Cardiovascular pharmacology

In vivo profile of the anticoagulant effect of 17β-amino-1,3,5(10)estratrien-3-ol

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The anticoagulant activity of 17β-amino-1,3,5(10)estratrien-3-ol (AE2) was established for the first time. Experiment 1: mice groups were treated with a single subcutaneous (s.c.) AE2 injection (0.5, 1, 2, 4, and 8 mg/100 g BW) or vehicle (propylenglycol; 0.5 ml/100 g). After 24 h, AE2 produced dose-dependent blood clotting time increases related to control, Emax = +121% (P < 0.01) finishing the sixth day. Experiment 2: four groups received a single s.c. administration of AE2 (4 or 8 mg/100 g BW) or 17β-estradiol (E2; 3 mg/100 g BW) or vehicle. After 24 and 48 h post-administration, the times of blood clotting, prothrombin, thrombin, and activated partial thromboplastin and fibrinogen concentrations were assessed. Both AE2 doses increased blood clotting and fibrinogen similarly, blood clotting time: 64, 94%; fibrinogen: 71, 107% (P < 0.01). Meanwhile, E2 decreased blood clotting 20% (P < 0.01) and thrombin 23% (P < 0.01) after 48 h. Experiment 3: for five consecutive days, mice received AE2 or E2 (0.1, 1, 10, 100, and 1000 pg/kg/day), or vehicle. Blood clotting time was assessed at 1, 2, 3, 4, 5, 8, and 11 days after treatment. AE2 at all doses were anticoagulant for 2–3 days after administration whereas E2 was procoagulant for 8–11 days. These opposite effects were: AE2 Emax = +29%; E2 Emax = −30%; (P < 0.01). AE2 is the parent compound of the 17β-aminosterogens, with the largest and longest anticoagulant effect until now reported.

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1. Introduction

Premenopausal women have lower incidence of cardiovascular disease compared with age-matched men (Hayward et al., 2000). This protection is mainly attributed to estrogens modulating action on lipid metabolism, vasculature, inflammation and blood coagulation processes (Guetta and Cannon, 1996; Cushman, 2002).

During menopause, estrogen production decreases and menopausal hormone therapy (MHT) is often prescribed, which is associated with an increased risk of venothrombotic episodes, (van Hylckama Vlieg et al., 2009; Canonico et al., 2010).

Estrogen therapy increases coagulation factors, decreases natural anticoagulants activity and those involved in fibrinolysis (van Hylckama Vlieg and Rosendaal, 2003; Gottsäter et al., 2001).

These alterations in the hemostatic balance may lead to a hypercoagulability state, favoring thrombogenesis (Rosendaal et al., 2001). Individualized MHT is recommended considering the patient’s risk factors and prescribing the lowest effective dose for the shortest possible duration (Santen et al., 2010).

In our search for alternative estrogens without thromboembolic risk, we have evaluated several 17β-aminosterogens in rodents. These compounds produce anticoagulant effects that contrast with the procoagulant effects elicited by estradiol (E2) (Rubio-Poo et al., 1985, Lemini et al., 2005a). Prodiame was the first AE reported as an anticoagulant, which possesses a diaminogroup substituent (−NH–CH2CH2–CH2–NH2) on C17β of the steroidal molecule (Rubio-Poo et al., 1983). Prodiame increased blood clotting times in mice, however higher doses produced a convulsive effect (Mandoki et al., 1991). Structural changes on the terminal amino group substituent in prodiame molecule led to prolamine (−CH2−NH–CH2CH2−OH), buame (−NH–CH2CH2–CH2−CH2), which retained the anticoagulant activity, and proacame (−NH–CH2CH2–CH2−COOH), which inhibited it (Mandoki et al., 1991).
2. Materials and methods

2.1. Reagents

All solvents and reagents used were of analytical reagent grade, without further purification and obtained from Baker Co. (México). Estradiol (E2, 1,3,5(10)-estratrien-3,17ß-diol) was obtained from Syntex S.A., Mexico City, Mexico. 17ß-amino-1,3,5(10)estratrien-3-ol (AE2) was synthesized from estrone obtained from Syntex S.A., Mexico City, Mexico. 17ß-aminoestrogens previously described, has no substituent on the amino group of steroid C-17β position (Lemini, et al., 1998). If AE2 has the same activity profile as the 17ß-aminoestrogens so far studied, it can be established that it is the main compound of the 17ß-aminoestrogens series and responsible for the observed anticoagulant effect. Therefore, our objective was to evaluate AE2 and E2 on mice blood clotting time, fibrinogen concentration, thromboplastin time, activated thromboplastin time, and prothrombin time.

2.2. Animals

All experimental studies were conducted in accordance to the Mexican National Protection Laws on Animal Protection and the General Health Law Related to Health Research (NOM-062-Z00-1999). Adult CD1 mice (25–32 g) were used for assessment of the anticoagulant activity, blood clotting time, and determination of hemostatic parameters. Mice were obtained from the animal facilities of the Faculty of Medicine, National Autonomous University of Mexico. The animals were kept at constant temperature (20–22 °C) in a room with 12 h–12 h light–dark cycle and maintained on standard chow (Nutricubos, Purina) and water ad libitum.

2.3. Blood clotting time

2.3.1. Experimental design

The experiments were performed in adult male CD1 mice. Animals were distributed among groups according to a balanced design based on body weight (6–8 animals per group in each experiment). Estrogens were dissolved in propylene glycol (vehicle) and administered subcutaneously (s.c.); control animals received vehicle only.

2.3.2. Blood clotting time assessment

Blood clotting time was measured according to previous reports (García-Manzano et al., 2002). Three experiments were performed:

Experiment 1. Five groups of mice were treated with a single s.c. injection of AE2 (0.5, 1, 2, 4, and 8 mg/100 g of body weight) or the vehicle (0.5 ml/100 g). In all groups, the temporal course of blood clotting time was assessed at 24, 48, 72, 96, 120, and 144 h after the last injection.

Experiment 2. Four groups of mice received a single s.c. administration of AE2 (4 and 8 mg/100 g) or E2 (3 mg/100 g of body weight), or the vehicle (0.5 ml/100 g). In all groups blood clotting time, fibrinogen concentrations, thrombin time, prothrombin time, and activated partial thromboplastin time were assessed at 24 and 48 h after the last injection.

Experiment 3. The mice received s.c. administrations of AE2 or E2 (0.1, 1, 10, 100, and 1000 μg/kg per day) or the vehicle (0.33 ml/100 g per day) for five consecutive days. The assessment of blood clotting time was performed at 1, 2, 3, 4, 5, 8, and 11 days after the last injection.

All the measurements of blood clotting time were blind and carried out following a balanced, Latin-square, block design, so that there were no differences in testing times among experimental groups. The experiments were performed at 22–24 °C and between 8.30 and 11.00 h. The blood clotting time data were normalized with respect to the vehicle group (100).

2.3.3. Blood collection

Animals were anesthetized with chloral hydrate (4% solution, 7 ml/kg) prior to blood withdrawal. Each animal was placed on supine position and immobilized. Arterial blood was collected from the iliac bifurcation with a plastic syringe and disposable-gauge needles, providing hemolysis-free blood samples (2–3 ml). Blood was immediately drawn out into plastic tubes containing 0.11 M sodium citrate (1:10, v/v). The samples were gently mixed for one minute and centrifuged at 2500 g for 10 min at 4–6 °C. Plasma samples were separated and stored at −70 °C until processing.

2.3.4. Determination of the hemostatic parameters

The coagulation screening tests, fibrinogen concentrations, thrombin time, prothrombin time, and activated partial thromboplastin time, were determined with modifications of the conventional clinical procedures described earlier (García-Manzano et al., 2002). For all clotting tests, Dade® Behring reagents were used and assayed according to instructions in the package insert. Reference curves and accuracy controls were set up using the corresponding control plasma of the manufacturer. Thrombin time, prothrombin time, and activated partial thromboplastin time were recorded using a Behring Fibrinrometer II (Dade® Behring). Clot formation was the end point of the reactions and results were reported in seconds. Fibrinogen concentration was evaluated in a mechanical fibrometer Fibrosystem Becton–Dickinson Mod 5–117 V. Bovine thrombin (100 IU/ml; Dade® Behring) was added to plasma samples (50 μl) to induce clot formation. The fibrinogen concentration was obtained from a reference curve calibrated with human plasma fibrinogen and reported in...
milligrams per deciliter (mg/dl). Thromboplastin time was determined using bovine thrombin (Dade® Behring) at a concentration of 5 IU/ml. Plasma samples (50 μl) were treated with 50 μl of bovine thrombin to start the reaction. Prothrombin determination was obtained using rabbit brain thromboplastin C plus (Dade® Behring). Plasma samples (50 μl) were pipetted into cuvettes and then 100 μl of thromboplastin C plus were added to activate the reaction. Activated partial thromboplastin time was assessed using the Actin FS reagent (Dade® Behring) containing purified soy phosphatides. Actin FS (100 μl) was added to plasma samples (50 μl). The mixture was incubated at 37 °C for 120 s, and 50 μl of 0.02 M CaCl₂ (37 °C) was added to activate the reaction.

2.4. Statistical analysis

Statistical significance between the control and treated groups was assessed by analysis of variance (ANOVA). The significance of differences among groups was estimated by the required test as appropriate (Zar, 1984). Results were expressed in means ± standard error (S.E.M.). P < 0.05 values were considered as the limit of significance. The differences related to the controls for all hemostatic parameters were obtained by the relation: [(Mean value treated group/Mean value control group) × 100] – 100. Analysis was performed using the Sigma Stat statistical software 3.1 Copyright® 1992–1995, Jandel Corporation. All experiments were performed twice and the samples of the evaluated parameters were assessed in triplicate.

3. Results

3.1. Dose-response relationship and temporal course of AE₂ effect on mice blood clotting time

Experiment 1. A dose-response curve was obtained by a single s.c. administration of different doses of AE₂ (0.5, 1, 2, 4, and 8 mg/100 g body weight) in male CD1 mice. AE₂ produced dose-dependent blood clotting time increases in treated animals (Fig. 2A). The maximum blood clotting time increase (Emax = +121%, P < 0.01) in relation to the control group was observed 24 h after the AE₂ administration of 8 mg/100 g. In both groups, the temporal evolution of their anticoagulant effects was measured and is shown in Fig. 2B. The anticoagulant effect gradually declined over the following five days after the administration. On the sixth day, anticoagulant activity showed no significant differences with respect to the control group.

3.2. Effect of AE₂ and E₂ on some mice blood coagulation parameters

In experiment 2, the blood clotting time and the coagulation parameters: fibrinogen concentration, thrombin time, prothrombin time, and activated partial thromboplastin time of animals treated with AE₂ (4 and 8 mg/100 g) or E₂ (3 mg/100 g) or vehicle were estimated 24 and 48 h after. The blood clotting times of the animals treated with AE₂ or E₂ compared to the control group are depicted on Fig. 3. In this assay, 24 h after the AE₂ injection with 4 or 8 mg/100 g, blood clotting time increased 64 and 94% (P < 0.01) respectively. At 48 h, the anticoagulant effects in the same groups increased to 43 and 63% (P < 0.01). E₂ administration produced no significant blood clotting times differences after 24 h, but was significantly reduced 20% after 48 h (P < 0.03) compared to the control group.

In these same animal groups, fibrinogen levels were measured and are shown in Fig. 4. After 24 h, AE₂ increased fibrinogen concentration in 71 and 107% (P < 0.001); decreasing to 41 and 87% (P < 0.001) 48 h later with the corresponding 4 and 8 mg/100 g doses. At both time determinations, E₂ produced a modest fibrinogen increase of 13% (P < 0.02). From 24 to 48 h, thrombin time was affected by both, AE₂ and E₂, but in an opposite manner (Fig. 5). AE₂ increased thrombin time in 21–29% (P < 0.02) and E₂ reduced it by 23% (P < 0.02). AE₂ modestly increased prothrombin time in 13–15% (P < 0.05) 48 h after both doses administration (Fig. 6). However, after 24 h, activated partial thromboplastin time increased 27–30% and 33–55% after 48 h (Fig. 7). In these assays, E₂ produced no significant changes.

3.3. Comparison between AE₂ and E₂ effects on mice blood clotting time

Experiment 3. Time course effects on blood clotting time after five days of s.c. administration of different doses (0.1, 1, 10, 100, and 1000 μg/kg) of AE₂ or E₂ to mice are shown in Fig. 8. AE₂ and E₂ showed opposite effects on blood coagulation in mice. AE₂ produced significant anticoagulant effects lasting from 48 to 72 h following the last administration, whereas E₂ induced only procoagulant effects. These opposite blood clotting times effects of the two steroids were similar in magnitude AE₂ Emax = 29%, Emax E₂ = −30% (P < 0.01) and independent from the administered dose.

The main difference between both steroids was the duration of their anticoagulant effect. The significant blood clotting times increases were observed with all the AE₂ doses. From the lowest dose (0.1 μg) significant anticoagulant effects were seen, lasting along 72 h in all cases and with the 1000 μg dose up to 96 h.

![Fig. 2. (A): Dose-response relationship of AE₂ (0.5–8 mg/100 g of body weight) on blood clotting time of male CD1 mice, 24 h after a single s.c. administration. (B): The blood clotting times of the animals treated with 8 mg/100 g dose of AE₂ (diamonds) or vehicle (open circles) were daily measured until the anticoagulant effect had waned and any significant changes could no longer be detected. The time course effects on blood clotting time lasted significantly for five days (Fig. 2B). *P < 0.05 vs. vehicle.](image-url)
Moreover, E2 showed procoagulant effects with all the administered doses. Emphasizing that the highest doses used, 100 and 1000 mg, produced the longest procoagulant effects; 100 mg a lasting from 24 h after the last administration significantly to eight days and the 1000 mg dose maintained the significant procoagulant effects for eleven days.

4. Discussion

This work reports for the first time the anticoagulant effect of the 17β-aminoestrol AE2. Our data indicate that AE2 is the prototype of the 17β-aminoestrogen series. The structural difference between AE2 and E2 is the presence of an amino (–NH2) in AE2 instead of the hydroxyl group (–OH) of E2 at C-17β position of the steroid. These functional groups (–NH2 and –OH) are isosteres (Mathison, et al., 1989) similar in their electronic structure, differing in their physicochemical properties and reactivity, which could significantly impact their biological activity.

High AE2 doses increased mice blood clotting time in a dose-dependent manner and produced opposite effects on blood clotting time to those induced by equivalent doses of E2. AE2 and E2 also produced opposite significant effects on thrombin time (Fig. 5), AE2 increasing it 29% and E2 decreasing it 23%. These effects are qualitatively in the same line with the blood clotting time results obtained with both steroids.
Our data agree with our previous results of acute treatment with high doses of the 17ß-aminoestrogens, prolame, butolame, and pentolame, on blood coagulation in male and ovariectomized rats (García-Manzano et al., 2002; Jaimez et al., 2000; Lemini et al., 2005a). The main response differences between AE₂ and the 17ß-aminoestrogens, prolame, butolame, and pentolame, on blood coagulation in male and ovariectomized rats (García-Manzano et al., 2002; Jaimez et al., 2000; Lemini et al., 2005a). The main response differences between AE₂ and the 17ß-aminoestrogens, prolame, butolame, and pentolame,
compared at equivalent doses were their efficacy and anticoagulant effect duration. An AE₂ single dose of 8 mg/100 g increased blood clotting time 120%, whereas 17β-aminoestrogens between 60–90%. AE₂ showed the greatest anticoagulant effect so far detected in CD1 mice and was significantly maintained for 5 days; whereas 17β-aminoestrogens, previously evaluated, had anticoagulant effect duration of 2 to 3 days (Lemini et al., 2005a).

These results confirm that the substituent on the amino group of AEs, prolame, butolame, and pentolame, influences and modifies the intrinsic physicochemical properties of each particular compound, probably changing their pharmacokinetic process pathways. The anticoagulant response and estrogenic effect variations among these compounds are probably due to liposolubility and polarity differences leading to changes in their transmembrane transport, biortransformation and individual elimination processes. Such structural modifications could also be leading to differences in their reactivity and activation at the receptor sites determining the magnitude and timing of their biological effect.

It is interesting to note that AE₂ percentage differences with respect to the control group on blood clotting time and fibrinogen concentration are close. This behavior was also observed in acute and sub-acute treatments in male rats with the AE pentolame, which also showed a direct increasing relationship between the blood clotting time and the fibrinogen concentration in a dose-dependent manner (Lemini et al., 1993; Lemini, 2009).

AE₂ action is probably linked to the stimulation of the mice hepatic synthesis of fibrinogen producing high levels (Emax 121%) of this glycoprotein. In the clinical practice, a prolonged thrombin time could be indicating dysfibrinogenemia due to fibrinogen dysfunctionality (Van Cott et al., 2002). In some individuals, the inherited defect in fibrinogen leads to the production of a dysfunctional molecule (dysfibrinogenemia) that is associated with bleeding or, in some cases, thrombosis or both (Verhovsek et al., 2008). Elevated fibrinogen concentration is more associated with prolonged reptilase times and less commonly with prolonged thrombin time (Van Cott et al., 2002). Our findings suggest that the fibrinogen concentration increases due to AE₂ administration could be associated with bleeding, since blood clotting time is clearly prolonged in a dose dependent manner in the treated mice. Thrombin time is a clotting time measure of the conversion of fibrinogen into fibrin. Thrombin cleaves fibrinogen to fibrinopeptides A and B, thereby converting fibrinogen into fibrin monomers, which then polymerize into a fibrin clot. It has been described that the thrombin time alteration could be due to impairment of fibrinopeptide release, fibrin polymerization, or thrombin inhibition (Colman et al., 2006). Some authors consider that the thrombin time assay tends to be more sensitive to fibrinogen deficiency and dysfunction than the prothrombin time assay. The prothrombin time parameter can detect deficiencies of factors X, VII, V and prothrombin, but only severe fibrinogen deficiencies (Verhovsek et al., 2008). We observed that prothrombin time was mildly affected by AE₂ (Emax 15%). According to this, the thrombin time increase observed (Emax 29%) after AE₂ administration could be indicating its action on fibrinogen dysfunction. Additionally, this idea is supported by the finding that the AE₂ effect on activated partial thromboplastin time (Emax 55%) was significantly greater than that elicited on thrombin time, which also suggests production of a dysfunctional fibrinogen action induced by this steroid. The activated partial thromboplastin time marker mainly detects severe fibrinogen dysfunction, and other deficiencies of the contact factors: prekallikrein, high molecular weight kininogen, factors XII, XI, X, IX, VIII, V, and prothrombin (Colman et al., 2006), which cannot be excluded as a possible AE₂ action. Given the complexity of the hemostatic system, and because the parameters here examined about the anticoagulant effect of AE₂ on mice were exploratory, it is certain that further research is essential to define the involved mechanisms in this anticoagulant effect of AE₂.

The opposite effects of AE₂ and E₂ on mice blood clotting time, fibrinogen concentration and thrombin time, and the inability of E₂ to produce significant changes in thrombin time and activated partial thromboplastin time indicate substantial differences in the action of these steroids on mice's hemostatic system. The noticeable observation of this work is that AE₂, like other 17β-aminoestrogens, produces its anticoagulant effects affecting importantly the common hemostasis pathway increasing fibrinogen concentration. Additionally, the obtained data highlight the importance of dosing during estrogen therapy, since it significantly affects hemostasis. Several clinical and experimental reports have found that low estrogen doses induce weaker responses on hemostasis and, therefore, the risk of thrombotic disease may be reduced (van Hylckama Vlieg et al., 2009; Tchaikovski and Rosing, 2010). Estrogen thrombotic events are complex and associated with the administration route, dose, and progestogen type in the formulation (van Hylckama Vlieg et al., 2009; Tchaikovski and Rosing, 2010; Canonico et al., 2011). The used dose and treatment duration are factors influencing estrogen thrombotic risk. The anticoagulant effects of AE₂ and procoagulant of E₂ on mice blood clotting time showed that the dose is related to the observed responses on blood clotting, but importantly also to latency and duration. Almost in all assays, higher doses produced earlier significant effects on blood clotting time. Repeated administration also led to less variability in hemostatic responses with greater significance than the corresponding single administration.

The observed anticoagulant effects in an in vivo model are probably linked to the steroids genomic mode of action. Responses to E₂ are mediated by estrogen, NR3A1 and NR3A2 subtypes receptors, belonging to the nuclear receptors family. The interactions between the ligand (E₂) with estrogen receptors activate transcription factors, regulating the synthesis of specific genes. The effects manifested through the genomic mode of steroids usually take long periods (Nilsson et al., 2001). However, we should consider that the effects here observed could also include membrane non-genomic mechanisms. Several studies have reported the involvement of both mechanisms in responses, like the estrogen cardioprotective effects, where the two estrogen NR3A1 and NR3A2 receptors and the membrane-bound receptor G protein-coupled estrogen receptor (GPER) are also involved in both, genomic and nongenomic, mechanisms (Deschamps and Murphy, 2009). At the membrane site, 17β-aminoestrogens can mediate part of their anticoagulant effects probably by nongenomic mechanisms. In vitro assays have shown the ability of 17β-aminoestrogens to produce platelet aggregation inhibition (De la Peña et al., 1993, 1994, González et al., 2009).

The structural substituent change at C17-OH in the E₂ molecule and by NH₂ in AE₂ strongly impacts on their biological activity changing their properties on blood coagulation and estrogenic effects. Many biologically active drugs with very high potency are related to specific action mechanisms. In this type of drugs even small changes in their chemical structure alter their biological activity. AE₂ alters blood clotting time in an opposite way to E₂ probably due to the NH₂ group reactive nature, conferring it the ability to alter hemostasis.

The 17β-aminoestrogens, prolame, butolame, and pentolame, are partial agonists of the estrogen NR3A1 and NR3A2 receptors with low affinity and low potency in relation to the natural hormone E₂ (Lemus et al., 1998; Jaimez et al., 2000). AE₂ has also low affinity for the estrogen receptors in cytosolic preparations of human breast cancer (Blickenstaff et al., 1985). However, its estrogenic effects on other estrogen targets are not known. In a parallel study, we evaluated the effects of AE₂ on some well-established estrogenic markers. AE₂ has very low uterotrophic effects in rats and mice, with doses from 0.1 to 100 μg/kg not
producing estrogenic effects. The receptor binding studies showed that AE2 had significantly lower binding affinity than the naturally occurring E2 and this response is mediated mainly by interaction with the estrogen NR3A1 receptor. AE2 estrogenic activity characterization data will be reported separately.

The present findings about the anticoagulant effect of AE2 suggest the need to extend its biological characterization. In this context, it will be important to compare pharmacokinetic processes of the 17β-aminoestrogens to establish the relationship with their pharmacodynamic processes in order to optimize effectiveness. Even though that in the present work no manifestation of toxicity was noticed in the treated animals, it will be necessary to establish any toxicity that might be presented by 17β-aminoestrogens to know their potential benefit and safety.

A deeper understanding about AE2’s biological activities could contribute to improve the understanding of estrogens’ action and mechanism on blood coagulation, bones and nervous system, where E2 has beneficial effects. Also, the evaluation of AE2 on other E2 targets may lead to the development of more specific estrogen compounds, such as those used for MHT in clinical applications, reducing rather than increasing the risk of thromboembolic events.

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