Vascular Disease in Mice With a Dysfunctional Circadian Clock

Ciprian B. Anea, MD; Maoxiang Zhang, PhD; David W. Stepp, PhD; G. Bryan Simkins, BS; Guy Reed, MD; David J. Fulton, PhD; R. Daniel Rudic, PhD

Background—Cardiovascular disease is the leading cause of death for both men and women in the United States and the world. A profound pattern exists in the time of day at which the death occurs; it is in the morning, when the endothelium is most vulnerable and blood pressure surges, that stroke and heart attack most frequently happen. Although the molecular components of circadian rhythms rhythmically oscillate in blood vessels, evidence of a direct function for the “circadian clock” in the progression to vascular disease is lacking.

Methods and Results—In the present study, we found increased pathological remodeling and vascular injury in mice with aberrant circadian rhythms, Bmal1-knockout and Clock mutant. In addition, naive aortas from Bmal1-knockout and Clock mutant mice exhibit endothelial dysfunction. Akt and subsequent nitric oxide signaling, a pathway critical to vascular function, was significantly attenuated in arteries from Bmal1-knockout mice.

Conclusions—Our data reveal a new role for the circadian clock during chronic vascular responses that may be of significance in the progression of vascular disease. (Circulation. 2009;119:1510-1517.)

Key Words: circadian rhythm ■ endothelium ■ remodeling ■ thrombus ■ vasculature

The cardiovascular system behaves rhythmically over the course of a day,1–5 coordinating tissue perfusion in accordance with oscillating metabolic and functional demands. These oscillations, which occur in blood vessels as variations in contractility and blood pressure, follow a distinctive temporal pattern—a circadian rhythm. Aberrations to circadian rhythm meet with pathological consequences. Shift work provokes a 40% increase in the risk of cardiovascular disease,4 and disturbance of daily blood pressure rhythms elevates the incidence of vascular disease.5,6 In addition, the onset of acute vascular events such as myocardial infarction7 and stroke8 also exhibits circadian variation. However, direct evidence to implicate the molecular components of circadian rhythms in the chronic progression of disease is lacking.

The molecular components that generate circadian rhythms—the circadian/biological clock—constitute a unique collaboration of genes and proteins that govern by virtue of transcriptional, translational, and posttranslational mechanisms. The transcriptional driving force is composed of the basic helix-loop-helix transcription factors Bmal1 and Clock (or Npas2). Bmal1/Clock or Bmal1/Npas2 heterodimerize to transactivate Period (Per) and Cryptochrome (Cry), the braking force of the loop, which then restrain Bmal1/Clock/Npas2 and, consequently, their own transcription. Further modification of the core complex, including phosphorylation and degradation, refines timing to the daily cycle.9 Indeed, vascular cells10,11 contain all necessary components of this unique molecular metronome. Although recent data have implicated the circadian clock in aspects of acute vascular function,12–14 no data directly implicate Bmal1 or Clock in the chronic process of vascular disease, and even less is known on the downstream mechanisms involved.

Methods

Animals

All animal studies were performed according to protocols approved by the Medical College of Georgia Institutional Committee for Use and Care of Laboratory Animals. Bmal1-knockout (Bmal1-KO) mice were housed under standard 12-hour light/dark (LD) conditions. Clock mutant (Clockmut) mice were held in either LD or constant dark (DD) conditions for 6 weeks (1 week acclimation, 5 weeks experimentation) as indicated. Bmal1-KO and littermate wild-type (WT) mice were formerly produced by gene targeting in 129Sv/J embryonic stem cells15 that we backcrossed 3 times to C57BL/6J. Clock heterozygote mutant mice that served as parental strains for mutants and control WTs in respective studies were purchased from The Jackson Laboratory (Bar Harbor, Me) (C57BL/
Vascular Disease and the Circadian Clock

Anea et al

6J-Clock<sup>fl/fl</sup>/J, stock No. 002923), formerly generated by ENU mutagenesis in C57BL/6J mice causing an A to T transversion at the third-base position of the 5’ splice donor site of intron 19 of Clock. We conducted studies in isolated aortic arteries.

Materials

P-endothelial nitric oxide synthase (eNOS) (1177), phosphoinositide-dependent kinase 1 (PDK1), and Akt1 polyclonal antibodies were purchased from Cell Signaling (Danvers, Mass); GAPDH monoclonal antibody from Ambion; P-Akt (threonine-308), eNOS, and plasminogen activator inhibitor-1 (PAI-1) antibodies from BD Transduction Labs; PAR4 peptide from Advanced ChemTech; and PPACK from Calbiochem.

Functional Studies in Isolated Aortic Arteries

Seven- to 10-week-old (young) Bmmall-KO mice or age-matched littermate WT controls were anesthetized with ketamine/xylazine and subsequently exsanguinated. Residual blood was removed by perfusing physiological saline by cardiac puncture. The thoracic aorta was carefully dissected free and excised from the aortic arch to the point of the diaphragm. To avoid damage to the endothelium, perivascular fat was carefully dissected, and the aorta was cut into rings (2-mm thickness) for placement into organ chambers containing Krebs buffer maintained under physiological conditions. The composition of Krebs-Henseleit solution (in mmol/L) was as follows: NaCl 118.3, KCl 2.5, MgSO<sub>4</sub> 1.2, KH<sub>2</sub>PO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 25, dextrose 5.6, equilibrated with 95% O<sub>2</sub>/5% CO<sub>2</sub> to maintain pH of 7.4 at 37°C. The rings were suspended by 2 tungsten wires (25-μm diameter) and mounted in a vessel myograph system (chamber size 6 mL, Multi Myograph, Danish Myo Technology [DPT, Atlanta, Ga]). Isometric tension was measured with a force transducer coupled to data acquisition system. A resting tension of 1.0 g was used throughout the experiments. After an equilibration period of 60 minutes (during which time Krebs-Henseleit solution was changed every 10 minutes and the resting tension was readjusted), rings were precontracted with phenylephrine until a plateau was reached. Vessels were then washed with Krebs-Henseleit solution and this was repeated at least 3 times to stabilize the tissue. Aortas were then precontracted with phenylephrine and concentration-dependent responses to the endothelium-dependent agonist acetylcholine (1×10<sup>−9</sup> to 5×10<sup>−7</sup> mol/L) and the endothelium-independent nitric oxide donor sodium nitroprusside (1×10<sup>−8</sup> to 10<sup>−4</sup> mol/L).

Flow-Dependent Vascular Remodeling and Femoral Artery Injury

The complete left common carotid artery ligation was performed as described previously. Briefly, the distal left common carotid artery and its bifurcation into the external and internal carotid were exposed with the use of blunt dissection. Nylon sutures (8-0) (USSC Sutures, Microsurgery Instruments Inc, Bellaire, Tex) were used to ligate the left common carotid artery, just proximal to the external/internal carotid artery bifurcation. For intravascular wire injury, the left femoral artery of mice was repeatedly cannulated (5 to 7 times) by a straight wire (0.38 mm in diameter; No. C-SP-15-15, Cook Inc, Bloomington, Ind) as described previously.6 Left common carotid artery ligation and femoral artery injury were performed at times of day as indicated. Incisions were closed (5-0 suture), and mice were left to recover for 5 weeks or 1 week, after which mice were euthanized at the same time of day at which the procedure was initiated.

Histomorphometry

After 5 weeks of flow reduction induced by left common carotid artery ligation, mice were anesthetized, exsanguinated, and perfused via the left ventricle with physiological saline. In processing vascular tissues for Western blotting, common carotid arteries or aortas were immediately dissected, flash-frozen, and stored at −80°C until further processing. In animal studies designed for histological/histomorphometric analysis of common carotid arteries, after saline infusion, mice were subsequently perfusion-fixed with neutral buffered formalin. Both right and left common carotid arteries were carefully excised and post-fixed overnight for morphometric studies or immediately embedded in frozen medium for cryo-temperate processing. Morphometric analysis of carotid arteries was performed with video microscopy as described. Perimeter (p) of the vessel lumen was taken as the circumference (C) of a circle and lumen diameter (D) determined from the equation D=πC/π, assuming that the vessel diameters were circular in vivo. To determine lumen area, the internal elastic lamina and patent lumen were circumscribed to derive a radius (R) value from the formula R=2C/π, and then internal elastic lamina area and luminal area (A) were calculated with the formula A=πR<sup>2</sup>. Thrombus area was derived from the difference of internal elastic lamina area and luminal area.

Whole Blood Analysis of Platelet Activation

Blood was collected via cardiac puncture after carbon dioxide asphyxiation. Approximately 500 to 600 μL blood was drawn through a 27-gauge needle into a 1-mL plastic syringe with 70 μL 3.8% sodium citrate and 10 μmol/L PPACK. Blood was immediately diluted 6-fold with a modified Tyrode’s buffer (137 mmol/L NaCl, 2.8 mmol/L KCl, 1 mmol/L MgCl<sub>2</sub>, 12 mmol/L NaHCO<sub>3</sub>, 0.4 mmol/L Na<sub>2</sub>HPO<sub>4</sub>, 5.5 mmol/L glucose, 1 mmol/L EDTA, 0.35% bovine serum albumin, 10 mmol/L HEPES, pH 7.4). P-selectin expression was monitored by flow cytometry (FACSCalibur, Becton Dickinson, San Jose, Calif). To assess platelet activation, blood was stimulated with PAR4 and subsequently incubated with bivalutamyl rat anti-mouse CD62P antibody or an isotype-matched control antibody followed by phycoerythrin-conjugated streptavidin (immunoreagents from BD Pharmingen, San Diego, Calif) to detect P-selectin by flow cytometry. Fluorescein isothiocyanate-conjugated rat anti-mouse CD41 antibody (BD Pharmingen) was used as a platelet identifier.

Statistical Analysis

The significance of differences was assessed by 2-way ANOVA analysis to test for all sources of variation (genotype versus age or genotype versus light cycle conditions), and subsequent tests were made to assess differences between paired right contralateral artery and left common carotid artery lumen diameter from individual animals by paired Student t tests. Platelet responses and immunoblot densitometry were analyzed for significance by unpaired Student t tests. Error bars show the calculated SEM. Concentration response curves of vascular function were analyzed by repeated-measures 2-way and 1-way ANOVA with a Bonferroni correction. Differences were considered significant at P<0.05.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Pathological Vascular Remodeling and Enhanced Vascular Injury in Mice With a Dysfunctional Circadian Clock

Chronic blood flow alterations that mimic the human condition of vascular disease can be induced experimentally in vivo by surgical ligation of the arterial circulation in animals. These “flow reduction models” simulate the structural adaptation that occurs in obstructed arteries (vascular remodeling) during atherosclerosis and hypertension. We conducted studies to determine whether disruption of the biological clock alters the response of blood vessels to a chronic reduction in blood flow. In young (aged 6 to 10 weeks) littermate control WT mice, reduction of blood flow in the left common carotid artery for a duration of 5 weeks induced wall thickening (Figure 1A) and inward remodeling (Figure 1B and 1C) of the left common carotid artery as shown previously.19 In contrast,
wall thickness of the remodeled left common carotid artery of young Bmal1-KO mice was further increased (Figure 1A) and lumen was impaired in its ability to narrow (inward remodeling) (Figure 1B and 1C), a response reminiscent of that in eNOS KO mice.20 The impairment in vascular remodeling in Bmal1-KO mice was striking; lumen diameters of the remodeled left common carotid artery of old WT (left) reveals a normal, inwardly remodeled vessel with patent lumen, whereas Bmal1-KO (right) exhibits an enlarged lumen undergoing remodeling around the site of thrombus formation (Figure 1E). In arterial regions that were thrombus free, aged Bmal1-KO mice exhibited a paradoxical increase in median diameter of male Bmal1-KO mice did not narrow (young, aged 7 to 10 weeks) or even exhibited a paradoxical increase in diameter (old, aged 25 to 30 weeks) (D) in regions that were free of thrombosis. E, Hematoxylin and eosin staining of remodeled left common carotid artery of old WT (left) reveals a normal, inwardly remodeled vessel with patent lumen, whereas Bmal1-KO (right) exhibits an enlarged lumen undergoing remodeling around the site of thrombus formation (Figure 1E). In arterial regions that were thrombus free, aged Bmal1-KO mice exhibited a paradoxical enlargement of lumen diameter after ligation relative to age-matched littermate controls (Figure 1D) reminiscent of stenosis-induced dilatation.

To determine whether the effects of Bmal1 on vascular remodeling were related to the circadian rhythm function of the endogenous clock, we extended our studies to mice harboring a mutation of the Clock gene (Clockmut). The advantage of this approach is that Clock mutant mice exhibit only a modest phase shift in circadian rhythm under standard LD conditions and become completely arrhythmic when housed under conditions of DD.16 Thus, by modifying circadian cues, we can directly assess the importance of these transcription factors to the relationship between biological rhythms and vascular function. In DD conditions, the left common carotid artery of Clockmut mice (n=4; *P<0.05, Clockmut in DD vs WT in DD) and a DD-dependent impairment in inward remodeling (B). C, Von Gieson staining of elastin fibers revealed enhanced intimal hyperplasia in the left femoral artery of male Clockmut mice undergoing intraluminal wire injury relative to injured arteries of male WT mice that were housed under the same conditions in DD (bar=50 μm). D, Morphometric analysis of femoral artery cross sections revealed an increase in neointimal area and intima-to-medial (I/M) ratio indicative of an exacerbated response to wire injury (n=7 to 10; *P<0.05).

Figure 2. Clock mutant mice exhibit pathological vascular responses. A, After acclimation to either LD or DD conditions, the left common carotid artery of WT and Clock mutant (MUT) mice (male, aged 15 to 20 weeks) was ligated for 5 weeks, and remodeled left common carotid artery thickness was measured, revealing a DD-dependent increase in wall thickness of left common carotid artery in Clockmut mice (n=4; *P<0.05, Clockmut in DD vs WT in DD) and a DD-dependent impairment in inward remodeling (B). C, Von Gieson staining of elastin fibers revealed enhanced intimal hyperplasia in the left femoral artery of male Clockmut mice undergoing intraluminal wire injury relative to injured arteries of male WT mice that were housed under the same conditions in DD (bar=50 μm). D, Morphometric analysis of femoral artery cross sections revealed an increase in neointimal area and intima-to-medial (I/M) ratio indicative of an exacerbated response to wire injury (n=7 to 10; *P<0.05).
different between WT and Clockmut mice, providing strong evidence for a direct link between light cycle- and circadian rhythm-dependent changes in vascular function.

We next sought to determine whether the circadian clock might also influence other types of vascular injury such as intimal hyperplasia in response to intraluminal vascular injury. Five weeks after wire injury to the femoral artery, Clock mutant mice housed in DD conditions exhibited a significant increase in arterial injury relative to WT mice, quantified as an increase in neointimal area and intima-to-medial ratio (Figure 2C and 2D). Although the extent of injury induced by arterial ligation (Figure I in the online-only Data Supplement) and wire injury was not affected by gender (Figure IIA and IIB in the online-only Data Supplement) or time of the initial insult (Figure IIC and IID in the online-only Data Supplement), wire injury in Bmal1-KO mice also induced a robust neointimal response relative to littermate WT control mice (Figure IIB and IID in the online-only Data Supplement), providing further evidence for the importance of clock function in the chronic vascular response to injury.

Bmal1-KO Mice Exhibit Intact Coagulative Responses

Thrombosis and vascular injury can occur because of a defect in the coagulation cascade localized to either the endothelium or blood platelets. To determine whether the injury in Bmal1-KO mice was due to a defect in the coagulative properties of blood, we assessed the platelet response by FACS analysis in isolated whole blood of mice. Baseline levels of total platelets measured by the platelet marker CD41 were not different between WT and Bmal1-KO mice (Figure 3A). Stimulating platelets in isolated whole blood with the thrombin receptor agonist PAR-4 induced a robust and significant increase in platelet activation as measured by P-selectin (cd62) expression. However, the level of activation was not different between Bmal1-KO mice and WT mice (Figure 3B), indicating that an intrinsic defect in platelet activation could not account for the increased thrombosis apparent in Bmal1-KO mice.

Endothelial Dysfunction in Bmal1-KO Mice and Clock Mutant Mice

Because the blood-borne response was intact, we next examined whether a defect in endothelial function might contribute to the impairments in vascular remodeling and thrombosis observed in Bmal1-KO mice. PAI-1, a prothrombotic enzyme that prevents fibrinolysis, was upregulated in the endothelium of remodelled arteries (Figure 3C) and livers (Figure 3D) of Bmal1-KO mice. Indeed, endothelial dysfunction is known to promote pathological vascular remodeling and thrombosis. To directly assess endothelial function, we conducted organ bath studies using isolated aortic rings from Bmal1-KO mice. Aortas from Bmal1-KO mice (Figure 4A, 4B) exhibited a severely impaired endothelium-dependent vasorelaxant response to acetylcholine relative to WT mice. Acetylcholine (5×10⁻⁶ mol/L) induced a robust 71±3.5% relaxation in WT mice versus only 17±3.3% relaxation in Bmal1-KO mice. Endothelial dysfunction was also observed in aortas isolated from Clock mutant mice that were acclimated to conditions of constant darkness. Percent relaxation to acetylcholine was only 56±11% in Clockmut mice versus 87±9.1% in WT mice also acclimated to DD conditions (Figure 4C). Importantly, the endothelial dysfunction in Clockmut mice was not apparent under LD conditions (Figure 4D), providing further evidence in support of a direct link between the biological clock and vascular integrity.

Smooth muscle cell responses to nitric oxide as measured by relaxation in response to sodium nitroprusside remained intact in Bmal1-KO mice (Figure 4E), providing further evidence of a direct impact of circadian rhythm on endothelial function. Finally, WT mice exhibited a variation in vascular function (measured by the response to acetylcholine; Figure 4F), consistent with the temporal variation observed in prior studies. Interestingly, dysfunction in Bmal1-KO mice was further exacerbated at time points of reduced endothelial function in WT mice (Figure 4F).

Akt-eNOS Signaling Is Blunted in Remodeled Arteries of Bmal1-KO Mice

The serine-threonine kinase Akt1 protects cells from apoptosis, increases nitric oxide production via phosphory-
translation of eNOS, and modulates vascular function. Previously, we assessed global expression patterns of circadian oscillating genes in the aorta and identified the serine/threonine kinase Akt1/PKB as a putative target of the circadian clock. Indeed, expression levels of Akt1 protein were significantly reduced in Bmal1-KO mice versus littermate control WT mice in left common carotid artery undergoing 5 weeks of vascular remodeling (Figure 5A). In addition, the phosphorylated form of Akt at threonine 308, an index of Akt activation, was dramatically blunted in the remodeled arteries (Figure 5A and 5B). Because phosphorylation of Akt1 on threonine 308 is dependent on the upstream kinase PDK1, we also assessed PDK1 expression. We found that protein expression of PDK1 was significantly decreased in Bmal1-KO mice relative to WT mice. In mice undergoing a shorter, 1-week ligation, we found that the contralateral control (unligated) right contralateral arteries of Bmal1-KO also exhibited substantially lower levels of P-Akt, Akt, and PDK (Figure 5C). Moreover, comparison of right contralateral arteries versus left common carotid artery after 1 week of ligation revealed that P-Akt and t-Akt were robustly upregulated in remodeled left common carotid arteries of WT mice, an effect that was still lower in Bmal1-KO mice but still upregulated nonetheless, which may reflect the impact of shear forces to regulate Akt.

Because Akt exerts its regulatory role in vascular function, at least in part, by phosphorylating and subsequently activating eNOS, we further examined levels of eNOS in the remodeled arteries undergoing long-term, 4-week ligation. Indeed, expression levels of phosphorylated eNOS were significantly attenuated in Bmal1-KO mice relative to WT mice (Figure 5A and 5B), consistent with impaired endothelium-dependent vasomotion, pathological remodeling, and enhanced vascular injury.
Seminal observations have led to the discovery of the circadian clock and its importance to daily rhythms in central and peripheral function. Central to activation of the circadian clock are the transcription factors Bmal1 and Clock. Bmal1 and Clock heterodimerize to bind E-box motifs within the promoter regions of downstream clock genes to induce the rhythmic changes in gene expression. On a functional level, disruption of Bmal1 or mutation of Clock in mice impairs circadian rhythms in daily activity. However, the biological significance of an aberrant endogenous clock in the long-term progression to vascular disease is unknown. In the present study, we demonstrate that Bmal1-KO and Clock mutant mice exhibit pathological responses to vascular injury, which may stem in part from pronounced endothelial dysfunction in the vasculature. As Bmal1-KO mice age, the chronic response to arterial ligation becomes more severe, evident as a susceptibility to thrombosis, consistent with recent studies of photochemical injury in Bmal1-KO mice. Indeed, aging itself is known to modify circadian clock function. Other studies have also demonstrated that the aging process is accelerated in Bmal1-KO mice, manifest as arthropathy and organ pathology by 35 to 40 weeks of age, in contrast to WT mice of the same age.

Changes in other physiological and metabolic parameters in response to disruption of circadian rhythm do not appear to account for the changes in vascular remodeling observed in Bmal1-KO mice. Bmal1-KO mice exhibited elevated levels of cholesterol and triglycerides relative to WT mice, as demonstrated previously; however these differences were age independent (Table I in the online-only Data Supplement) and modest relative to mouse models of hypercholesterolemia. In apolipoprotein E knockout mice, endothelial function is preserved in the face of striking 10- to 20-fold increases in cholesterol and is impaired only at sites of plaque formation in cholesterol-fed apolipoprotein E knockout mice. Thus, as it applies to our studies, it would be highly unlikely that the <1.5-fold elevations in cholesterol in Bmal1-KO (which are atherosclerosis free on a regular chow diet) would contribute to the endothelial dysfunction and impaired remodeling we observe.

In prior studies, we have shown that responses to insulin are improved in Bmal1-KO mice, which would not readily explain endothelial dysfunction because insulin resistance is intimately linked to compromised endothelial function. Finally, Bmal1-KO mice are not hypertensive but actually hypotensive and lack the nighttime spike in blood pressure.

These findings are the first to demonstrate that the endogenous clock genes can orchestrate the progression to chronic vascular disease. In addition, the acute endothelial dysfunction we observed in Bmal1-KO and Clock mutant mice is also consistent with observations in mutant mice of another core clock component, Per2. Further indication that the vascular effects of Bmal1 and Clock are dependent on overall clock function is that the development of vascular disease (as evidenced by wall thickening and impairment in inward remodeling) only occurred when Clockmut mice were acclimated to free running conditions (DD) versus normal rhythmicity (LD). Thus, we have established a relationship between light cycle and deterioration of vascular integrity that indicates that the vascular phenotype is not a mere consequence of Clock mutation or Bmal1 deficiency and secondary effects therein but is dependent on the integrity of circadian rhythms.

Previously, we have demonstrated that eNOS knockout mice exhibit aberrant remodeling, a phenotype strikingly similar to that which we now demonstrate in Bmal1-KO mice. PDK and its downstream target Akt1, which plays a key regulatory step in controlling eNOS activity, were attenuated in both naive and remodeled common carotid arteries of Bmal1-KO mice. Moreover, 1 week of left common carotid artery ligation revealed robust upregulation of P-Akt in remodeled left common carotid artery of WT mice relative to right contralateral arteries of WT mice, similar to in vitro observations in sheared endothelial cells, hinting at a complex manner of Akt regulation involving both the circadian clock and shear stress. The reduction in Akt signaling and subsequent vascular pathology observed in Bmal1-KO mice is consistent with the established protective role for Akt and eNOS in the regulation of vascular function. In contrast to the blunting in Akt, PAI-1, which is prothrombotic and antifibrinolytic, was increased in Bmal1-KO mice. Indeed, ample evidence links the regulation of PAI-1 to the molecular clock. PAI-1 oscillates with a circadian rhythm, and its expression is regulated by 2 clock components Bmal1 and Revverbo. Moreover, PAI-1 transgenic mice exhibit spontaneous age-dependent coronary thrombosis, a phenotype similar to the injury-induced thrombosis we observed in Bmal1-KO mice. Thus, biological oscillation driven by the circadian clock can influence events in an extended temporal process that, when perturbed, may precipitate a state of endothelial dysfunction, pathological vascular remodeling, and thrombosis that ultimately may forge a path to vascular disease.

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None.

References


**CLINICAL PERSPECTIVE**

Vascular physiology exhibits a profound circadian rhythm, evident as 24-hour variations in blood pressure and vascular contractility. In addition, acute cardiovascular events also exhibit a distinct timing; heart attacks and strokes occur most frequently in the morning. However, evidence for the involvement of the transcription factors that generate circadian rhythms in the progression to vascular disease is lacking. This study demonstrates that the circadian transcription factors Bmal1 and Clock influence not only acute vascular responses but also the long-term adaption of arteries as occurs during vascular remodeling and vascular injury. We found that mice with genetic disruption of the Bmal1 gene (Bmal1-knockout) or mutation of the Clock gene (Clockmut) exhibited pathological vascular remodeling of the common carotid artery after vessel ligation and pronounced intimal hyperplasia of the femoral artery after intraluminal wire injury. Moreover, the extent of injury was conditioned by aging, progressing to thrombosis in remodeled common carotid arteries of Bmal1-knockout mice. This was further accompanied by an increase in the profibrinolytic and prothrombotic molecule plasminogen activator inhibitor-1, a known target of the circadian clock. Conversely, mechanisms protective of endothelial function were impaired, evident as attenuated vascular responses to acetylcholine and attenuation in protein expression of the Akt–endothelial nitric oxide synthase pathway. These data provide direct evidence for the circadian clock in vascular remodeling and vascular injury that may ultimately be of significant importance in the progression to vascular disease.
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Supplemental Figure 1. Aberrant vascular remodeling in mice with a dysfunctional biological clock. Male Bmal1-KO and respective littermate wild-type controls (WT) (10-15 weeks of age) underwent LC ligation at indicated times of day. After 5 weeks, common carotid arteries were analyzed by histomorphometry. Inward remodeling was quantified as the percent change in LC diameter relative to RC. (N=5-7/group; *p<0.05 versus corresponding time).
Supplemental Figure 2. Increased vascular injury in Bmal1-KO and Clockmut mice. Endovascular injury was performed in the left femoral artery of mice at indicated times. Intima to medial ratio (I/M) was quantified as an index of vascular injury. I/M ratio was increased in Clockmut and Bmal1-KO mice relative to wild-type controls, but was gender (A, B) and time independent (C, D). (N=6-10 per 5-7/group, *p<0.05)
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<td>65.8±6.4</td>
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**Supplemental Table 1. Body weight and Lipid profiles in Bmal1-KO mice.** Plasma serum was isolated via the saphenous vein at a single time point (2 PM) and triglycerides and cholesterol were measured as previously described.40 (N number is shown in parentheses, *p<0.05 by one-way ANOVA versus corresponding WT age and gender control, †p<0.05 by unpaired t-test versus corresponding WT age and gender control)