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Whole blood thrombin generation in Bmal1-deficient mice

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Summary

The Calibrated Automated Thrombogram (CAT) assay that measures thrombin generation (TG) in platelet-poor and -rich plasma, is increasingly being recognised as a more sensitive tool to determine the overall function of the haemostatic system. We developed a method enabling the measurement of TG in a small aliquot of blood. The objective was to validate this assay in mouse blood and to examine the rate and extent of TG in a mouse model of premature aging. TG was assayed in blood from 20- to 28-week-old *brain and muscle ARNT-like protein-1* (Bmal1)-deficient (knockout, KO) mice and wild-type (WT) littermates. Bmal1-KO mice are known to display symptoms of premature aging. TG was initiated by adding calcium, tissue factor and a thrombin specific substrate. After TG, the samples were prepared for scanning electron microscopy (SEM). The intra-assay variations (%) in

mouse blood of the endogenous thrombin potential (ETP), peak height, lag time, time-to-peak and velocity index were 10% or less (n=24). We found that Bmal1-KO mice have a significantly ($p<0.001$) higher ETP (437 ± 7 nM.min; mean $\pm$ SD, n=7) when compared with WT mice (ETP= 220 ± 45 nM.min; mean $\pm$ SD, n=5). The peak heights also differed significantly ($p=0.027$). By applying SEM we found that Bmal1 deficient mice display a denser fibrin network with smaller pores compared to WT mice. In conclusion, the whole blood TG assay in mice revealed to be reproducible. As a proof-of-principle we have shown that the whole blood TG assay is capable of detecting a prothrombotic phenotype in Bmal1-KO mice.

Keywords

Thrombin generation, Bmal1-KO mice, aging

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Introduction

In the research field of thrombosis and haemostasis, the use of mouse models to investigate human coagulation disorders is getting increasingly popular (1-5). Plasma coagulation tests like the activated partial thromboplastin time (aPTT) and the prothrombin time (PT) are performed on regular basis. However, it has been demonstrated that these tests lack the sensitivity to detect conditions of hypo- and especially hypercoagulability (6-9). The Calibrated Automated Thrombogram (CAT) assay that measures thrombin generation (TG) in platelet-poor and platelet-rich plasma, is increasingly being recognised as a more sensitive tool to determine the overall function of the haemostatic system (10-14). Tchaikovski et al. (4) adapted this assay to measure TG in mouse plasma enabling the demonstration of factor (F)V Leiden-, oral contraception- and pregnancy-induced hypercoagulability. In addition, the plasma tests require relatively large amounts of mouse blood and do not account for the presence of blood cells.

Until recently, it was not possible to measure TG in whole blood due to technical problems that lead to highly erratic signals related to quenching of the fluorescent signal and red blood cell

sedimentation and retraction (15, 16). We recently developed a method in which these problems were overcome enabling the measurement of TG in a small aliquot of blood. The advantage of the use of this whole blood CAT (WB-CAT) assay is that it enables us to include the effect of blood cells on TG, thereby getting one step closer to physiological conditions compared to plasma. A more practical advantage is that it omits centrifugation steps, thereby also reducing the time needed to perform the assay and the experimental errors that can occur. We recently published the technical validation of this WB-CAT assay demonstrating its utility (17, 18). An additional advantage of the WB-CAT is the small sample volume necessary to perform the assay (less than 100 μ l), rendering it needless to sacrifice the mice.

In this paper we have studied the prothrombotic phenotype in *Brain and muscle ARNT-like protein-1* (Bmal1) clock gene knockout (KO) compared to wild-type (WT) mice using our WB-CAT assay. The Bmal1-KO mice have an impaired circadian behaviour and demonstrate loss of rhythmicity in the expression of their clock genes. They are known to have a reduced lifespan and display symptoms of premature aging (19-21). Plasma clotting assays revealed a discrepancy in test results. Fibrinogen levels and FVII

activity were increased and PT was decreased in Bmal1-KO mice, while the aPTT was prolonged (19, 22, 23).

The goal of this study was to demonstrate that the WB-CAT assay can also be applied on mouse blood. As a proof-of-principle we have shown that the WB-CAT assay is capable of detecting a prothrombotic phenotype in Bmal1-KO mice.

Materials and methods

Mice

For the first methodical part of the study, whole blood was taken from C57Bl/6 mice (Charles River, Sulzfeld, Germany) to validate the WB-CAT assay ($n = 6$). For the second part of our study, male Bmal1-KO mice and WT littermates were generated in the animal facility of the Centre for Molecular and Vascular Biology (KU Leuven, Leuven, Belgium) as previously described (23). Their age varied between 22 and 28 weeks ($n = 12$). All animals were kept in 12-hour night/day temperature controlled cages 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 Committees of the KU Leuven or Maastricht University and performed in accordance with the *National Institutes of Health Guide for the Care and Use of Laboratory Animals* (1996) (23).

Blood sampling

Mice were anaesthetised with subcutaneous injection of ketamine and xylazine (0.1 mg/g and 0.02 mg/g body weight). Subsequently, blood was taken via retro-orbital puncture using a glass capillary with a length of 2 cm that was filled with sodium citrate (3.8%). The first three drops of citrate/blood were discarded. The following drops were collected on 3.8% sodium citrate (1/10 volume) in a 0.5 ml Eppendorf tube and mixed immediately to prevent clotting. The red blood cell and platelet count were measured using a Cell-

Dyn 3200R counter (Abbott Diagnostics, Louvain-La-Neuve, Belgium).

Whole blood thrombin generation

Whole blood TG was performed in a 96-well plate as previously described (17). Briefly, the total volume of blood needed for TG and calibration measurement in triplicate is 60 μ l. For TG measurement, 30 μ l of citrated whole blood was mixed with 10 μ l thrombin specific substrate (P2Rho; 1.8 mM) and activated with 20 μ l TF and CaCl₂ (1.5 pM and 50 mM, respectively). For the calibration measurement we mixed 30 μ l of citrated whole blood with 10 μ l thrombin specific substrate (P2Rho; 1.8 mM) and 20 μ l α 2M-thrombin (300 nM thrombin activity). Hirudine (50 nM) was also added to the calibrator wells in order to prevent thrombin formation that could affect the linearity of the calibration curve. Immediately after activation, 5 μ l of the mixture was added to the wells containing the porous filter paper discs and the wells were covered with 40 μ l of mineral oil (USB Corporation) to prevent evaporation. Samples were run for 50 minutes (min) and fluorescence was recorded every 5 seconds with a Fluoroskan Ascent microplate fluorometer with $\lambda_{ex} = 485$ nm and $\lambda_{em} = 538$ nm (Thermolabsystems, Helsinki, Finland). All procedures were performed at 37°C.

Scanning electron microscopy

Following TG, the filter paper disks were fixated with glutaraldehyde, dehydrated with ethanol/HMDS and coated with gold using a Rotary-Pumped Sputter Carbon Coater (Quorum Technologies, East Grinstead, West Sussex, UK). Samples were analysed using a Phenom G2 pro desktop scanning electron microscopy (SEM, Phenom World, Eindhoven, The Netherlands).

Statistical analysis

The Mann-Whitney U test was used for statistical analysis of the data using SPSS 19.0 for windows (SPSS, Chicago, IL, USA). A p-value below 0.05 was considered to be significant.

Results

TF dependency

TG was measured in mouse blood that was activated with various TF concentrations in the presence of P2Rho (300 μ M) and CaCl₂ (16.7 mM). The values of the thrombogram parameters are depicted in ► Table 1. Substantial thrombin generation was already seen in the condition without TF, possibly resulting from the presence of endogenous TF or contact activation. However, addition of CTI (50 μ g/ml) to the blood collection tube partly reduced contact activation as indicated by a minor prolongation of the lag time (5.7 vs 7.8 min) and time-to-peak (10.6 vs 13.3 min) without affecting the other parameters. When the same experiment was performed with mouse plasma, CTI (50 μ g/ml) completely diminished

Table 1: Tissue factor dependency of thrombin generation parameters in C57Bl/6 mice.

	ETP	Peak	Lagtime	TTP	VI
	(nM*min)	(nM)	(min)	(min)	(nM/min)
0 pM TF	310 $\pm$ 26	24 $\pm$ 0.4	5.7 $\pm$ 0.1	10.6 $\pm$ 0.1	4.9 $\pm$ 0.2
0 pM TF + CTI	339 $\pm$ 14	22 $\pm$ 0.7	7.8 $\pm$ 1.6	13.3 $\pm$ 2.0	40 $\pm$ 0.4
0.25 pM TF	284 $\pm$ 26	30 $\pm$ 2.5	4.3 $\pm$ 0.3	7.4 $\pm$ 0.3	9.8 $\pm$ 0.9
0.5 pM TF	285 $\pm$ 12	44 $\pm$ 4.3	3.0 $\pm$ 0.2	5.3 $\pm$ 0.3	19 $\pm$ 2.5
1 pM TF	320 $\pm$ 11	55 $\pm$ 3.1	2.2 $\pm$ 0.1	4.2 $\pm$ 0.1	27 $\pm$ 2.0
2 pM TF	345 $\pm$ 14	68 $\pm$ 7.4	1.5 $\pm$ 0.1	3.2 $\pm$ 0.1	42 $\pm$ 8.5
2.5 pM TF	332 $\pm$ 16	80 $\pm$ 2.1	1.1 $\pm$ 0.1	2.7 $\pm$ 0.1	52 $\pm$ 2.6

Data represent the mean $\pm$ SD (N=3); TF, tissue factor; CTI, corn trypsin inhibitor; ETP, endogenous thrombin potential; TTP, time-to-peak; VI, velocity index.

Table 2: Intra-assay variation of whole blood thrombin generation in a C57Bl/6 mouse.

	Mean	SD	CV
ETP (nM*min)	309.7	37.1	12.0
Peak (nM)	40.3	3.1	7.6
Lagtime (min)	2.6	0.2	7.4
TTP (min)	5.0	0.3	6.2
VI (nM/min)	16.8	1.7	10.1

Blood was activated with 0.5 pM TF and 16.7 mM CaCl₂. SD, standard deviation; CV, coefficient of variation; ETP, endogenous thrombin potential; TTP, time-to-peak; VI, velocity index; The sample was tested 24 times in one single run.

thrombin generation. Moreover, in the absence of exogenous TF, thrombin generation was neither observed. These findings indicate that the presence of blood cells diminishes the inhibitory effect of CTI and that the resulting TG is likely not due to the presence of endogenous TF. With increasing TF concentrations, a dose-dependent increase in peak height and decrease in lag time and time-to-peak was observed. The ETP was not dependent on the TF concentration in this assay.

Precision of the WB-CAT assay in mouse blood

The intra-assay variation of the WB-CAT assay was tested for 24 replicates. Blood was taken from three different WT mice and activated with 0.5 pM TF, 300 μ M P₂Rho and 16.7 mM CaCl₂. The mean intra-assay variation of the ETP, peak height, lag time, time-to-peak and velocity index from one representative mouse are shown in ►Table 2. Except for the ETP, all the parameters have an acceptable coefficient of variation (CV $\leq$ 10%).

Comparing TG in Bmal1-KO and WT mice

TG was measured in blood of WT and KO mice in the presence of 300 μ M P₂Rho, 0.5 pM TF and 16.7 mM CaCl₂. The mean age of the WT and KO mice was 24.2 and 25.2 weeks, respectively (p=0.48). ►Figure 1 shows the mean TG curve of the WT group (black line) and the KO group (interrupted line). The mean values of the TG parameters for each group are depicted in ►Table 3. The lag time, time-to-peak and velocity index did not differ significantly between the two groups (p-values were 0.45, 0.145 and 0.742 respectively). However, significant higher TG peak height (30.1 nM vs 45.8 nM; p=0.027) and ETP (220 nM.min vs 437.6 nM.min; p<0.001) values were found in the KO mice. The higher TG in the KO mice correlates with the prothrombotic phenotype seen in these mice. Red blood cell count was found to be significantly (p<0.001) higher in the KO mice (9914x10³/ μ l $\pm$ 724x10³/ μ l) compared to WT mice (7900x10³/ μ l $\pm$ 334x10³/ μ l). Also the platelet count of KO mice was significantly (p<0.005) higher (1281x10³/ μ l $\pm$ 157x10³/ μ l) compared to that of WT mice 942x10³/ μ l $\pm$ 152x10³/ μ l).

Figure 1: Representative TG curves of Bmal1-KO and WT mice. Samples were activated with 0.5 pM TF and 16.7 mM calcium. The full and interrupted lines represent the WT and KO mice, respectively. Both curves are the mean of three measurements.

Visualisation of fibrin network

At the end of the TG assay, the samples were fixated and analysed by scanning electron microscopy. Representative images are shown in ►Figure 2 (5000x magnification). The images in the upper part are clots from WT mice, while images of the lower part represent those from the KO mice. While the fibrin structure in WT clots is more localised, the fibrin formation is seen throughout the whole paper matrix in KO clots. In addition, much more fibrin is present in clots of KO versus WT mice.

Discussion

It is becoming increasingly apparent that global blood clotting tests like thrombin generation assays are more sensitive to detect conditions of hypo- and especially hypercoagulability when compared to conventional assays only measuring clotting times. Since transgenic mouse models have been very helpful in increasing our insights in the process of blood coagulation, it is appropriate to develop a method by which TG can be measured in mouse blood.

Table 3: Whole blood thrombin generation parameters in Bmal1-KO and WT mice.

	WT (n=5)		KO (n=7)		
	Mean	SD	Mean	SD	P-value
ETP (nM*min)	220.3	45.1	437.6	78.3	<0.001
Peak (nM)	30.1	3.7	45.8	14.3	0.027
Lagtime (min)	2.5	0.8	2.2	0.5	0.45
TTP (min)	4.8	0.9	5.9	1.6	0.145
VI (nM/min)	13.7	3.3	15.0	9.6	0.742

Blood samples of Bmal1-KO and WT mice were activated with 0.5 pM TF and 16.7 mM CaCl₂. SD, standard deviation; ETP, endogenous thrombin potential; TTP, time-to-peak; VI, velocity index; KO, n=7; WT, n=5.

Figure 2: Representative SEM pictures of the fibrin network of Bmal1-KO and WT mice. TG was activated as described in Figure 1. After fixation SEM pictures were taken of the clots of Bmal1-WT (A) and KO-mice (B) of which three representative pictures are shown. Magnification was 5000x.

TG tests have been reported for platelet-poor and platelet-rich murine plasma. However, a major drawback of these assays is the requirement of relatively large amounts of plasma implying that the animal has to be sacrificed. In addition, under the conventional CAT conditions, mouse plasma appears to be highly reactive hampering reliable TG measurements (1, 4). These problems were solved by diluting the plasma samples. However, dilution of plasma dramatically distorts the balance between pro- and anticoagulant activity of the sample (11). Our assay proved to be reproducible and requires only a small amount of blood sample (60 μ l per assay) and enables us to perform TG in blood collected at different time points from the same mouse. Moreover, the usage of whole blood eliminates the need to separate the analyte with a centrifugation step thereby reducing the number of pre-analytical variables and the experimental errors. Finally, measuring TG in the presence of blood cells might bring the assay closer to physiology. It is advised to use a low TF concentration (0.5 pM) to keep the assay sensitive for the coagulations factors of the intrinsic coagu-

lation pathway and to prevent clotting before the measurement is actually started.

As proof-of-principle we measured TG in the Bmal1 clock gene KO mice, which display symptoms of premature aging (21-23). These KO mice exhibit imbalances in the haemostatic mechanism, i.e. higher plasma levels of procoagulant factors VII, VIII, fibrinogen and raised platelet count on the one hand and decreased endothelial anticoagulants (thrombomodulin and protein C receptor) on the other hand. It was concluded that, with aging, progressive endothelial dysfunction, rising platelet counts, and high factor VII further enhance thrombogenicity and provoke priapism (23). In this paper we showed that Bmal1-KO mice have a higher TG and fibrin formation compared to their Bmal1-WT littermates. This is confirming the results of Wolberg et al. who established that the fibrin formation is dependent on the thrombin concentration. A low thrombin concentration leads to a loosely woven fiber network with thick fibrin fibres, whereas high thrombin concentrations lead to a compact, dense network of thin fibrin fibres

What is known about this topic?

- Studies have shown that hypo- and hypercoagulable conditions can be detected by measuring thrombin generation with the Calibrated Automated Thrombogram (CAT) assay in plasma.
- We developed a CAT-based assay (WB-CAT) able of measuring thrombin generation in whole blood.
- Bmal1-KO mice have a prothrombotic phenotype, although coagulation clotting tests reveal opposite results, as the aPTT is prolonged, while the PT is shortened.

What does this paper add?

- We showed that the WB-CAT assay is also feasible and reproducible in whole blood of mice. The advantage of this assay is that only very small amounts of blood are needed, so more conditions can be tested (e.g. different coagulation triggers and concentrations). The number of mice needed to conduct a study will decrease since blood samples can be taken repeatedly in time from the same mouse.
- As a proof of principle we looked at thrombin generation in Bmal1-KO mice and we could demonstrate with our WB-CAT assay a higher ETP and Peak height in these mice compared to their wild-type littermates.

(24-26). We also confirmed a higher platelet count in Bmal1-KO mice compared to WT mice of the same age. However, earlier studies from our group demonstrated that the whole blood platelet count does not contribute to the extent of TG in whole blood (17). Additionally, we established a higher red blood cell count in KO compared to WT mice. Based on our previous study it is not likely that an increased haematocrit affects TG in our assay (17). This is in contrast to the findings of Whelihan et al. who did find a haematocrit-dependent TG in mixtures containing only plasma and red blood cells (27-29). However, our experimental conditions were completely different (lower TF concentration; creation of a thin layer of blood by means of a porous matrix) which may provide an explanation for the observed differences.

The present findings of our study showed that our whole blood thrombin generation assay is also applicable on mouse blood and has a good reproducibility. As a proof-of-principle we have shown that the whole blood TG assay is capable of detecting a prothrombotic phenotype in Bmal1-KO mice.

Conflicts of interest

None declared.

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