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Effect of estetrol, a selective nuclear estrogen receptor modulator, in mouse models of arterial and venous thrombosis

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ABSTRACT

Estetrol (E4) is a natural estrogen synthesized exclusively during pregnancy by the human fetal liver, and the physiological role of this hormone is unknown. Interestingly, E4 was recently evaluated in preclinical and phase II-III clinical studies in combination with a progestin, with the advantage to not increase the circulating level of coagulation factors, at variance to oral estradiol or ethinylestradiol. Here, we evaluated the effect of E4 on hemostasis and thrombosis in mouse. Following chronic E4 treatment, mice exhibited a prolonged tail-bleeding time and were protected from arterial and also venous thrombosis *in vivo*. In addition, E4 treatment decreased *ex vivo* thrombus growth on collagen under arterial flow conditions. We recently showed that E4 activates uterine epithelial proliferation through nuclear estrogen receptor (ER) α. To analyze the impact of nuclear ERα actions on hemostasis and thrombosis, we generated hematopoietic chimera with bone marrow cells deficient for nuclear ERα. E4-induced protection against thromboembolism was significantly reduced in the absence of hematopoietic nuclear ERα activation, while the increased tail-bleeding time was not impacted by this deletion. In addition to its "liver friendly" profile described in women, our data shows that E4 has anti-thrombotic properties in various mouse models. Altogether, the natural fetal estrogen E4 could represent an attractive alternative to classic estrogens in oral contraception and treatment of menopause.

Estrogens have reproductive and non-reproductive effects such as impact on bone (Manolagas et al., 2013), eating behavior (Hirschberg, 2012), cardiovascular diseases (dos Santos et al., 2014) and breast cancers (Chang, 2011). Low estrogen levels after menopause can negatively impact women health. The most common menopausal complaints are vasomotor symptoms (hot flushes and night sweats), vulvovaginal atrophy and loss of bone mineral density. These symptoms are usually relieved by the administration of an estrogen. However, hormone replacement therapy (HRT) is associated with adverse effects such as an increased incidence of thromboembolic events (Rossouw et al., 2002; Scarabin, 2014). The identification of new safer estrogenic compounds for HRT that would selectively preserve the beneficial effects of estrogens on the bone and uro-genital system while reducing their unwanted side effects is largely needed. Recent preclinical and clinical studies add evidence for considering estetrol (E4) as a potential candidate (Mawet et al., 2015; Abot et al., 2014; Apter et al., 2016; Duijkers et al., 2015; Coelingh Bennink et al., 2016).

This hormone (estra-1,3,5(10)-trien-3,15a-16a,17b-tetrol) was discovered in 1965 (Hagen et al., 1965) and is naturally produced from 17β-estradiol (E2) and estriol (E3) by the human fetal liver during pregnancy and reaches the maternal circulation through the placenta (Hagen et al., 1965; Gurpide et al., 1966). E4 was initially considered essentially as a weaker estrogen agonist compared to E2, as higher doses of E4 are required to elicit estrogenic activities in the brain (Tskitishvili et al., 2014; Pluchino et al., 2014; Holinka et al., 2008), bone (Coelingh Bennink et al., 2008a), uterus and vagina (Abot et al., 2014; Holinka and Gurpide, 1979; Heegaard et al., 2008), ovulation inhibition (Visser and Coelingh Bennink, 2009; Coelingh Bennink et al., 2008b), atheroma prevention (Abot et al., 2014) and vasorelaxation (Hilgers et al., 2012). In addition, E4 is active in a hot flushes rat model

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(Holinka et al., 2008). However, pre-clinical studies revealed that E4 has some antagonistic properties toward the proliferative effect of E2 in breast tumor tissues (Gerard et al., 2015; Visser et al., 2012). Furthermore, at variance to E2 that elicits endothelial NO synthase activation and accelerates endothelial healing, E4 does not induce these effects, but rather prevents the E2 actions (Abot et al., 2014). Thus, E4 acts as an estrogen agonist in some tissues and as an estrogen antagonist in others, and thus share properties of selective estrogen receptor modulators (SERM).

E4 binds the two nuclear steroid receptors: estrogen receptors (ER) α and ERβ, with a four-to five-fold preference for the ERα and with a four-fold lower affinity compared with ethinylestradiol (EE) and E2 (Coelingh Bennink et al., 2008c; Visser et al., 2008). In addition to the classic genomic actions of ER, estrogens have been found to induce rapid effects occurring within minutes following administration. These effects are mediated through a subpopulation of receptors associated with the plasma membrane, a process usually termed “membrane-initiated steroid signaling” (MISS), “nongenomic” or “extranuclear” effects (Watson et al., 2005). Using combined genetic and pharmacological approaches, we recently proposed that E4 modulates ER actions in a tissue-specific manner through a selective nuclear, but not membrane, ERα activation (Abot et al., 2014; Benoit et al., 2017).

Very interestingly, E4 was recently evaluated, in a phase II clinical trial, as an oral contraceptive in combination with a progestin (Kluft et al., 2017). At variance to EE or E2, E4 (at the daily dose of 20 mg) was found to have little or no effect on sex hormone-binding globulin, angiotensinogen or coagulation factors (Kluft et al., 2017). This “liver friendly” aspect of E4 represents another facet of this original molecule, which would not increase the risk of thromboembolic events according to our current knowledge (Mawet et al., 2015; Kluft et al., 2017). Using mouse models, we previously reported that a chronic high physiologic dose of E2 decreased platelet responsiveness, increased tail-bleeding times and protected animals against collagen/epinephrine-induced thromboembolism, through hematopoietic ERα, and independently of hematopoietic ERβ (Valera et al., 2012). The aim of the present study was to define the impact of E4 on several models of arterial and venous thrombosis in mouse. We found that E4 treatment increased mouse tail-bleeding times, protected from both arterial and venous thrombosis and induced a resistance to acute thromboembolism. While E4 had no significant impact on platelet aggregation following stimulation in suspension, *ex vivo* flow-based adhesion studies conducted in whole blood under arterial flow conditions on a collagen matrix showed that E4 treatment reduced platelet adhesion. Finally, we explored the role of hematopoietic nuclear ERα in hemostasis and thromboembolism following E4 treatment.

1. Materials and methods

1.1. Materials

Collagen reagent HORM® (type I fibrils from equine tendon) suspension was purchased from Takeda, DIOC6 was from Life Technologies, fibrinogen from Sigma-Aldrich and $FeCl_3$ from Mallinckrodt Chemical. E4 was supplied by Mithra Pharma (Liège, Belgium) and was dissolved in 60% ethanol and 40% PBS.

1.2. Mice

Female C57BL/6J mice were purchased from Charles River. ERα-AF2^0 mice have been described previously (Billon-Gales et al., 2011). All procedures were performed in accordance with the principles established by the National Institute of Medical Research and were approved by the local Ethical Committee of Animal Care. Mice were anesthetized by intraperitoneal injection of ketamine (25 mg/kg) and xylazine (10 mg/kg) and ovariectomized at 4 weeks of age. Treatments were started approximately 2 weeks after ovariectomy to allow for complete recovery from surgery. Ovariectomized mice were implanted subcutaneously with osmotic minipumps releasing either vehicle or E4 ($6\ mg{\cdot}kg^{-1}{\cdot}d^{-1}$). The treatment doses of E4 were chosen according to results from previous *in vivo* studies (Coelingh Bennink et al., 2008a; Visser and Coelingh Bennink, 2009; Benoit et al., 2017). Mice were euthanized after a 3-week E4 treatment period.

1.3. Bone marrow transplantation

Two weeks after ovariectomy, recipient mice were lethally irradiated (9.2 Gy, γ source) then intravenously reconstituted with bone marrow cells from mice mutated for the activation function 2 (AF2) of ERα (the mice deleted for AF2 of ERα were designated ERα-AF2^0 and the WT littermates were designated ERα-AF2$^{+/+}$). Three weeks later, transplanted mice were implanted or not with an E4 osmotic minipumps ($6\ mg{\cdot}kg^{-1}{\cdot}d^{-1}$). Enrofloxacine (Baytril, Bayer HealthCare Animal Health Division) was added to their drinking water for 3 weeks after bone marrow transplantation. The efficiency of the bone marrow transplantation was confirmed by PCR.

1.4. Tail-bleeding time

After mice anesthesia, we measured bleeding time following 3-mm tail-tip transection. Blood drops were removed every 15 s with the use of a paper filter. If bleeding did not recur within 30 s of cessation, it was considered stopped. Experiments were terminated after 30 min if no cessation of blood flow occurred.

1.5. Thromboembolism and histochemical analysis of mouse lung

Acute systemic vascular thromboembolism was induced by injecting a mixture of collagen (0.4 mg/kg) and epinephrine (60 μg/kg) into the right jugular vein of anesthetized mice. Mice were euthanized 10 min after injection of the mixture. Lungs were excised and formalin-fixed. Paraffin sections (5 μm thick) were stained with hematoxylin-eosin and analyzed histologically for the presence of thrombi. Transthoracic echocardiography were performed as previously described (Valera et al., 2017).

1.6. Inferior vena cava thrombosis: stasis and stenosis models

Mice were anesthetized and after laparotomy, intestines were exteriorized and sterile saline was applied during the whole procedure to prevent drying. After gentle separation from aorta, inferior vena cava (IVC) was ligated by a 8.0 polypropylene suture immediately below the renal veins to obtain complete blood stasis as previously described (Wrobleski et al., 2011). Mice were euthanized after 24 h. For stenosis, IVC ligation was performed over a 30-gauge needle placed outside the vessel and then the needle was removed. This procedure has been shown to decrease vascular lumen by about 90% and avoid endothelial injury (Brill et al., 2011). Mice were euthanized after 48 h. In both cases, the thrombi formed in the IVC were carefully dissected, weighted and removed for formalin fixation and paraffin embedding immunohistochemistry.

1.7. Carotid artery thrombosis

The right and left carotids were dissected free from surrounding tissues. Two flow probes were connected to a Transonic model T403 flow meter (Transonic System; Emka Technologies) to record the blood flow (milliliters per minute) of the carotids. $FeCl_3$ was used to induce vascular injury. A 1 × 4-mm strip of paper saturated with 7% $FeCl_3$ solution was applied to the adventitial surface of the left carotid for 2 min then removed. The right carotid was used as an internal control. Blood flow was then monitored continuously throughout the procedure (IOX software).