Chapter 6

Long-term estrogen treatment of mice with a prothrombotic phenotype induces a sustained increase in thrombin generation without affecting tissue fibrin depositions

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Dear editors,

In thrombin generation assays, the resulting area under the curve (endogenous thrombin potential; ETP) is considered to have a predictive value. A low ETP value is associated with a bleeding tendency, whereas an increased value represents a hypercoagulable state. Regarding this latter category, it has been shown that women using oral contraceptives, which are associated with an increased risk for venous thrombosis, have higher ETP values than non-users.[1]

The estrogen in oral contraceptives, often 17α-ethinylestradiol, is considered to be the predominant thrombogenic component. We have previously shown that oral ethinylestradiol (EE) in mice has profound effects on the plasma levels of pro- and anticoagulant factors.[2] Now, we report the overall effect of these estrogen-induced alterations on the hemostatic balance by assessing thrombin generation and determine the net effect on a spontaneous thrombotic phenotype, i.e. fibrin depositions in lung tissues of the prothrombotic factor V Leiden ($FV^{Q/Q}$) and thrombomodulin proline mutant ($TM^{pro/pro}$) mice.

Hereto, ovariectomized $FV^{Q/Q}$ and $TM^{pro/pro}$, and wild-type mice as a reference, were orally treated with 1 µg EE/day or a vehicle for either 10 days or 10 weeks (n=12 mice per group). After the last EE or vehicle administration, mice were exsanguinated and platelet-poor plasma was obtained.[3] In order to determine the estrogen-induced effects on fibrin depositions, lungs of 10-week treated mice were isolated as previously described.[4,5]

To evaluate the effects on the overall plasma coagulability after 10 days of treatment, we assessed thrombin generation by means of the Calibrated Automated Thrombogram method using 1 pM tissue factor to trigger 1:6 diluted mouse plasma.[6] Oral EE treatment resulted in significantly increased ETP values in $FV^{Q/Q}$ (fig. 1A) and $TM^{pro/pro}$ mice (fig. 1B), which was comparable to the effects observed in wild-type animals (448±30 nM*min vs. 930±41 nM*min, $p<0.001$). The thrombin generation curves in figures 1A and 1B show that the inhibition of thrombin activity, i.e. the tail of the curve, is predominantly affected in EE-treated mice, which largely determines the differences in ETP values between the vehicle- and EE-treated groups. Since antithrombin is the main inhibitor of thrombin activity, we measured the antithrombin activity levels in plasma (Coamatic antithrombin kit, Chromogenix) and found that estrogen administration caused a significant 15% reduction in antithrombin activity levels, which negatively correlated with the ETP values (Pearson r=-0.51, p=0.002).

To determine the relation between thrombin generation and a spontaneous thrombotic phenotype, $FV^{Q/Q}$ and $TM^{pro/pro}$ mice were daily treated with vehicle or 1 µg EE/day for 10 weeks. During these 10 weeks, none of the mice died or showed signs of abnormalities. In addition, careful inspection of the mice upon sacrifying revealed no signs of macrovascular thrombosis. Thrombin generation curves and ETP values after 10 weeks of treatment showed a comparable pattern to the 10-day treatment, with ETP values of 446±40 nM*min vs. 746±98 nM*min ($p<0.01$) for $FV^{Q/Q}$ mice and 683±75 nM*min vs. 901±70 nM*min ($p<0.05$) for $TM^{pro/pro}$ animals, indicating a sustained increase in thrombin generation during the 10 weeks of oral ethinylestradiol treatment.

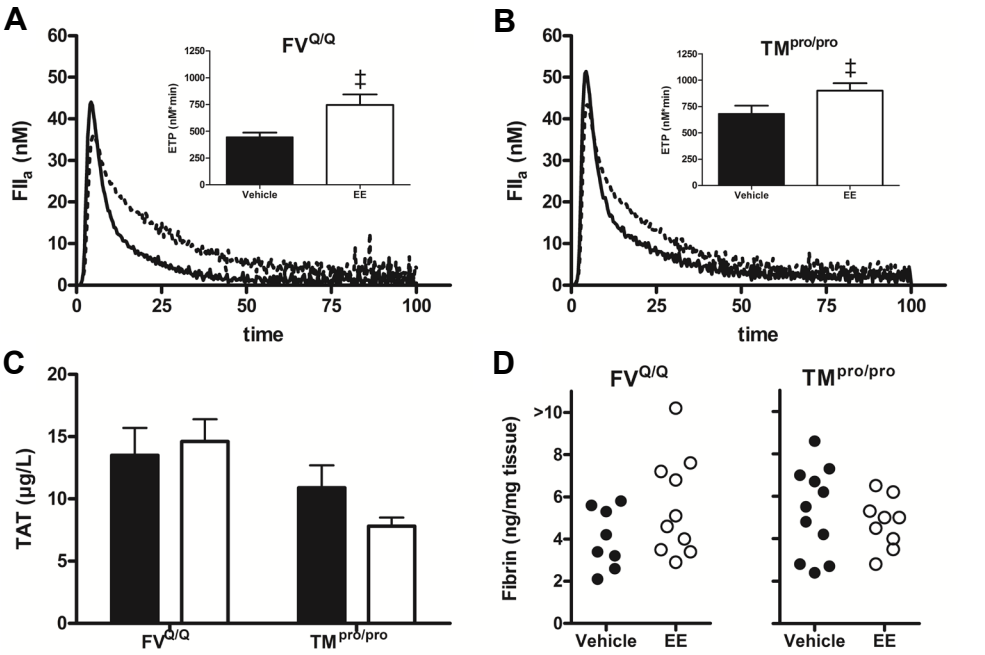

Figure 1: Factor V Leiden (A) and thrombomodulin proline mutant (B) mice were orally treated with 1 µg ethinylestradiol (EE; dashed line) or a vehicle (solid line) per day for 10 days after which thrombin generation was determined in 1:6 diluted plasma triggered with 1 pM tissue factor. The resulting endogenous thrombin potential (ETP) values are presented in the inserts. Plasma thrombin-antithrombin (TAT) complexes (C) and fibrin concentrations in lung homogenates of $FV^{Q/Q}$ and $TM^{pro/pro}$ (D) were determined after 10 weeks of treatment. Data are presented as mean ± standard error of the mean of n=12 mice per group (A-C) or as individual measurements (D). ‡$p<0.001$ as compared to vehicle-treated animals.

As a biochemical marker for a thrombotic phenotype, fibrin depositions in lung homogenates were determined via Western blotting.[5] Although fibrin depositions of $FV^{Q/Q}$ and $TM^{pro/pro}$ mice were in the lower range of detection (2-10 ng/mg tissue), they were higher than fibrin concentration present in wild-type mice, which did not exceed the detection threshold of 2 ng/mg. However, oral estrogen administration for 10 weeks did not result in significant increased fibrin depositions in either the $FV^{Q/Q}$ (4.0±0.5 ng/mg vs. 5.5±0.7 ng/mg) or $TM^{pro/pro}$ mice (5.3±0.6 ng/mg vs. 4.8±0.4 ng/mg; figure 1C). In addition, plasma thrombin-antithrombin complex analyses (Enzygnost TAT micro, Dade Behring) did also not differ between vehicle- and EE-treated animals (figure 1D), which supports the absence of an effect on fibrin depositions despite a sustained increased potential to generate thrombin upon estrogen treatment.

Previous studies have shown that the $FV^{Q/Q}$ status converts to a spontaneous perinatal lethal phenotype when combined with mice carrying gene deletions in anticoagulant genes that in itself are not lethal, like tissue factor pathway inhibitor and protein Z.[7,8] In addition, microvascular thrombosis in lungs of $TM^{pro/pro}$ mice proved highly responsive to hypoxia,[5] showing that these models are capable of displaying enhanced fibrin deposition upon risk factor exposure. On the other hand, with respect to oral ethinylestradiol administration, we have previously shown that short-term EE exposure was not able to aggravate the thrombogenicity in a stasis-induced thrombosis model,[9] which is in line with these current observations.

From the data presented here, we conclude that despite the fact that long-term oral ethinylestradiol administration can induce a sustained increase in thrombin generation in factor V Leiden and thrombomodulin proline mutant mice, this does not translate into a spontaneous macrovascular or microvascular thrombotic phenotype, which argues against the use of these mice in studying the effects of estrogens on thrombosis.

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