Matrix Metalloproteinases 2 and 9 Dysfunction Underlie Vascular Stiffness in Circadian Clock–Mutant Mice


Objective—To determine if elasticity in blood vessels is compromised in circadian clock–mutant mice (Bmal1-knockout [KO] and Per-triple KO) and if matrix metalloproteinases (MMPs) might confer these changes in compliance.

Methods and Results—High-resolution ultrasonography in vivo revealed impaired remodeling and increased pulse-wave velocity in the arteries of Bmal1-KO and Per-triple KO mice. In addition, compliance of remodeled arteries and naïve pressurized arterioles ex vivo from Bmal1-KO and Per-triple KO mice was reduced, consistent with stiffening of the vascular bed. The observed vascular stiffness was coincident with dysregulation of MMP-2 and MMP-9 in Bmal1-KO mice. Furthermore, inhibition of MMPs improved indexes of pathological remodeling in wild-type mice, but the effect was abolished in Bmal1-KO mice.

Conclusion—Circadian clock dysfunction contributes to hardening of arteries, which may involve impaired control of the extracellular matrix composition. (Arterioscler Thromb Vasc Biol. 2010;30:00-00.)

Key Words: circadian rhythm ■ extracellular matrix ■ matrix ■ metalloproteinases ■ peripheral arterial disease ■ prostaglandins ■ vascular biology ■ vascular stiffness

The cardiovascular system is governed and characterized by 24-hour rhythms. Recent observations have begun to establish that the genetic components that underlie circadian rhythm (the circadian clock) impart a significant influence in the regulation of the vascular system. Mice with a mutation of circadian clock components, including the integral component and transcription factor, Bmal1, exhibit acute vascular dysfunction1,2 and aberrant chronic vascular responses in angiogenesis,3 vascular remodeling, and injury.4 The vascular defects in circadian-mutant mice may stem, at least in part, from impairments in endothelial function.1,4 Indeed, endothelial mediators control the structural and mechanical properties of arteries by influencing smooth muscle cellularity and of extracellular matrix turnover to control elasticity and stiffness,5,6 whose respective dysfunction is significantly correlated with the onset of vascular disease.7 In the current study, we examine if elasticity in arteries and arterioles is compromised in Bmal1-knockout (KO) mice and if matrix metalloproteinases (MMPs) might confer these changes in compliance.

Methods

Animals

All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved and monitored by the Medical College of Georgia, Augusta, Institutional Animal Care and Use Committee. Studies were conducted in 2 mouse models of circadian dysfunction having distinct mutations in the circadian clock; mice with disruption of the Bmal1 gene (Bmal1-KO) and disruption of all the Period gene isoforms (Period-1, Period-2, and Period-3/Per-triple KO [TKO]) and respective littermate control mice were used. Mice were housed under standard 12-hour light/dark conditions. Bmal1-KO and littermate wild-type (WT) mice were formerly produced by gene targeting in 129Sv/J embryonic stem cells, which we backcrossed 5 times to C57BL/6J. Animals were anesthetized by intraperitoneal injection of ketamine and xylazine.

Ultrasonographic Imaging

Mice were imaged in a supine position on a pad (THM100 Mouse Pad) with an integrated temperature sensor, a heater, and ECG electrodes. Appendages were secured to ECG pads to allow constant monitoring of heart rate and body temperature. Body temperature was maintained at 37.5°C. Dépilatory cream (Nair) was used to remove fur from the region of interest, and medical ultrasonographic acoustic gel (Other-Sonic) was used as a coupling fluid between the real-time microvisualization scan head and the skin. Ultrasonographic imaging was performed using a commercially available system (Vevo 770). By using B-mode imaging, the real-time microvisualization 706 scan head was positioned and held immobile using a system (VisualSonics Vevo Integrated Rail System II) to view the mouse common carotid arteries (right common carotid artery [RC] and left common carotid artery [LC]) and heart. The real-time microvisualization 706 scan head is a 40-MHz scan head with a 6-mm focal length and lateral and axial resolutions of 68.2 and 38.5 μm, respectively.

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Pulse-Wave Doppler Measurement
Arterial blood flow was measured using pulse-wave (PW) Doppler mode. After a brief stabilization period, a baseline recording of PW Doppler was obtained from the common carotid artery 3 to 4 mm before the external and internal carotid artery branch point to measure blood flow velocity. Doppler velocity measurement was made at the smallest possible angle of incidence between the Doppler beam and the assumed blood flow direction in the carotid vessel. For diameter measurement at locations where the flow velocity was measured, M-mode recording was performed in the section with the targeted vessels perpendicular to the ultrasonographic beam. From PW Doppler mode, peak flow velocity and velocity time interval were calculated and averaged for 3 consecutive cardiac cycles. From the M-mode recording, the diameters of the vessel were measured for 3 cardiac cycles and averaged to an overall average vessel diameter (D). The total blood volume (V) passing the measurement location per cardiac cycle flow at each location was calculated as follows: \( V = TV \left( \pi D^4 / 4 \right) \).

PW Velocity
Vascular stiffness was determined by measuring PW velocity with a PW Doppler probe. An aortic PW was acquired at 2 aortic sites (descending and abdominal aorta) 4 cm apart. All data were acquired at a depth of approximately 2 to 4 mm and approximately 5 to 6 mm, respectively, with the Doppler probe. PW velocity (measured in meters per second) was calculated as the quotient of separation distance and the time difference between pulse arrivals, as measured from R-peaks of the ECG.

Flow-Dependent Vascular Remodeling
The complete LC ligation was performed as previously described. Briefly, the distal LC and its bifurcation into the external and internal carotid were exposed using blunt dissection. The 8 to 0 nylon sutures were used to ligate the LC, just proximal to the external and internal carotid artery bifurcation. Incisions were closed (5 to 0 suture), and the blood flow and lumen diameter were assessed using a system (Vevo 770) over the next 4 weeks.

Passive Mechanics Studies
Common carotid arteries were excised from mice and incubated in calcium-free Krebs buffer (values in mmol/L: NaCl 18, sodium bicarbonate 23.8, potassium chloride 4.7, magnesium sulfate 1.2, KH2PO4 1.18, and glucose 11.1) with potassium cyanide bicarbonate 23.8, potassium chloride 4.7, magnesium sulfate 1.2, KH2PO4 1.18, and glucose 11.1) with potassium cyanide. Vessels were then mounted on the glass pipettes of a pressure myograph in Krebs buffer heated to 37°C and gassed with 21% O2, 5% CO2, and (13 mmol/L) for 1 hour to deplete vessels of myogenic tone. Vessels were fit with an exponential regression equation to determine the pressure response curves using the following equations:

\[ \text{Circumferential wall stress} = (P \times 1D) / 2WT \]

\[ \text{Circumferential wall strain} = (I - ID_0) / ID_0 \]

where ID is the inner diameter, P, intralumenal pressure; WT, wall thickness; and ID0, ID at 0 mm Hg. Individual stress-strain curves were fit with an exponential regression equation to determine the slope coefficient, or \( \beta \)-coefficient, an indicator of vessel stiffness.

Histomorphometry
After 4 weeks of flow reduction induced by LC 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 studies designated for histological or morphometric analysis of common carotid arteries, after saline infusion, mice were subseqent perfusion fixed with neutral-buffered formalin. Both RCs and LCs were carefully excised and either postfixied overnight for morphometric studies or immediately embedded in frozen medium for cryotome processing using sections proximal and distal to the point of ligation. Morphometric analysis of common carotid arteries was performed using videomicroscopy, as described. The perimeter of the vessel lumen was taken as the circumference (C) of a circle, and lumen diameter (D) was determined from an equation (D = C/\( \pi \)), assuming that the vessel cross-sections were circular in vivo. To determine stenosis area, the internal elastic lamina and patent lumen were circumscribed to derive a radius (R) value from a formula (R = 2C/\( \pi \)) and then internal elastic lamina area and luminal area (A) were calculated using a formula (A = \( \pi R^2 \)). The stenotic area was derived from the difference of internal elastic lamina area and luminal area.

Batimastat Treatment
Batimastat in this study was suspended in sterile PBS/0.01% Tween 80 and administered IP at a daily dose of 35 mg/kg (previously tolerable and effective) from the day of arterial ligation until the
day of euthanization, when the common carotid arteries were isolated for morphometric analysis.

**Gelatin Zymography**

The common carotid arteries were dissected, pulverized under liquid nitrogen, and prepared using zymogram sample buffer. Equal amounts of tissue homogenate from ligated LCs and nonligated RCs were loaded on SDS-PAGE gels containing 10% gelatin. Lytic bands corresponding to molecular weights of MMP-9 and MMP-2 were observed and quantified by densitometry.

**In Situ Gelatinase Zymography**

Both RCs and LCs were excised and immediately embedded in frozen medium for cryotome processing. In situ zymography was performed on frozen sections with a kit (EnzCheck Gelatinase/Collegenase Assay Kit). Gelatinase activity was seen as an increase in fluorescence proportional to proteolytic activity. Specificity for MMPs was established by loss of signal in the presence of 1,10-phenanthroline, a general metalloproteinase inhibitor.

**Murine Endothelial and Smooth Muscle Cell Isolation**

Aortas were isolated from 6- to 8-week-old mice, perfused with collagenase type II, and incubated for 60 minutes. Endothelial cells were flushed out with media and subsequently plated and cultured. The remaining aorta was cut longitudinally and in small segments and inserted in a cell culture dish plate bathed in DMEM with 10% fetal bovine serum. After smooth muscle cells migrated onto the plate, the aortic pulverized were removed and the residual smooth muscle cells were cultured until 80% confluence was obtained. The murine vascular endothelial and smooth muscle cells were then lysed and subsequently used for Western blotting.

**Western Blotting**

After 4 days of complete carotid artery ligation, remodeled arteries were dissected, pulverized under liquid nitrogen, and extracted using ice-cold lysis buffer. Samples were loaded on 10% SDS-PAGE gel and transferred onto nitrocellulose membrane. MMP-9 was detected with rabbit anti–mouse MMP-9 polyclonal antibodies, followed by enhanced chemiluminescence. Signals on x-ray films were quantified by using software (Image J).

**Image Analysis**

PW Doppler images and M-mode diameters were digitally stored and transferred to a computer for off-line analysis. To reduce variability, imaging parameters were held constant throughout each experiment, with focus and depth optimized at the beginning of the study for each animal. All studies were performed using approximately the same scan plane, as determined by anatomic markers.

**Figure 2.** Flow velocity and transit blood flow volume in WT and Bmal1-KO mice. A and C, Doppler flow velocity in common carotid arteries was determined by echo ultrasonography using PW D-mode imaging (see Methods section) in RC (A) and LC (C) before and 4 weeks after ligation. B and D, Transit blood flow was then determined in the RC (B) and LC (D) in weekly intervals up to 4 weeks postligation. In B, contralateral unligated RC did not display significant changes in WT vs Bmal1-KO mice before and after surgical ligation of LC (n=6 per group). In D, blood flow in the LC underwent a dramatic decrease after ligation, which was constant and not different in WT vs Bmal1-KO mice over 4 weeks after ligation (n=6 per group).

**Figure 3.** Increased PW velocity in the great arteries of Bmal1-KO mice. A and B, PW velocity was assessed noninvasively by ultrasonography (see Methods section) in the aortas of Bmal1-KO (A) and TKO (B) mice (n=4). *P<0.05, unpaired t test.
Statistical Analysis
Statistical analysis was performed using computer software (GraphPad Prism version 4.02 for Windows). Data were compared using an ANOVA, followed by a Tukey posttest or an unpaired t test, used as indicated. Data are expressed as mean±SEM. P<0.05 was considered statistically significant.

Results
Ultrasonicographic Analysis of Remodeling in Bmal1-KO Mice
Chronic blood flow alteration was induced in the LC by ligation at the external and internal carotid artery bifurcation of mice. Common carotid arteries were then visualized in vivo in anesthetized mice by ultrasonography. As demonstrated in prior studies by histomorphometric quantification of arterial cross-sections,10 ligation of the LC in WT mice induced inward remodeling relative to the RC (Figure 1A), as seen by a diminution in the vessel diameter imaged by ultrasonography. In addition, complete cessation of flow in the branches downstream of the ligation was confirmed because there was no detectable blood flow to image in the external and internal carotid downstream of the ligated LC; in the unligated contralateral RC, the external and internal carotid arteries were clearly visible in WT mice. In Bmal1-KO mice, the LC postligation was enlarged relative to the response observed in WT mice. Subsequently, the course of vascular remodeling of the lumen (inward remodeling) to ligation was monitored by ultrasonography in WT and Bmal1-KO mice for 4 weeks. As early as 1 day postligation, lumen diameter narrowed in WT mice (Figure 1B), consistent with observations describing an acute vasoconstrictive response during the process of remodeling, as previously shown.11,12 LC lumen robustly narrowed by 4 weeks postligation, to 0.143±0.020 mm from an initial diameter of 0.289±0.011 mm.

Despite ligation of the LC, Bmal1-KO mice did not inward remodel (Figure 1C), with lumen diameter remaining unchanged over 4 weeks. We further examined hemodynamics in the WT

Figure 4. Increased vascular stiffness in remodeled arteries and arterioles of Bmal1-KO mice. A, Pressure-diameter relationships were assessed ex vivo in remodeled LCs 4 weeks after ligation. B, Wall thickness was also assessed in the remodeled LCs (n=4 to 7). C, Circumferential wall stress and strain were calculated from measurements made over a range of pressures in passive calcium-free conditions. Arteries were preincubated for 1 hour in KCN, 13 μmol/L, solution to eliminate the myogenic tone. In the LC undergoing 4 weeks of ligation of Bmal1-KO mice, there was a leftward shift in the stress/strain curve, indicating a decrease in LC compliance. D, Arterioles in proximity to the femoral artery were also isolated and stress/strain curves were determined, indicating a decrease in compliance in Bmal1-KO mice. E and F, β Coefficients derived from stress/strain relationships were significantly increased in the remodeled arteries (E) and arterioles (F) of Bmal1-KO mice. *P<0.05 for LCs from Bmal1-KO mice (n=5); n=7 for control WT mice.

Figure 5. Increased vascular stiffness in arterioles of TKO mice. A, Circumferential wall stress and strain were determined in isolated arterioles proximal to the femoral artery from TKO mice. B, β Coefficients derived from stress/strain relationships were significantly increased. *P<0.05 (n=6).
and Bmal1-KO mice. In contralateral RC, there were no major differences in transit blood volume between WT and Bmal1-KO mice (Figure 2A and B), whereas ligation robustly suppressed this index of blood flow in a fashion that was comparable between WT and Bmal1-KO mice (Figure 2C and D).

**Increased Vascular Stiffness in Arteries and Arterioles of Bmal1-KO and Per-TKO Mice**

We next assessed PW velocity in naïve large arteries of Bmal1-KO and Per-TKO mice in vivo by assessing the transit time of the flow velocity waveform in an arterial segment.13 PW velocity was increased in aortas of Bmal1-KO (Figure 3A) and Per-TKO (Figure 3B) mice. Because increased PW velocity is an indicator of arterial stiffness,14,15 we sought to more directly measure vascular compliance by assessing stress versus strain curves in ex vivo pressurized conduit arteries. In these pressurized vessels, the LC inner or lumen diameter (Figure 4A) of WT mice was significantly smaller than the LC of Bmal1-KO over the entire range of pressures studied, consistent with live imaging from ultrasonographic B-mode imaging. In addition, wall thickness was increased in the LC of Bmal1-KO mice relative to WT mice (Figure 4B), further evidence of an impaired inward remodeling response accompanied by medial hypertrophy in these mice with disrupted clocks. When plotting diameter versus pressure from WT mice undergoing LC ligation, the resultant stress strain curves in LCs from Bmal1-KO exhibited a

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**Figure 6.** Increased expression and activity of MMP-9 and MMP-2 in Bmal1-KO mice. Increased gelatinolytic activity in carotid artery postligation. Equal amounts of tissue homogenate were loaded on SDS-PAGE gels containing gelatin. A, Gelatinolytic activity associated with MMP-9 was equally induced at day 3 after ligation in both Bmal1-KO and WT ligated common carotid arteries. After 3 weeks of ligation, gelatinolytic activity associated with MMP-9 and MMP-2 was significantly increased in Bmal1-KO vs WT littermate controls (n=4). Remodeled arteries were dissected, and protein lysates were isolated and separated by SDS-PAGE electrophoresis from littermate control WT mice vs Bmal1-KO mice at a single point (12 pm). B and C, Expression levels of MMP-9 (B) and MMP-2 (C) were significantly increased in remodeled LCs relative to WT mice. Changes were quantified by densitometry. *P<0.05 vs WT (n=4 WT, and n=4 Bmal1-KO).
leftward shift (Figure 4C) versus WT mice, indicative of a decrease in compliance. To determine if Bmal1 might also modify elasticity of blood vessels across the vascular tree, we assessed mechanical properties of resistance vessels from Bmal1-KO mice (second-degree femoral artery blood vessels). The arterioles from Bmal1-KO mice also exhibited a leftward shift in the stress/strain curves relative to WT arterioles (Figure 4D). Thus, the derived index of vascular stiffness, the [$\beta$]-coefficient/elastic modulus,16,17 was increased in both remodeled LC (Figure 4E) and naïve (Figure 4F) arterioles, further indicating an increase in wall stiffness in blood vessels from Bmal1-KO, which was also observed in arterioles of Per-TKO mice (Figure 5).

**MMP Dysfunction in Bmal1-KO Mice**

Because the extracellular matrix exerts an important influence on the elasticity of blood vessels18,19 and the evolution of cardiovascular disease,20,21 we then examined if metallopro-
and Table), and wall thickness (Figure 8D) in WT mice, but reduced neointima (Figure 8A and B), stenosis (Figure 8C), and vessel compliance (Figure 7A), whereas COX-2 and an attenuation of COX-1 expression in endothelial cells (Figure 7A), whereas COX-2 and an attenuation of COX-1 expression (data not shown). This was accompanied by an increase in MMP-2 activity in remodeled arteries. This was further reflected as an elevation in protein expression of MMP-2 and MMP-9 gelatin degradation, after 3 days' incubation in fixed isolated arteries (1) to further demonstrate impairments in the control of the vasculature.1,3 Herein, we demonstrate large-vessel and resistance-vessel stiffening in mice with genetic disruption of 2 distinct circadian clock components, Bmal1 and the Period isoforms. In addition, live pulsating vessels, assessed by ultrasonography and pressurized vessels ex vivo, corroborate recent observations in Bmal1-KO mice observed in fixed isolated arteries1 to further demonstrate impairments in the response to arterial ligation, reflected as an inability to inward remodel.

In aortas of Bmal1-KO and Per-TKO mice, PW velocity was increased in vivo. In addition, pressurized and remodeled common carotid arteries of Bmal1-KO mice ex vivo also exhibited a reduction in compliance relative to their counter-part WT mice, despite comparable reduction in flow in the ligated LC of WT and Bmal1-KO mice. Moreover, elasticity of remodeled arteries of WT mice was no different than that of their unligated contralateral vessel (data not shown). However, the response to injury, remodeling, and angiogenesis is impaired in mice with a mutation of circadian clock components.1,3 Herein, we demonstrate large-vessel and resistance-vessel stiffening in mice with genetic disruption of 2 distinct circadian clock components, Bmal1 and the Period isoforms. In addition, live pulsating vessels, assessed by ultrasonography and pressurized vessels ex vivo, corroborate recent observations in Bmal1-KO mice observed in fixed isolated arteries1 to further demonstrate impairments in the response to arterial ligation, reflected as an inability to inward remodel.

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### Table. Effect of Batimastat on Lumen Diameter During Remodeling in Bmal1-KO Mice*

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<th>Variable</th>
<th>Preligation</th>
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*The end-diastolic lumen diameter was measured live in vivo through echo ultrasonographic imaging using M mode (see the Methods section). Vascular remodeling, evident as a lumen diameter reduction, was seen by 1 week after arterial ligation in WT mice treated with vehicle control (LC part of table). Batimastat treatment in WT mice accelerated a constrictive response, demonstrated as a significant reduction in lumen diameter 1 day postligation. However, by 3 weeks after ligation, constrictive remodeling in LC WT mice treated with batimastat was attenuated, exhibiting increased lumen diameter vs LC WT mice treated with vehicle (n=4 per group, P<0.05 by unpaired t test). Bmal1-KO mice exhibited no inward remodeling but exhibited outward remodeling that was significant at 2 weeks in the vehicle-administered group and in an analogous manner to WT, became accelerated, albeit outwardly, occurring with significance at 4 days and 2 weeks postligation in the batimastat-treated group (LC part of table) (n=4 per group, P<0.05 by 1-way ANOVA vs corresponding preligation lumen diameter). Batimastat and Bmal1 mutation did not affect lumen diameter occurring with significance at 4 days and 2 weeks postligation in the batimastat-treated group (LC part of table) (n=4 per group, P<0.05 by 1-way ANOVA vs corresponding preligation lumen diameter). Batimastat and Bmal1 mutation did not affect lumen diameter in control contralateral RCs (RC part of table).

†P<0.05.
moderately hypotensive. Indeed, the intrinsic composition of the vasculature is important in its elastic properties. Thus, the observed vascular stiffness in Bmal1-KO mice is consistent with the enhanced collagen deposition that has been demonstrated in remodeled arteries of Bmal1-KO mice. Although genes relevant to the extracellular matrix oscillate with a circadian rhythm, to our knowledge, this is the first demonstration of a defect in the matrix associated with circadian gene mutation. Both MMP-2 and MMP-9, which are important in extracellular matrix turnover, were upregulated in remodeled arteries of Bmal1-KO mice. In support of a putative influence of circadian rhythm in metalloproteinase regulation, it was previously reported that circulating MMP-9 is elevated in the light period (relative to the dark period) in human patients with prior myocardial infarction; however, additional compelling evidence is scarce. Previously, it was demonstrated that Akt and NO signaling are blunted in Bmal1-KO mice, which may, in part, contribute to the impairment in metalloproteinases.

References


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Supplemental Material.

Figure I. Increased gelatinase activity in arteries of Bmal1-KO mice by in situ zymography. WT and Bmal1-KO mice underwent ligation of the LC for 4 weeks. At 4 weeks, common carotid arteries were harvested and freshly processed (not perfusion fixed) for histological staining by in situ gelatinase zymography. In situ gelatinase zymography of RC and LC in mice undergoing 4 weeks of arterial ligation revealed an increased gelatinolytic activity (bar=10 µm).
Figure II. Immunohistochemical detection of MMP-9. Immunohistochemical detection of MMP-9 in mice undergoing 4 weeks of arterial ligation revealed an increase in expression in the LC and RC Bmal1 KO versus WT. Controls were incubated with secondary antibody alone, in the absence of immune primary (bar=30 µm).
Figure III. Collagen staining by Sirius Red. WT and Bmal1-KO mice underwent ligation of the LC for 4 weeks. At 4 weeks, common carotid arteries were harvested by perfusion fixation and processed for histological staining by Sirius red. Sirius red staining reveals an increase in collagen (arrow) in the remodeled artery of Bmal1-KO mice (bar=10 μm).