Progression of the Prothrombotic State in Aging Bmal1-Deficient Mice

Bianca Hemmeryckx, Cor E. Van Hove, Paul Fransen, Jan Emmerechts, Alexandre Kauskot, Hidde Bult, H. Roger Lijnen, Marc F. Hoylaerts

Objective—The goal of this study was to examine the functional relationship between aging endothelium and thrombogenicity in a mouse model of premature aging.

Methods and Results—Coagulation tests and factors, blood cell counts, aorta endothelial function, aorta gene expression, and FeCl3-induced thrombosis in mesenteric blood vessels were analyzed in 10- to 30-week-old brain and muscle ARNT-like protein-1 (Bmal1)–deficient (knockout [KO]) mice and wild-type littermates. Ten-week-old KO mice manifested shortened prothrombin times (9.7 versus 11.3 seconds in wild-type) and elevated plasma fibrinogen (264 versus 172 mg/dL). At 30 weeks, factor VII (198% versus 149%), and platelet counts (2049 versus 1354 K/μL) were increased in KO mice. Gene deficiency reduced the vasoactive nitric oxide production at 10 and 30 weeks and tended to reduce and increase the protein expression of thrombomodulin and von Willebrand factor, respectively, with aging. Shortened venular and arteriolar occlusion times on FeCl3-induced injury in 10-week-old KO mice confirmed higher thrombogenicity, culminating in priapism, observed in 60% of 25- to 30-week-old KO males.

Conclusion—Endothelial dysfunction and a hypercoagulable state cause early arterial and venous thrombogenicity in Bmal1 KO mice. With aging, progressive endothelial dysfunction, rising platelet counts, and high factor VII further enhance thrombogenicity, provoking priapism. (Arterioscler Thromb Vasc Biol. 2011;31:2552-2559.)

Key Words: circadian rhythm ▪ coagulation ▪ elderly ▪ endothelial function ▪ thrombosis
Table 1. Effect of Age and Bmal1 Deficiency on Blood Characteristics and Hemostasis Parameters

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>KO</th>
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<th>KO</th>
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<tr>
<td></td>
<td>10 W</td>
<td>30 W</td>
<td>10 W</td>
<td>30 W</td>
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<tr>
<td>Blood characteristics</td>
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<tr>
<td>WBC (#/µL)</td>
<td>3179±411</td>
<td>3701±349</td>
<td>5236±622†</td>
<td>3457±601</td>
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<tr>
<td>Neutrophils (#/µL)</td>
<td>443±50</td>
<td>657±90</td>
<td>920±148‡</td>
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<td>Lymphocytes (#/µL)</td>
<td>2583±352</td>
<td>2631±217</td>
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<td>Monocytes (#/µL)</td>
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<td>48±9</td>
<td>52±12</td>
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<td>Eosinophils (#/µL)</td>
<td>6±3</td>
<td>8±2</td>
<td>1±1</td>
<td>8±2*</td>
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<tr>
<td>Basophils (#/µL)</td>
<td>113±19</td>
<td>176±27</td>
<td>169±22</td>
<td>76±21†</td>
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<tr>
<td>Platelet No. (K/µL)</td>
<td>1341±63</td>
<td>1354±54</td>
<td>1639±117</td>
<td>2049±56†</td>
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<td>Red blood cells (#/µL)</td>
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<td>8.0±0.3</td>
<td>7.6±0.3</td>
<td>8.8±0.6*</td>
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<td>Hemoglobin level (mg/dL)</td>
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<td>13.8±0.5</td>
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<td>Hemostasis parameters</td>
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<td>aPTT (s)</td>
<td>35±1</td>
<td>34±1</td>
<td>42±2†</td>
<td>41±2†</td>
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<td>PT (s)</td>
<td>11.3±0.2</td>
<td>10.1±0.2*</td>
<td>9.7±0.2†</td>
<td>9.3±0.2†</td>
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<tr>
<td>FVII (%)</td>
<td>138±8</td>
<td>149±12</td>
<td>150±11</td>
<td>198±13†</td>
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<td>FVIII (%)</td>
<td>109±10</td>
<td>128±15</td>
<td>145±5†</td>
<td>125±11</td>
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<tr>
<td>Fibrinogen (mg/dL)</td>
<td>172±6</td>
<td>189±10</td>
<td>264±8†</td>
<td>309±32†</td>
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</table>

Data are mean±SEM. No. of animals per group ≥9. WT indicates wild-type; KO, knockout; W, wk; WBC, white blood cells; aPTT, activated partial thromboplastin time; PT, prothrombin time; F, factor.

*P<0.05 vs WT. †P<0.05 vs WT mice (Mann–Whitney).

further increase thrombogenicity in the elderly by enhancing platelet activation, as a result of vascular deficiency.

Brain and muscle ARNT-like protein-1 (Bmal1) clock gene–deficient mice with an accelerated aging phenotype are characterized by a prothrombotic tendency in response to experimentally inflicted injury. In addition, these mice display reduced NO bioavailability. We have selected the prematurely aging Bmal1 gene–deficient mouse model to investigate the relationship between vascular aging and thrombogenicity by focusing on risk factors for arterial as well as venous thrombosis. Therefore, in addition to performing time-lapse analysis of primary and secondary hemostatic parameters, we have studied vascular expression profiles, morphology, and function, in young and prematurely aged Bmal1 knockout (KO) mice. We noted the existence of a hypercoagulable state in young mice, progressing toward a prothrombotic state, associated with spontaneous priapism in prematurely aged mice.

Methods

Animal Model
Breeding couples of mice heterozygous for Bmal1 (100% C57BL/6J) were kindly provided by Dr K. Esser (University of Kentucky, Lexington, KY). Male Bmal1-deficient KO mice and wild-type (WT) littermates at the age of 10 weeks (body weight: WT 20.5 to 27 g; KO 17.0 to 25.0 g) to 30 weeks (body weight: WT 23.0 to 33.0 g; KO 17.3 to 25.0 g) were generated in the animal facility of the Center for Molecular and Vascular Biology (Katholieke Universiteit Leuven, Leuven, Belgium) and genotyped as described elsewhere. All animals were kept in microisolation cages in a temperature- and light-controlled (12-hour night/day cycle) environment and had free access to drinking water and standard chow (KM-04-k12, Muracon, Carfil, Oud-Turnhout, Belgium; 13% kcal as

fat, caloric value 10.9 kJ/g ad libitum. All animal procedures were approved by the Ethical Committee of the Katholieke Universiteit Leuven and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (1996).

Procedures
The protocols for the mouse manipulations, blood and plasma assays, isometric tension measurements, in vivo thrombosis and inflammation model, histology and immunohistochemistry and the subsequent microscopy analysis, quantitative real-time polymerase chain reaction, and statistical analysis can be found online in the supplemental materials (available online at http://atvb.ahajournals.org).

Results

Hematologic Changes Associated With Aging of Bmal1+/– Mice
The white blood cell count and platelet numbers did not change from 10 to 30 weeks in WT mice (Table 1). Bmal1 gene deficiency increased neutrophil numbers 2-fold at 10 weeks (P<0.05) and slightly at 30 weeks (1.2-fold, P>0.05). In addition, at 10 weeks, KO mice showed increased white blood cell count, and at 30 weeks, they showed higher red blood cell and platelet counts, as well as a trend toward increased hemoglobin blood levels, compared with WT mice (Table 1). Eosinophil and lymphocyte numbers were normal in 30-week-old KO mice but not in 10-week-old KO mice (Table 1). The density of VWF-positive megakaryocytes in 30-week-old KO bone marrow was significantly higher than in age-matched control bone marrow, in agreement with the elevated platelet numbers (Figure 1). Despite high platelet numbers at 30 weeks in KO mice, platelet aggregation assays revealed a normal platelet response to the agonist ADP (0.5 to
2 μmol/L) when tests were executed at comparable platelet numbers (data not shown).

Plasma coagulation tests and coagulation factors (accepted risk factors in venous thrombosis) revealed no changes in the activated partial thromboplastin time (aPTT) in WT mouse plasma from 10 to 30 weeks. No consistent changes were found for plasma fibrinogen, FVII, and FVIII levels (Table 1). In contrast, Bmal1 gene deficiency was associated with a prolongation of the aPTT and a shortening of the prothrombin time by 1.6 seconds in 10-week-old mice and 0.8 seconds in 30-week KO mice, compared with their age-matched controls. At 30 weeks, KO mouse plasma FVII was higher than at 10 weeks. Fibrinogen was elevated in KO mouse plasma, independently of age, whereas FVIII seemed transiently elevated at 10 weeks. These findings are compatible with a hypercoagulable state in KO mice, aggravating with aging. Serum levels of the inflammatory factor C-reactive protein were somewhat higher in Bmal1-deficient mice versus WT mice at 10 weeks (KO 171±20 ng/mL, n=8; WT 139±10 ng/mL, n=12; P=0.18) and at 30 weeks (KO 206±23 ng/mL, n=4; WT 143±11 ng/mL, n=14; P=0.06), findings that explain, in part, why aPTT is prolonged, despite shortened prothrombin time values and elevated fibrinogen (see below).

Smooth Muscle and Endothelial Cell Function in Bmal1−/− Mice

The effect of Bmal1 deficiency on aging smooth muscle cell (SMC) and endothelial cell function was tested functionally in thoracic aortic rings isolated from 10- and 30-week-old KO and WT mice. SMC contraction and vasorelaxation were studied in organ chambers in the presence of the nitric oxide synthase (NOS) inhibitors Nω-nitro-L-arginine and Nω-nitro-L-arginine methyl ester (LNA/LNAME, black curves) of both genotypes at 10 weeks (KO:○; wild-type [WT] ●) and 30 weeks (KO:●; WT:■) during contraction induced by phenylephrine (PE). Tracings in the presence of LNA/LNAME (+LNA/LNAME, red curves, same labels as defined above) are shown. B, Increase in maximum PE force for WT at 30 weeks (○) and its attenuation by Bmal1 deficiency. C, Concentration response curves induced by diethylamine NONOate (DEANO) in aortic rings contracted with 3 μmol/L PE in the presence of LNA/LNAME. D, Relaxation curves induced by acetylcholine (ACh) in rings constricted with 3 μmol/L PE. Results show mean±SEM, n=10. *P<0.05 vs 10 W; †P<0.05 vs 10 W; ‡P<0.01 vs WT; 2-way ANOVA followed by Bonferroni post hoc tests.

Figure 1. Effect of Bmal1 deficiency on megakaryocyte density in bone marrow sections. A, Megakaryocytes in paraffin-embedded bone marrow sections stained with a rabbit antihuman von Willebrand factor (VWF) antibody (magnification ×400). The arrow indicates a VWF-positive megakaryocyte (brown). WT indicates wild-type; KO, knockout. Scale bar=20 μm. B, Megakaryocyte density in 30-week-old Bmal1 KO and WT mice (n=2). Data are mean±SEM. †P<0.05 vs WT (Mann–Whitney).

Figure 2. Effect of age and Bmal1 deficiency on aortic smooth muscle cell and endothelial cell function. A, Tension in control rings (without Nω-nitro-L-arginine/Nω-nitro-L-arginine methyl ester [LNA/LNAME], black curves) of both genotypes at 10 weeks (KO:○; wild-type [WT]●) and 30 weeks (KO:●; WT:■) during contraction induced by phenylephrine (PE). Tracings in the presence of LNA/LNAME (+LNA/LNAME, red curves, same labels as defined above) are shown. B, Increase in maximum PE force for WT at 30 weeks (○) and its attenuation by Bmal1 deficiency. C, Concentration response curves induced by diethylamine NONOate (DEANO) in aortic rings contracted with 3 μmol/L PE in the presence of LNA/LNAME. D, Relaxation curves induced by acetylcholine (ACh) in rings constricted with 3 μmol/L PE. Results show mean±SEM, n=10. *P<0.05 vs 10 W; †P<0.05, ‡P<0.01 vs WT; 2-way ANOVA followed by Bonferroni post hoc tests.
(Figure 2B). The effect of NOS inhibition increased with age in WT mice but not in Bmal1-deficient mice, and basal NO release was significantly attenuated by Bmal1 deficiency at the age of 30 weeks.

Subsequently, acetylcholine (ACh) was given to activate eNOS activity by stimulating endothelial muscarinic receptors. In WT aortic rings, ACh caused almost complete relaxation that tended to be more pronounced at 30 weeks. At 10 weeks, Bmal1 deficiency led to a significant reduction of amplitude (Figure 2D) and sensitivity (pD2 values: WT 7.41 ± 0.07, KO 7.17 ± 0.08, n = 10, P < 0.05). These findings confirm that endothelial NO bioavailability was significantly reduced in 10-week-old Bmal1-deficient mice and that endothelial NO production is significantly reduced in 10- and 30-week-old Bmal1-deficient mice, and that endothelial NO production elicited by ACh further declined with age in 30-week-old KO mice.

### Bmal1 Gene Deficiency Mildly Influences Vascular Pro- and Anticoagulant Pathways During Aging

More detailed investigation of endothelial cell aging showed a 1.5-fold drop of the transcription of the procoagulant vascular tissue factor (initiator of physiological coagulation) with aging in KO aorta extracts (Table 2). Likewise, mRNA for VWF (a risk factor for arterial thrombosis) appeared to be produced to a lesser degree (1.6-fold) in aging KO aorta extracts. Aortic VWF protein expression, on the contrary, increased slightly with age for Bmal1 KO mice when its expression was scored on immunohistochemical staining of aorta cross-sections (score at 10 weeks: 2.7 ± 0.2; at 30 weeks: 3.1 ± 0.2; P = 0.13); in WT mice, the opposite trend was observed (score at 10 weeks: 3.4 ± 0.2; at 30 weeks: 2.6 ± 0.4; P = 0.18). Plasma VWF antigen levels, however, were not influenced by age or genotype (WT 116 ± 9% at 10 weeks and 147 ± 20% at 30 weeks versus KO 143 ± 12% at 10 weeks and 153 ± 8% at 30 weeks). On the other hand, a mildly (1.6-fold) reduced transcription of the endothelial anticoagulant protein thrombomodulin (TM) was observed in aging KO aorta extracts, accompanied by a 2-fold drop in the transcription of the endothelial protein C receptor (Table 2). We observed a similar trend for the aortic TM protein expression, evaluated via scoring with age in KO mice (score at 10 weeks: 2.2 ± 0.4; at 30 weeks: 1.3 ± 0.5), albeit that relative staining intensities varied strongly in 30-week sections (P < 0.37). Age did not influence the intensity and homogeneity of the aortic endothelial protein C receptor staining of WT (score at 10 weeks: 2.7 ± 0.2; at 30 weeks: 2.8 ± 0.2; P = 0.48) and KO mice (score at 10 weeks: 2.7 ± 0.2; at 30 weeks: 2.7 ± 0.3; P = 0.94).

### Vascular Inflammatory Changes in Bmal1 KO Mice

Baseline leukocyte rolling over endothelium was hardly affected by activation with A23187 of the mesenteric endothelium, both when analyzed at 10 and 30 weeks. On average, lower numbers of rolling cells were found on Bmal1 KO than WT mouse endothelium at all ages (46 ± 7 rolling cells in WT mice versus 14 ± 2 rolling cells in KO mice, P < 0.05, when analyzed per 5 subsequent frames; see supplemental materials). These findings confirm the absence of a proinflammatory vascular phenotype in mesenteric blood vessels, even suggesting a reduced inflammatory vigilance on Bmal1 KO endothelium, despite higher numbers of circulating leukocytes and somewhat higher plasma C-reactive protein levels in young KO mice (Table 1).

### Increased Arterial and Venous Thrombotic Risk in Bmal1 KO Mice

In view of the existence of an age-dependent plasma hypercoagulable state, reduced NO bioavailability, modified anticoagulant properties in KO mice, and elevated platelet numbers (a risk factor in arterial thrombosis), we have investigated the arterial and venous thromboembolic risk separately in aging Bmal1-deficient mice via controlled vascular injury-induced thrombosis studies. Arteriolar and venular occlusion times were measured in 10-week-old and 20- to 25-week-old WT and KO mice. The 30-week-old KO mice did not survive anesthesia and could not be analyzed; therefore, thrombosis studies were done in slightly younger animals. Figure 3 illustrates that Bmal1 deficiency shortened arteriolar as well as venular occlusion times convincingly at the age of 10 weeks, compatible with early arterial and venous thrombogenicity in KO mice. In 20- to 25-week-old KO mice, enhanced thrombogenicity was only suggested but not formally demonstrated because of the large experimental variability.
Arterial and venous thrombosis during aging can be attributed to age-induced changes in structure and function of the vessel wall, to age-associated alterations in blood flow, and to a shift toward a hypercoagulable state. However, the functional relationships between aging endothelium and enhanced risk, especially for venous thromboembolism, are not fully understood. Because there is increasing evidence that arterial and venous thrombosis share many risk factors, we performed a detailed analysis of arterial and venous thrombogenicity and of their classical risk factors, in association with functional vascular changes in 10- and 30-week-old Bmal1 KO and WT mice, to assess the role of the aging vasculature in thrombogenicity evolution.

**Hematologic Changes Associated With Aging of KO Mice**

Kondratov et al found more than a 2-fold rise in the percentage of monocytes and neutrophils in aged KO mice. We found a modest neutrophil elevation but did not notice any change in monocyte numbers, but our KO mice had higher numbers of functional platelets at 30 weeks compared with WT mice, in accordance with more VWF-positive megakaryocytes in the bone marrow. This finding is at variance with results by Somani et al, who did not find platelet changes. These differences may relate to more trivial factors, such as different housing environment, a differential night-day regimen, or the blood isolation procedure. As Bmal1 is a clock gene, exposing KO mice to 2 weeks of constant darkness up to the time of analysis may influence the rhythmicity of several biological parameters.

Aging men develop mild anemia; in the present study, we found that both erythrocytes and platelets went up at 30 weeks in Bmal1 KO mice. This suggests hematopoietic effects in the myeloid erythroid precursor lineage. Because increased oxidative stress in Bmal1-deficient mice causes premature aging of several (but not all) organs, upregulated ROS with age may be responsible for enhanced megakaryopoiesis and erythropoiesis in the bone marrow numbers of aging KO mice, as has been suggested by O’Brien et al for megakaryocyte maturation.

So far, secondary hemostasis has not been investigated in Bmal1 KO mice. We found that Bmal1 gene deficiency triggers a hypercoagulable state at 10 weeks, evidenced by a shortened prothrombin time and elevated plasma fibrinogen levels (P < 0.05). In addition, this state worsened with age because of further rising FVII levels (a risk factor for venous thrombosis) at 30 weeks (P < 0.05). The shortened prothrombin time in KO mice can be explained by increased fibrinogen and FVII levels. In contrast, the prolonged aPTT may result from interference of phospholipid binding inflammatory factors, such as C-reactive protein, measured in the present study and found to be elevated, or serum amyloid protein, which is claimed to be a more important acute phase protein in the mouse. Also, in recent mouse studies, similar apparently conflicting aPTT prolongations have been reported.

**Smooth Muscle and Endothelial Cell Function in Bmal1−/− Mice**

Aging of arterial vessels is accompanied by a drop in SMC proliferation and reduced contractile responses to vasoactive
agents. Endothelial dysfunction or a reduction in NO-mediated vasodilation is elicited as a consequence of increases in ROS levels. Anea et al already showed that Bmal1 deficiency attenuates concentration-dependent responses to the vasodilator ACh but that relaxant responses to the endothelium-independent NO donor sodium nitroprusside are similar between 10-week-old KO and WT mice. We have expanded this observation by investigating the contractility of aortic SMCs and the basal and stimulated (endothelium-dependent and -independent) NO production in these mice as a function of age. We observed that Bmal1 deficiency was not accompanied by defects of the contractile function of aortic SMCs, as indicated by (1) normal contractile responses to the α1-adrenoceptor agonist PE when eNOS activity was blocked by high concentrations of Nω-nitro-L-arginine/Nω-nitro-L-arginine methyl ester, and (2) normal responsiveness to exogenous NO released by the NO donor diethylamine NONOate. However, endothelium-dependent vasomotor responses differed for KO mice. Both basal NO release, assessed by the rise of PE contraction induced by the NOS inhibitors Nω-nitro-L-arginine and Nω-nitro-L-arginine methyl ester, and ACh-muscarinic receptor-induced, endothelium-dependent relaxations were suppressed in aortic rings of Bmal1-deficient mice, particularly at 30 weeks. These defects
in endothelial function are not attributed to alterations in eNOS mRNA levels or to mRNA expression or activity of enzymes involved in generation (NADPH oxidase 4, xanthine dehydrogenase) or degradation (catalase, superoxide dismutase 1, glutathione peroxidase 1) of ROS (data not shown). The defect in basal NO production is possibly linked to a posttranscriptional level of regulation of eNOS activity. Anea et al. attributed the reduction in ACh-mediated relaxation in 10- to 15-week-old KO mice to a blunted Akt signaling in remodeled arteries. The serine-threonine kinase Akt is involved in cellular survival, stimulates NO production by phosphorylating eNOS on serine residue 1177, and thereby influences vascular function. Therefore, a reduction in Akt signaling results in less phosphorylation of eNOS on Ser1177, a lower NO release, and endothelial dysfunction.

**Bmal1 Gene Deficiency Mildly Influences Vascular Pro- and Anticoagulant Pathways During Aging**

Endothelial dysfunction can cause a shift toward a procoagulant, proinflammatory, and prothrombotic state favoring thrombotic occlusion. At the gene transcription level, we noticed that the aged arterial endothelium of Bmal1 KO mice became less anticoagulant, because the transcription of the anticoagulant genes TM and endothelial protein C receptor dropped significantly. At the protein level, we observed trends toward an increase of aorta VWF and a drop in aorta TM expression with age in Bmal1 KO mice, tilting the hemostatic balance toward elevated vessel wall-dependent thrombogenicity. Plasma VWF antigen levels, however, were not influenced by age in these mice. The small reduction in TM expression with age in KO animals can be multifactorial: (1) TM is a Clock-Bmal2-controlled gene, the expression of which could be attenuated by age as for Bmal1, *Period 1*, and *Period 2* in vascular endothelial cells; and (2) Bmal1-deficient endothelium may be exposed to disturbed shear stress in less elastic arteries, resulting in decreases in TM mRNA, as suggested by Malek et al.

**Vascular Inflammatory Changes in Bmal1 KO Mice**

Plasma fibrinogen and the neutrophil and platelet counts were increased in 30-week-old Bmal1 KO mice compared with WT mice, at first suggestive of a systemic inflammatory phenotype. However, despite lower basal NO production in aortic segments, the endothelium of KO mice did not manifest a proinflammatory state, as fewer leukocytes were found rolling on the (A23187-activated) endothelium of arterioles and venules in the mesenteric circulation of these mice. Therefore, Bmal1 deficiency may not only influence the vascular expression of adhesion molecules but also affect granulocyte function itself and cause a defective inflammatory vigilance, which would also explain the poor thrombus inflammation found in priapism.

**Increased Arterial and Venous Thrombotic Risk in Bmal1 KO Mice**

Substantially shortened occlusion times were found on FeCl₃ challenge in 10-week-old KO mice, both in arterioles and in venules. Because of the progressive rise with age of procoagulant FVII, an attenuated anticoagulant defense system, and weaker NO synthesis, combined with a doubling of the platelet counts at 30 weeks, we expected to see an aggravation of the prothrombotic state and a further decline of the arterial and venous occlusion times in KO versus WT animals following FeCl₃ injury. This could not be confirmed experimentally, but old KO males spontaneously develop priapism, illustrative of the progressive deterioration of the prothrombotic state. Priapism, the development of thrombosis in the penile blood vessels, leading to erectile dysfunction, depends on both the occurrence of prothrombotic risk factors and vascular dysfunction, especially a deregulation of the NO pathway, both present in 30-week-old Bmal1-deficient mice. Histological analysis of Bmal1 KO penile sections demonstrated the presence of platelet and fibrin-rich thrombi in the venous sinus without significant infiltration of inflammatory cells, ie, linking priapism to thrombosis and illustrating how primary and secondary hemostatic risk factors cooperate to cause thrombosis in the penile venous sinus. Priapism has also been associated with increased oxidative stress. Indeed, whereas young Bmal1-deficient mice do show increased ROS levels in several organs, with increasing age, ROS levels in spleen and kidney further increase in Bmal1 KO mice but not in WT mice. Thus, the lack of Bmal1 induces an increase in ROS production, causing a substantial reduction in the average life span of these mice. Because we did not notice changes in the mRNA of pro- and antioxidant genes and in the level of 3-nitrotyrosine-containing proteins in Bmal1 KO versus WT arteries (independent of age), it is not certain that the endothelial dysfunction in the penile blood vessels, implicated in thrombus formation and priapism, is caused by enhanced oxidative stress. However, in view of the reduced NO production in Bmal1 KO mice, the involvement of ROS cannot be excluded.

In conclusion, the present model of premature aging spontaneously develops a hypercoagulable state, characterized by high plasma fibrinogen, FVIII, and FVII levels. However, because of additional age-induced circulatory changes, such as high platelet numbers and vascular changes, resulting from attenuated NO and anticoagulant factor synthesis, the arterial and venous thrombogenicity in aged Bmal1 KO mice is severely upregulated, culminating in obstructive thrombus formation in penile venous sinuses and priapism. Hence, rapidly aging Bmal1 KO mice represent an in vivo model of spontaneous and accelerated thrombogenicity, which may be applicable in various intervention studies.

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**Disclosures**

None.
References


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PROGRESSION OF THE PRO-THROMBOTIC STATE IN AGEING BMAL1 DEFICIENT MICE

Bianca Hemmeryckx¹, Cor E. Van Hove², Paul Fransen², Jan Emmerechts¹, Alexandre Kauskot¹, H. Bult², H. Roger Lijnen¹, and Marc F. Hoylaerts¹

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SUPPLEMENTAL MATERIAL

I. Mouse manipulations

After a 4h fast, mice were anesthetized by intraperitoneal injection of 60 mg/kg sodium pentobarbital (Nembutal, Abbott Laboratories, North Chicago, IL) and blood was retrieved from the retro-orbital sinus on 3.8% sodium citrate. Plasma was collected after centrifuging blood twice at 10,000 rpm for 10 min and was stored at -80° C.

Anesthetized mice were perfused with saline for 10 min to remove blood from the organs, and the aorta was isolated, weighed and used for RNA isolation, organ bath culture or histological and immunohistochemical analysis. RNA was extracted from the whole aorta, from both carotid arteries and from both iliac arteries. Aorta tissues were immediately snap
frozen in liquid nitrogen and stored at -80°C. For immunohistochemical staining and organ bath measurements, only the thoracic portion of the aorta was used. The femur was removed, cleaned, and processed for immunohistochemistry. All animal procedures were approved by the Ethical Committee of the KU Leuven, and performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals (1996).

II. Assays

The differential total blood cell count was performed on a Cell-Dyn® 3200 R counter (Abbott Diagnostics, Louvain-la-Neuve, Belgium). The activated partial thromboplastin time (aPTT), the prothrombin time (PT) and plasma levels of factor (F)VII and FVIII were determined using Synthasil (Instrumentation Laboratory, Zaventem, Belgium) and Innovin and deficient plasma (Siemens Healthcare Diagnostics, Marburg, Germany) on a KC10 ball coagulometer (Amelung, Germany). Plasma fibrinogen levels were measured on an automated coagulation analyzer (BCS XP system, Siemens). Plasma von Willebrand Factor (VWF) antigen was measured using a specific home-made ELISA. Briefly, plates were coated with a rabbit anti-human VWF antibody (1/500; DAKO, Heverlee, Belgium) overnight at 4°C. Plates were subsequently blocked with bovine serum albumin (BSA, 1% in phosphate buffered saline (PBS)) for 2 hrs at RT and washed three times with PBS supplemented with 0.002% Tween 80. Standard samples and 1/30 diluted plasma samples (in PBS with 0.1% BSA) were applied to the coated plate for 3 hrs at RT. Plates were then washed five times with wash buffer. Bound VWF antigen was subsequently detected with a horse radish peroxidase (HRP)-conjugated rabbit anti-human VWF antibody (P0226, dilution 1/8000 in PBS with 0.1% BSA; DAKO), applied for 1.5 hrs at RT. After a final wash, substrate buffer supplemented with 0.1 mg/mL ortho-phenylenediamine dihydrochloride (OPD) and 3x10⁻³ % hydrogen peroxide were added to each well to colorimetrically detect HRP activity. After 1 hr, the reaction was stopped with 50 µl hydrogen sulfate and the plate was read at 490 nm in a ELISA microplate.
reader (model EL808, Biotek, Colmar Cedex, France). A standard curve was obtained from a murine plasma pool for FVII and FVIII and from Standard Human plasma (Siemens) for fibrinogen. Serum C reactive protein (CRP) levels were determined using a commercially available ELISA (USCN Life Science, Wuhan, China).

III. Isometric tension measurements

Segments (2 mm) were mounted between two parallel tungsten wire hooks in 10 ml-organ baths containing Krebs solution (37°C, 95% O₂/5% CO₂, and pH 7.4) with 118 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 25 mM NaHCO₃, 0.025 mM CaEDTA and 11.1 mM glucose. Indomethacin (10 µM) was added to avoid vasomotor interferences due to prostanoids (1). Isometric tension was measured with force transducers (EMKA technologies) connected to a data acquisition system (Powerlab 8/30, ADInstruments, Spechbach, Germany) and reported in mN. Segments were gradually stretched until a stable loading tension of 14 mN was attained. One segment was incubated with a combination of 300 µM NΩ-nitro-L-arginine methyl ester (LNAME) and 300 µM NΩ-nitro-L-arginine (LNA) to study smooth muscle cell function; in the adjacent segment nitric oxide synthase (NOS) activity was not inhibited. One hour after isolation of the aorta, both rings were exposed to cumulative concentrations of phenylephrine (PE, 3 nM - 30 µM). The difference in maximum responses between adjacent rings without and with LNA/LNAME was determined as an index of basal release of NO (2). The PE curve was immediately followed by cumulative injection of diethylamine NONOate (DEANO, 1 nM - 3 µM) in rings with eNOS inhibitors, or acetylcholine (ACh, 3 nM - 10 µM) in rings without eNOS inhibitors.

Responses to vasodilators were expressed as percentages of the initial contractions. Maximal responses and pD₂ (negative logarithm of concentration causing 50% of maximal response) were determined for each segment using four parameter logistic regression.
IV. In vivo thrombosis and inflammation model

Thrombotic occlusion times were measured in mesenteric arterioles and venules, as previously described, upon FeCl\(_2\)-induced vascular lesions (3, 4). The procedure to investigate vessel inflammation by inducing leukocyte rolling over the endothelium of the mesenteric arteries is similar: instead of inducing vascular injury by a paper soaked in 5% FeCl\(_3\), a paper was used impregnated with 30 \(\mu\)M of the endothelial cell activator A23187 (Sigma-Aldrich, Bornem, Belgium), diluted in Tyrode buffer (10 mM HEPES, 3 % human albumin, 11.1 mM glucose, 273 mM NaCl, 5.4 mM KCl, 23.8 mM NaHCO\(_3\), 0.86 mM Na\(_2\)H\(_2\)PO\(_4\), pH 7.4) supplemented with 1 mM CaCl\(_2\) and 1 mM MgCl\(_2\). After a 1 min application, the impregnated paper was carefully removed. Movies were taken (200 frames at a rate of 2 frames/s) of the mesenteric artery before, 1 min and 10 min after the application of A23187. The number of rolling leukocytes (i.e. the number present in the view field for at least 5 frames) were determined in 9 frames (3 at the start, middle and end of the movie). Neither for the wild-type (WT), nor for the Bmal1-deficient (KO) mice, age-dependent effects were noticed; therefore the data of all observations (3 time points) were averaged per group. In this model, heparin largely inhibits venular thrombosis, without affecting arteriolar thrombosis (4), validating the use of venular occlusion times, as a model of venous thrombosis.

V. Histology and immunohistochemistry

Femur tissues were fixed in 2% paraformaldehyde (PFA) in Phosphate-Buffered Saline (PBS) for 24 hrs and subsequently decalcified by immersion in a daily fresh 0.5 M EDTA solution for 10 days. Prior to isolation of penile tissues, animals were perfused twice with saline and 1% PFA solution; tissues were removed and processed as described for femur. Aorta tissues were processed as described for adipose tissue (5). After rinsing in PBS, both femur, penile and thoracic aortic tissues were embedded in paraffin, dehydrated in ethanol
(70%-96%-100%) and cleared in xylene. Sections were cut using a HM360 microtome (Microm, Walldorf, Germany) and were 10 µm longitudinal and were deparaffinized in xylene, followed by decreasing series of ethanol concentrations and distilled water. The first microscopic glass of every series of 8 µm thick aortic and penile and 10 µm thick bone marrow paraffin sections was stained with hematoxylin and eosin (H&E), the second one with a Martius Scarlet Blue stain (MSB) (6). Megakaryocytes in bone marrow sections, aortic sections and blood vessels in penile sections were stained for VWF by initially exposing antigens by trypsin digestion (25 µg/ml in 0.1% CaCl₂) for 7 min. Endothelial protein C receptor (EPCR) and thrombomodulin (TM) antigens in aortic tissues were released by immersion in Target Retrieval solution (S1699, DAKO) for 20 min at 95°C. Subsequently, endogenous peroxidase activity was blocked by a 20 min incubation in methanol containing 0.3% H₂O₂ (Merck, Overijse, Belgium) at RT. Aspecific binding to the biotin-labeled secondary goat anti-rabbit and rabbit anti-goat (RAG) antibodies (Abs, DAKO) and to the peroxidase-labeled RAG Ab (DAKO) was blocked by incubating sections, before application of the primary rabbit anti-human VWF Ab (1/500; A0082; DAKO), goat anti-mouse (GAM) affinity purified EPCR Ab (1/1000), and GAM TM Ab (1/9640, G409) with 20% pre-immune rabbit or goat serum (DAKO) for 45 min. The primary and secondary Abs (1/300) were diluted in Tris-NaCl-blocking buffer (TNB) (0.1 M Tris-HCl pH 7.5; 0.15 M NaCl, 0.5% blocking reagent (TSA BT kit, NEL700001, Perkin Elmer, Boston, MA) and applied O/N and 45 min at RT, respectively. The secondary Abs have been preincubated with 10% or 20% (EPCR) pre-immune mouse serum (DAKO) O/N at 4°C. The biotin label on the bone marrow and aortic sections was detected by avidin-biotin-peroxidase complex (Vector Laboratories Ltd, Peterborough, United Kingdom). The peroxidase signal was then amplified using the Tyramide Signal Amplification kit (Perkin Elmer, Boston, MA). VWF, EPCR and TM signals were then visualized with 3, 3’-diaminobenzidine (DAB) (Sigma-Aldrich) at RT. Sections
were then run through a series of increasing percentages of ethanol and finally xylene. The sections were mounted with DEPEX (VWR, Leuven, Belgium). Negative control included a section incubated with buffer alone.

VI. Microscopy analysis

H&E, MSB, EPCR, TM and VWF stainings were analyzed with a Zeiss AxioPlan 2 Imaging microscope (Zeiss, Jena, Germany) with normal light and pictures were taken with Axiovision Rel. 4.6. (Zeiss) at x100 (H&E) and x400 (H&E, MSB, EPCR, TM, VWF) magnification. To measure the density of VWF-positive megakaryocytes in bone marrow sections of 30W old KO and WT animals (n = 2), 7-8 fields per animal were randomly chosen and the total field area and number of brown-colored VWF-positive megakaryocytes were determined. The megakaryocyte density was then defined as the ratio of megakaryocyte number divided by field area. The data for all animals in a given group were averaged. The intensity and homogeneity of the EPCR, VWF and TM staining were scored by two independent investigators (EPCR: r = 0.45, p < 0.0001; VWF: r = 0.66, p < 0.0001; TM: r = 0.84, p < 0.0001 for investigator 1 versus 2) using the following criteria: 0 = no staining; 1 = light and heterogeneous staining; 2 = strong and heterogeneous staining; 3 = light and homogeneous staining and 4 = strong and homogenous staining. A third investigator randomly choose 6 sections per animal. The 6 measurements of each investigator were then taken to calculate an average score for each staining, for each animal. Subsequently, an average score for each staining was calculated by averaging all scores for each animal in one group.

VII. Quantitative real-time PCR (RT-PCR)

Taqman gene expression assays (Applied Biosystems, Lennik, Belgium) were used to analyze mRNA levels of the following genes in aorta tissue by quantitative RT-PCR:
endothelial and inducible nitric oxide synthase (eNOS, Mm00435217_m1; iNOS, Mm01309902_m1), TM (Mm00437014_s1), EPCR (Mm00444092_m1), tissue factor (TF, Mm00438853_m1), and VWF (Mm00550376_m1). Protocols for mRNA isolation and cDNA synthesis have been described earlier (7). RNA concentrations were measured spectrophotometrically using the NanoDrop2000 machine (Nanodrop Technologies, Wilminton, DE). Data were obtained as cycle threshold (Ct) values and expressed as copy number of target mRNA relative to 10^5 copies of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

VIII. Statistical analysis

GraphPad Prism 4.03 software was used to determine statistical differences (GraphPad, La Jolla, CA). Data are represented as mean ± SEM, or as median ± 25-75% confidence interval, when indicated. Differences between two groups were analyzed via non-parametric Mann-Whitney U-tests. Correlation analysis was performed using the non-parametric Spearman rank correlation test. Differences in frequency of KO animals displaying priapism between two age groups (20-25W versus 25-30W) were calculated by the Fisher’s exact test. Normally distributed concentration response curves in the vasodilatation tests were analyzed as a function of age with a two-way ANOVA, and other data of the isometric tension measurements with one-way ANOVA.

IX. References


