EXPERIMENTAL STUDY

Estrogen increases coagulation factor V mRNA levels via both estrogen receptor- α and - β in murine bone marrow/bone

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Abstract

Objectives: Both oral estrogen-based hormone-replacement therapy and contraceptives increase the risk of venous thromboembolism. Several circulating factors involved in coagulation/fibrinolysis are expressed mainly in the liver whilst some are expressed in extrahepatic tissues, including bone marrow. The aim of this study was to identify estrogen-responsive target genes involved in the pathogenesis of estrogen-induced venous thromboembolism.

Methods: Ovariectomized mice were treated with 17 β -estradiol and possible effects on the expression of genes related to coagulation/fibrinolysis were investigated using DNA microarray analyses.

Results: None of the selected genes was regulated by 17 β -estradiol in the liver. Interestingly, 17 β -estradiol increased mRNA levels of coagulation factor V in the bone marrow/bone. Furthermore, this stimulatory effect of 17 β -estradiol on coagulation factor V expression can be mediated via both estrogen receptor- α and - β .

Conclusions: The expression of bone marrow-derived, but not liver-derived, coagulation factor V is increased by estrogen treatment in mice. The pathophysiological importance of this finding for estrogen-induced venous thromboembolism remains to be determined.

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Introduction

Both estrogen-based hormone-replacement therapy (HRT) and oral contraceptives increase the risk of venous thromboembolism (VTE) (1–4). The increased risk is thought to be due to the estrogen component of these treatments but no clear molecular mechanism behind estrogen-induced VTE has yet been identified. Several factors involved in coagulation/fibrinolysis are generally regarded to be expressed mainly in the liver but some are also expressed in the bone marrow. Most previous studies on the effects of estrogen on hemostatic balance have focused on circulating liver-derived factors. It has been shown that oral contraceptives regulate the levels and activity of multiple circulating hemostatic factors. Activated protein C (APC) sensitivity as well as the plasma concentration and activity of antithrombin III, protein S and plasminogen activator inhibitor-I (3–6) are reduced, while circulating levels of fibrinogen, prothrombin and coagulation factor VIII (4) have been reported to be increased by estrogen-based treatments. The aim of the present study was to identify estrogen-regulated target genes in liver and/or bone marrow of potential importance for the pathogenesis of estrogen-induced

VTE. The mRNA levels of several hemostatic balance-related genes, i.e. the coagulation cascade, the anticoagulant system and the fibrinolytic system, were analysed in liver and bone marrow/bone, using the DNA microarray technique.

Materials and methods

Animals

Before ovariectomy, the mice were anesthetized with a mixture of ketamine hydrochloride (75 mg/kg; Ketalar; Pfizer AB, Täby, Sweden) and medetomidine (1 mg/kg, i.p.; Dormitor Vet.; Orion Pharma AB, Sollentuna, Sweden).

Short-term estrogen treatment Eight-week-old ovariectomized female C57Bl/6 mice were injected s.c. with 2.3 μ g 17 β -estradiol benzoate/mouse (Sigma, St Louis, MO, USA). The mice were killed 10, 24 and 48 h after the injection.

Long-term estrogen treatment Wild-type (WT) and estrogen receptor (ER)-inactivated mice were bred as previously described (7–10). The mice were ovariectomized

at 2 months of age and then injected s.c. with 2.3 µg 17β-estradiol benzoate/mouse per day (Sigma) for 5 days/week for 3 weeks. Control mice received injections of vehicle (olive oil; Apoteksbolaget, Göteborg, Sweden). The Ethics Committee of Göteborg University approved this study.

DNA microarray analysis

RNA was isolated from the liver and bone marrow/bone (humerus) as described elsewhere (11, 12). For microarray analysis of the long-term estrogen treatment experiment, the RNA samples were pooled into two pools per animal group. The RNA was reverse transcribed into cDNA, labeled and analysed by DNA microarray (MG-U74A Array; Affymetrix, Santa Clara, CA, USA).

Bioinformatics

Scanned output files were analysed using Affymetrix micro array suite version 5.0 software as described elsewhere (11). For the long-term estrogen treatment experiment, comparisons were made between the two vehicle-treated and the two estrogen-treated GeneChips, generating a total of four comparisons. To be regarded as regulated by estrogen in WT mice, the gene had to be expressed at a detectable level (according to the Affymetrix algorithm) in all pools and at least two of the four comparisons had to be considered to be increased or decreased according to Affymetrix algorithms. The average fold increase or decrease should be at least 1.6-fold.

Real-time PCR analysis

The confirmatory real-time PCR analyses were run on samples from individual animals using the ABI Prism 7700 sequence detection system (PE Applied Biosystems, Stockholm, Sweden) as described elsewhere ($n = 4-8$) (11). The sequences of primers and FAM-labeled probes were: coagulation factor V (FV) forward primer: 5'-TCG-AGAACAAGATAAACCTAAAACCAT; reverse primer: 5'-AGCTGGCGCTTTCATCCA and probe: 5'-AGACAGG-AAGACCCACATGATGAAGCAC; coagulation factor X (FX) forward primer: 5'-GGCCGAGTCCACACTGAT; reverse primer: 5'-TCTCATGCGTGCGTCCAA and probe: 5'-CCGCTCACGATGCCCCGTCTTCT. 18S rRNA was used as internal standard.

Statistical analysis

All real-time PCR data are expressed as means ± S.E.M. Statistical significance was assessed by Student's *t*-test. A value of $P < 0.05$ was considered statistically significant.

Results

In order to identify estrogen-induced genes of potential interest for the hemostatic balance, 35 genes involved in coagulation/fibrinolysis and present on Affymetrix Chips MG-U74A were selected (Table 1). These genes were, as expected, in general more highly expressed in the liver than in bone marrow/bone (13, 14) (Table 1). Coagulation factors V, VII, IX, X, XII and XIII were highly expressed in the liver while only FV and FX were expressed at detectable levels in the bone marrow/bone (Table 1). Expression analysis of FV mRNA levels in different tissues demonstrated that FV was highly expressed in liver and moderately expressed in bone marrow/bone while only low expression was found in muscle, kidney, fat and heart (Fig. 1A).

Long-term 17β-estradiol treatment of ovariectomized mice did not regulate the expression of any of the selected genes in the liver. In contrast, and unexpectedly, FV and FX mRNA levels in bone marrow/bone were increased by long-term 17β-estradiol treatment (Table 1). Real-time PCR analysis on individual mice ($n = 6-7$) confirmed the microarray finding that 17β-estradiol strongly increased mRNA levels of both FV and FX in bone marrow/bone (Fig. 1B).

The recent and interesting finding of a functional bone marrow-derived megakaryocytically produced pool of platelet FV in mice prompted us to further characterize the observed effects of 17β-estradiol on FV expression in bone marrow/bone (15). In order to investigate whether FV is a primary or secondary target gene for the effect of 17β-estradiol in bone marrow/bone, a short-term 17β-estradiol experiment was performed. Ovariectomized mice were given a single injection of 17β-estradiol and FV mRNA levels were analyzed 10, 24 and 48 h after the injection using real-time PCR analyses on individual mice ($n = 6-7$). FV mRNA levels were significantly increased 24 h after the 17β-estradiol treatment (Fig. 1C).

To establish the ER specificity for the stimulatory effect of 17β-estradiol on FV mRNA levels, the effect of long-term 17β-estradiol treatment was compared in ovariectomized WT, ERα^{-/-}, ERβ^{-/-} and ERα^{-/-}β^{-/-} mice. FV mRNA levels were increased by 17β-estradiol treatment in WT, ERα^{-/-} and ERβ^{-/-} but not in ERα^{-/-}β^{-/-} mice (Fig. 1D). Thus, the stimulatory effect of 17β-estradiol on FV mRNA levels is dependent on ER expression but can be mediated both via ERα and via ERβ.

Discussion

Both oral estrogen-based HRT and contraceptives increase the risk of VTE. Hitherto, the effect of oral contraceptives on the hemostatic balance has been focused on changes in plasma concentration and the changes in activity of different factors involved in the process.

Table 1 Expression of coagulation system-related genes in bone and liver.

Gene	GeneBank accession no.	Liver		Bone marrow/bone	
		Exp.	Reg.	Exp.	Reg.
Anticoagulant system					
Thrombin receptor	L03529	—		—	
Anticoagulant protein C	AF034569	****	N	—	
Protein C receptor, endothelial	L39017	—		*	N
Protein S	L27439	**	N	**	N
Tissue factor pathway inhibitor 2	D50586	**	N	—	
Tissue factor pathway inhibitor	AF004833	—		*	N
Coagulation factor II (thrombin)	X52308	****	N	—	
Coagulation factor II (thrombin) receptor-like 1	Z48043	—		—	
Coagulation factor II (thrombin) receptor-like 3	AF080215	—		—	
Thrombomodulin	X14432	—		*	N
Fibrinolysis					
Plasminogen	J04766	****	N	—	
Tissue plasminogen activator	J03520	—		—	
Urokinase-type plasminogen activator	M17922	*	N	—	
Plasminogen activator inhibitor	M33960	—		—	
Plasminogen activator inhibitor, type II	X16490	—		—	
Plasminogen activator inhibitor 3	U67878	—		—	
Tetranectin (plasminogen-binding protein)	X79199	—		—	
α -2 antiplasmin	Z36774	****	N	—	
Clotting factors					
Coagulation factor III	M26071	—		—	
Coagulation factor V	U52925	***	N	**	I(2.3-fold)
Coagulation factor VII	U66079	***	N	—	
Coagulation factor VIII	L05573	—		—	
Coagulation factor IX	M23109	***	N	—	
Coagulation factor X	AF087644	***	N	*	I(3.2-fold)
Coagulation factor XII	X99571	****	N	—	
Coagulation factor XIII	D10071	**	N	—	
Others					
Fibrinogen-like protein 2	M16238	—		*	N
Glandular kallikrein	X13215	—		—	
Kallikrein	V00829	—		—	
Kallikrein 3	M58588	**	N	—	
Kallikrein 5	Y00500	—		—	
Kallikrein 8	X03994	—		—	
Glandular kallikrein	J00389	—		—	
Kallikrein-binding protein	X61597	—		—	

DNA microarray analyses were performed on mRNA prepared from liver and bone marrow/bone from ovariectomized mice treated with vehicle or 17 β -estradiol according to the Materials and methods. Exp., the average mRNA expression (signal according to Affymetrix); —, not detectable; *0–500; **501–2000; ***2001–8000; **** > 8000. Estrogen-induced regulation (Reg.) of mRNA levels of genes expressed at detectable levels is indicated by N, no change and I, increased by estrogen according to the criteria given in the text.

The coagulation cascade factors are predominately regarded as being exclusively synthesized in the liver. However, significant expression of FV in bone marrow/bone is supported by previous studies, demonstrating a clear expression in bone marrow-derived megakaryocytes (16, 17). Furthermore, recent bone marrow transplant models as well as transgenic mouse studies have demonstrated that the murine platelet FV pool is derived entirely from synthesis within bone marrow-derived megakaryocytes, while the circulating plasma FV pool is liver derived (15, 18).

In this study, we have shown that FV mRNA is increased in the bone marrow/bone by long- as well as short-term 17 β -estradiol treatment in female ovariectomized mice. The presence of both a short-term and long-term stimulatory effect of 17 β -estradiol indicates that 17 β -estradiol directly regulates FV mRNA

levels. A direct regulatory effect of estrogen on FV gene expression is supported by the presence of half-palindromic estrogen response element motifs in the promoter of this gene (19). No effect of estrogen on the circulating FV levels has been reported, which can probably be explained by the fact that circulating FV is mainly liver derived (20, 21), together with the present finding that 17 β -estradiol does not regulate liver production of FV. Nevertheless, the present finding of estradiol-induced FV expression in bone marrow/bone might be of importance, as this bone marrow-derived pool of FV has recently been shown to have a functional role in the coagulation cascade (15). FV has a central role in both the procoagulant and the anticoagulant pathways. In addition, APC resistance, the most common inherited coagulation deficiency, is in most cases caused by a single point (R506Q) mutation in the FV

Figure 1 Expression and regulation of FV and FX mRNA levels as determined by real-time PCR analysis on individual animals. (A) Expression of FV mRNA levels in different tissues. (B) Effect of long-term 17β-estradiol treatment on FV and FX mRNA levels in bone marrow/bone ($n = 6-7$). (C) Effect of short-term 17β-estradiol treatment on FV mRNA levels in bone marrow/bone ($n = 7$). (D) Effects of long-term 17β-estradiol treatment on FV mRNA levels in bone marrow/bone of WT, ERα^{-/-}, ERβ^{-/-} and ERα^{-/-}β^{-/-} ovariectomized mice ($n = 4-8$). BM, bone marrow/bone. Values are given as % of vehicle treatment and expressed as means $\pm$ S.E.M. ** $P < 0.01$ (Student's t -test).

gene (22). One may therefore speculate that estrogen-induced high levels of bone marrow-derived FV might be involved in the pathogenesis of VTE.

In conclusion, mRNA levels of FV are strongly increased by 17β-estradiol in bone marrow/bone, but not in the liver. This effect of 17β-estradiol is mediated both via ERα and ERβ and one may speculate that FV regulation might be involved in the pathogenesis of estrogen-induced VTE.

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