved systolic blood pressure rhythms in TAC mice and after captopril injection at sleep or wake time.

may have occurred because the ACE inhibitors are administered at times when they would have had their smallest effects (i.e., during daytime); however, the radiotelemetry diurnal BP measurements indicated that this did not occur and was not a likely explanation for the benefits on cardiac remodeling.

Finally, we have hypothesized that cardiovascular growth and remodeling are rhythmic and occur predominantly during sleeping hours (1,27); these data support that hypothesis. In the murine heart, neither sleep-time nor wake-time captopril altered the cyclic expression profile of the core circadian clock genes, *per2* and *bmal,* consistent with there being a diurnal target underlying the beneficial response. It is worth noting, however, that angiotensin II has been reported to shift *per2* and *bmal* gene expression in cultured vascular smooth muscle cells in vitro (28). Although a similar shift was not observed in our TAC model in vivo, the possibility exists that human clinical samples from heart disease where angiotensin II levels are altered could still show minor differences.

Conclusions

Transverse aortic constriction is a commonly used and useful experimental model in rodents for studying the adverse effects of cardiac remodeling leading to heart failure. Models such as these allow the exploration of novel

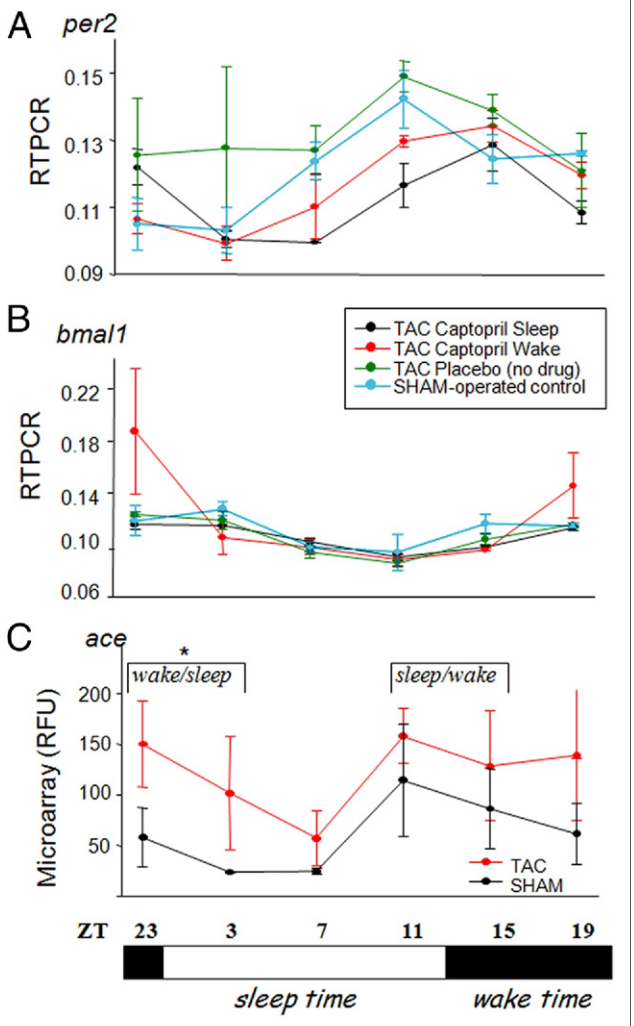

Figure 6 **Diurnal Gene Expression in Heart**

Core molecular circadian clock genes cycle in a predictable manner for all experimental groups, thus facilitating comparisons between transverse aortic constriction (TAC) mice with similar endogenous molecular physiology, but given captopril drug at different treatment (sleep vs. wake) times. **(A)** Real-time polymerase chain reaction (PCR) of the core circadian clock gene *mper2*. Diurnal expression pattern peaks at the sleep–wake barrier in all groups. **(B)** Real-time PCR of *mbmal*; diurnal expression troughs at sleep–wake barrier time. The complementary gene rhythms of *mper2* and *mbmal* are consistent with their expression on opposite arms of the 24-h transcription/translation autoregulatory feedback loop. **(C)** Diurnal expression of angiotensin converting enzyme mRNA rhythm in TAC **(red line)** versus sham **(black line)** murine heart significantly increases ($p < 0.05$) across wake to sleep time. The image is derived from microarray data validated as described (22); this image has not been published previously. Mean ± SEM, n = 3 mice/group. **Open bar** = lights on, animal sleep time; **solid bars** = lights off, animals awake.

approaches to clinical therapy. The application of chronotherapeutic principles to human disease would of course require tailoring; administration of captopril to rodents in the morning should mimic the treatment of humans at night (i.e., both sleep-time treatments). Moreover, we anticipate that future studies will show similar chronotherapeutic variation in the effectiveness of ACE inhibition to benefit myocardial remodeling after myocardial infarction and in congestive failure. This would open the possibility for more effective antagonism of RAAS in patients in whom therapy has been limited by side effects through the use of shorter acting agents. In addition, our observations may help to explain the success of the HOPE (Heart Outcomes Prevention Evaluation) trial in humans (29,30); in this study, the ACE inhibitor ramipril was only given at bedtime. Similarly, in the CAMELOT (Comparison of Amlodipine vs Enalapril to Limit Occurrences of Thrombosis) trial (31), patients were treated in the morning with either amlodipine, a Ca2+ channel blocker with a very long pharmacological half-life, or enalapril, an ACE inhibitor with a shorter half-life. Amlodipine had a far greater benefit in reducing adverse cardiovascular events. The diurnal timing of the therapy did not appear to be a consideration in this trial; our results suggest that enalapril may have been equally effective in such patients if it had been given instead at sleep time.

Our results also suggest that disregard for diurnal rhythms may explain differences in responses to therapy seen between nocturnal animal models, such as rodents during drug development versus human patients in clinical trials; in rodents, new agents are usually tested during the day (rodent sleep time) for clinical trial administration to humans also during the day, when they are not asleep, but active and awake. Contemporary therapeutic strategies usually target a pathway or receptor over the full 24 h using either multiple doses or slow release or long half-life drugs. These are often taken in the morning for convenience with little consideration for biological rhythms and varying benefit. Drug administration at a chronobiologically inappropriate time (or perhaps using a drug with a long half-life) may unnecessarily expose the patient to the risk of adverse effects with minimal benefit.

These experiments demonstrate that there is significant potential for improving on therapeutic outcome even with drugs with a long history of therapeutic experience, such as ACE inhibitors. Investigating the diurnal risk/benefit profile of cardiovascular drugs is a fruitful area for contemporary research. We hope that clinical extension of these studies will provide new opportunities for the effective treatment of cardiovascular disease.

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Key Words: angiotensin-converting enzyme inhibitors ■ cardiac remodeling ■ chronotherapy ■ circadian ■ diurnal.

APPENDIX

For an expanded Methods section, please see the online version of this article.