17β-Estradiol is the Most Active Component of the Conjugated Estrogen Mixture Active on Uremic Bleeding by a Receptor Mechanism

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ABSTRACT

We have reported previously that a mixture of conjugated estrogens which is effective in shortening the prolonged bleeding time in uremic patients is also effective on bleeding time in a rat model of uremia. With the present study we took advantage from such a rat model of chronic uremia and decided to identify the component(s) of the conjugated estrogen mixture responsible for shortening the bleeding time. Moreover, we wanted to clarify whether estrogen effect on primary hemostasis is due to a receptor mechanism and can be neutralized by specific estrogen receptor antagonists such as tamoxifen or clomiphene. Both estrone sulfate and 17β-estradiol, but not equilin, were effective in shortening the prolonged bleeding time of uremic rats. 17β-Estradiol was the most active component of the mixture, reproducing the time course of bleeding time shortening of the entire mixture (effect lasting 48 hr). The effect of estrone sulfate injection lasted only 24 hr. Tamoxifen and clomiphene pre-treatment prevented the shortening of bleeding time induced by conjugated estrogen mixture and its active components. These findings indicate that 17β-estradiol is the key compound of the conjugated estrogen mixture effective on bleeding time shortening and that the effect of estrogens on primary hemostasis is mediated by a receptor mechanism.

Conjugated estrogens were introduced recently in the management of bleeding associated with uremia (Liu et al., 1984; Livio et al., 1986) and are of particular value in patients undergoing major surgery given their long-lasting effect on primary hemostasis (Remuzzi, 1988). At the moment it is unknown which component of the conjugated estrogen mixture is the active one in shortening the prolonged bleeding time of uremics. Such studies are difficult to be performed in humans. Recently, the availability of a rat model of chronic renal insufficiency in which the bleeding time is prolonged and can be normalized by conjugated estrogens (Zoja et al., 1988), allowed us to approach the problem in the experimental animal. Of interest is that in the rat model like in humans the effect of conjugated estrogens on bleeding time is long-lasting. Despite their long-lasting effect on primary hemostasis, the plasma half-life of conjugated estrogens is very short in patients with uremia (Viganò et al., 1988), suggesting that they may act through a receptor mechanism which would imply entering the cell, binding high affinity receptors and translocating of the complex to the nucleus (Jensen et al., 1972; O'Malley and Means, 1974).

The aims of the present study were 1) to identify the active component of conjugated estrogen mixture responsible for the shortening of bleeding time in uremia and 2) to establish whether such an activity is neutralized by specific receptor antagonists.

Materials and Methods

In order to identify which component(s) of the conjugated estrogen preparation are responsible for the shortening of the prolonged bleeding time in uremia, we studied the effect on bleeding time of the major components of the mixture, estrone sulfate, equilin sulfate and 17β-estradiol. A model of chronic uremia that develops in rats after extensive renal mass ablation and that is associated with an impairment of primary hemostasis was used (Zoja et al., 1988). The doses of estrone sulfate, equilin sulfate and 17β-estradiol to be administered were decided after quantifying the percentage of each component in the estrogen mixture by high pressure-liquid chromatography method (Viganò et al., 1988) and accounted for 59, 29 and 5%, respectively. Then, it was calculated the amount of each component present in 12 mg/kg of conjugated estrogen mixture, i.e., the minimum dose effective in shortening the prolonged bleeding time of uremic rats (Zoja et al., 1988). It accounts for 7.2 mg/kg of estrone sulfate, 3.6 mg/kg of equilin sulfate and 0.6 mg/kg of 17β-estradiol.

Experimental protocol. Male Sprague-Dawley rats (Charles River Italia S.p.a., Calco, Italy) weighing 325 to 350 g at the start of the experiment were used. Animals were fed a standard rat chow (Altromin Rieper, Vandoies, Italy) and had free access to tap water. Renal mass was reduced by removing the right kidney and infarcting approximately five-sixths of the left kidney by ligation of two or three extrarenal branches of the main renal artery according to the method of Olson et al. (1982) with the rats under ether anesthesia. Four weeks after the
were premarin, Ayerst Italiana which were injected i.v. with 12 mg/kg of conjugated estrogens (Emo-
Co., St Louis, MO); group 3, 12 rats, which were injected i.v. with 3.6 mg/kg of equilin sulfate (Wyeth-Ayerst Laboratories, Rouses Point, N.Y.); group 4, 12 rats, which were injected i.v. with 0.6 mg/kg of 17β-
estriadiol (Sigma). All the compounds were dissolved in 1 ml of 2% benzilic alcohol solution. An additional group of five rats, group 5, received i.v. 1 ml of vehicle alone. Twenty five additional rats underwent sham operation: laparotomy and manipulation of renal pedicles but without destruction of renal tissue. Four weeks after the sham procedure, rats were subdivided as follows: group 6, five rats, which were injected i.v. with 12 mg/kg of conjugated estrogens; group 5, seven rats, which were injected i.v. with 7.2 mg/kg of estrone sulfate; group 8, five rats, which were injected i.v. with 3.6 mg/kg of equilin sulfate; group 9, five rats, which were injected i.v. with 0.6 mg/kg of 17β-
estriadiol; group 10, five rats, which were injected i.v. with vehicle alone.

Bleeding times were measured before (time 0) and 24, 48 and 72 hr after drug or vehicle injection. Serum creatinine levels were assayed 4 weeks after surgical procedure.

Dose-effect study of estrogen receptor antagonists on bleeding time in uremic rats treated with estrogens. To evaluate the effect of estrogen receptor blocking on the shortening of prolonged bleeding time induced by estrogens in rats with uremia, we gave two receptor antagonists to uremic rats, namely tamoxifen and clomiphene. These compounds are well known to bind to cytoplasmic estrogen receptors, and the modified complex is translocated into the nucleus. A competition for estrogen binding sites results in a diminished amount of estrogen receptor available and explains their antiestrogenic activity (Murad and Haynes, 1985). Experiments were carried out in 42 rats with renal mass reduction with the aim of establishing the minimum dose of the two estrogen receptor antagonists able to influence the shortening of bleeding time induced by estrogen injections. To this purpose, 4 weeks after the ablative procedure, groups of six rats each received p.o. 1, 2, 3 and 4 mg/kg of tamoxifen (Nolvadex, ICI-Pharma S.p.a., Milan, Italy) and 1, 2 and 3 mg/kg of clomiphene (Clomid, Gruppo Lepetit, S.p.a., Milan, Italy) in 1 ml of 0.5% carboxy methyl cellulose. Bleeding times were measured before and 24 hr after estrogen receptor antagonists in order to evaluate possible influences of estrogen receptor antagonist administration itself on bleeding time. Subsequently, conjugated estrogens (12 mg/kg) were injected i.v. and 24 hr later bleeding time test was again performed. Four additional groups of six uremic rats each were studied to evaluate the effect on bleeding time of the tamoxifen and clomiphene administrations at the dose of 3 mg/kg followed by estrone sulfate or 17β-estradiol injections.

Bleeding time. We performed bleeding time in unanesthetized animals to avoid possible influence of anesthesia on blood-vessel interplay and interactions between anesthetics and drugs to be administered (Dejana et al., 1979). Rats were placed in a plastic cylinder with several openings on one of which the rat's tail emerged. A standardized Simplate II device (General Diagnostic, Milan, Italy) was used. The device was applied longitudinally on the dorsal part of the tail between 6 and 9 cm from the tip, taking care to avoid large veins. Tails of rats were left in the air and the animals were maintained at room temperature. Bleeding time was measured from the moment the tail was incised until bleeding stopped completely (no rebleeding within 30 sec). Bleeding time was expressed in seconds.

Serum creatinine. Serum creatinine was measured by the alkaline picrate method (Bonsnes and Taussky, 1945).

Statistical analysis. Results are expressed as mean ± S.D. Data were subjected to one- or two-way analysis of variance as appropriate (Linton and Gallo, 1975). Significance level of differences between individual group means was established using Tukey's test. Statistical significance level was defined as P < .05.

Results

Animals with renal mass reduction developed renal insufficiency. Four weeks after surgical ablation serum creatinine levels ranged from 0.88 to 1.26 mg/dl. In sham-operated animals renal function was unchanged (serum creatinine range, 0.47–0.60 mg/dl). These data are in agreement with our previous study (Zoja et al., 1988).

Effects of the components of conjugated estrogen preparation on bleeding time in uremic rats. Table 1 shows the effect of conjugated estrogens, estrone sulfate, equilin sulfate, 17β-estradiol or vehicle on bleeding time in uremic and sham-operated animals at different time intervals from injection. Basal values of bleeding time of uremic rats were significantly (P < .01) longer than those of sham-operated animals thus confirming our previous data (Zoja et al., 1988) indicating that in rats as in humans the development of renal failure is associated with an acquired defect of primary hemostasis. When uremic animals were given conjugated estrogen preparation, bleeding time values became significantly (P < .01) shorter than basal values at 24 and 48 hr after injection. Seventy-two hours after conjugated estrogen treatment bleeding times returned to preinjection values. Injection of estrone sulfate was followed by a significant (P < .01) shortening of bleeding time at 24 hr in comparison with basal values. Forty-eight hours after estrone sulfate injection bleeding time returned to preinjection values. Equilin sulfate did not affect the prolonged bleeding times of uremic rats. Indeed, bleeding time values at all time intervals considered after injection were not different from the basal ones. 17β-estradiol reduced the prolonged bleeding time reproducing the pattern observed after conjugated estrogen preparation injection. Thus, bleeding time values remained significantly (P < .01) shorter than basal values until 48 hr and returned to preinjection values within 72 hr. The injection of vehicle alone had no effect on bleeding time in uremic rats. Neither conjugated estrogen preparation nor estrone sulfate, equilin sulfate, 17β-estradiol or vehicle had any effect on the bleeding time in sham-operated rats at any time interval considered.

Dose-effect study of estrogen receptor antagonists on bleeding time in uremic rats treated with conjugated

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<td>Effects of the components of conjugated estrogen preparation or vehicle on bleeding time in uremic or sham-operated rats</td>
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<td>Values are expressed as mean ± S.D. The doses of injected drugs are given under &quot;Materials and Methods.&quot;</td>
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<td>Sham-operated rats</td>
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* P < .01 vs. the respective basal value.
**Estrogen preparation.** To evaluate whether estrogen effect on bleeding time was mediated by a receptor mechanism we administered p.o. to uremic rats two well known estrogen receptor antagonists, tamoxifen and clomiphene. As shown in figure 1, 24 hr after different doses of tamoxifen administration, when the peak plasma concentration of tamoxifen has been reached (Fromson et al., 1983), bleeding time values were not different from the basal ones. After the 24-hr bleeding time determination was performed rats were injected with conjugated estrogen preparation and bleeding time measured 24 hr later, when the effect of conjugated estrogens on bleeding time was maximum (Zoja et al., 1988). Figure 1 shows that the bleeding time shortening induced by conjugated estrogen injection was not affected when uremic rats were pretreated with 1 and 2 mg/kg of tamoxifen. In contrast, the administration of 3 and 4 mg/kg of tamoxifen did block conjugated estrogen effect on bleeding time, suggesting that a receptor mechanism may account for the hemostatic effect of conjugated estrogens. Such blocking was confirmed by the results obtained with the use of the other receptor antagonist, clomiphene. As shown in figure 2, bleeding time shortening induced by conjugated estrogen was obtained in uremic rats pretreated with 1 and 2 mg/kg of clomiphene. When uremic rats were given 3 mg/kg of clomiphene no effect on bleeding time was observed after conjugated estrogen injection.

**Effect of estrogen receptor antagonists on bleeding time in uremic rats treated with estrone sulfate or 17β-estradiol.** The minimum dose of tamoxifen and clomiphene (3 mg/kg) able to prevent the shortening of bleeding time induced by estrogen preparation was administered to uremic rats before estrone sulfate or 17β-estradiol. As shown in figure 3, in uremic rats pretreated with either receptor antagonist, estrone sulfate and 17β-estradiol did not shorten the bleeding time.

**Discussion**

Bleeding is still a potentially fatal complication of uremia (Rabiner, 1972; Livio et al., 1985). Previous studies have documented that a mixture of conjugated estrogens is effective both in shortening the skin bleeding time (Liu et al., 1984; Livio et al., 1986), the best laboratory hallmark of clinical bleeding (Steiner et al., 1979), and in maintaining a prolonged hemorrhage-free period in patients with chronic renal failure and recurrent gastrointestinal bleeding (Bronner et al., 1986). The present study was designed first to document which of the components of the conjugated estrogen mixture is effective in shortening the bleeding time. To that purpose we used an animal model of chronic uremia described recently (Zoja et al., 1988). The interest of this model rests on the observation that uremic animals, like humans, have a prolonged bleeding time and that estrogen injection shortens the bleeding time, the
effect being long-lasting in rats (Zoja et al., 1988), as documented previously in humans (Livio et al., 1986). Our present findings indicate that both estrone sulfate and 17β-estradiol but not equilenin are effective in shortening the bleeding time in uremic rats. However, whereas 17β-estradiol reproduced exactly the time course of bleeding in uremic rats, the estrone sulfate injection induced a comparable shortening of the bleeding time at the beginning but the effect lasted only for 24 hr. Thus, 17β-estradiol appears the key component in the conjugated estrogen mixture that accounts for the observed effect on primary hemostasis in vivo.

We also investigated whether estrogens influence primary hemostasis by a receptor-mediated mechanism, given the dissociation between the short plasma estrogen half-life and the long-lasting effect on bleeding time. Actually the various components of the conjugated estrogen mixture have a half-life of few hours in uremic blood (Viganò et al., 1988), whereas the effect on bleeding time lasts 14 days or more (Livio et al., 1986; Viganò et al., 1988). A possibility to reconcile these findings relates to the events that follow estrogen entry to the cells bearing specific receptors. Thus, estrogens enter the cell and bind high affinity receptor proteins on the cytosol (Jensen et al., 1972; O'Malley and Means, 1974). The estrogen protein complex is translocated into the cell nucleus, with subsequent induction of specific mRNA and certain specific but unknown proteins (Gorski and Gannon, 1976).

Estrogen activity can be blocked by antiestrogens. These compounds have some structural similarities to estrogens that may explain their action (Murad and Haynes, 1985). Indeed, antiestrogens such as tamoxifen and clomiphene, two well known nonsteroidal agents useful in the treatment of human breast cancer (Fisher et al., 1989) and of infertility (Marshall, 1978), respectively, compete for estrogen binding sites leading to a diminished amount of estrogen receptor available for endogenous hormone activity and causing estrogen blockade and inhibition (Marshall, 1978; Heel et al., 1978).

Here we found that estrogen receptor antagonists, tamoxifen and clomiphene, prevented the shortening of bleeding time induced by conjugated estrogen mixture or its active components in uremic rats. These findings strongly suggest that the effect of estrogens on primary hemostasis in uremia is mediated by a receptor mechanism, but do not contribute to clarify the nature of the receptor bearing cell responsible for the effect.

Actually, among cells involved in primary hemostasis, platelets do not possess specific binding for estrogens (Chang et al., 1982). This is not surprising in as much as platelets are anucleate cells which contain no DNA and direct effects of steroid hormones, which affect gene expression, would therefore not be expected. Indeed, Chang and co-workers (1982) by studying 12-lypoxigenase activity in rat platelets stimulated by 17β-estradiol, were unable to detect any specific binding of estradiol to platelets.

Whether endothelial cells actually have estrogen receptors is still an open issue. Indeed, it has been reported that rabbit endothelial cells possess estrogen receptors (Colburn and Buonassisi, 1978) and that ovarian hormones may influence the role of the endothelium in various physiological and pathophysiological conditions. It was also found that estrogen binding sites are present in canine vascular tissues (Horwitz and Horwitz, 1982), in rabbit arteries (Malinow et al., 1959) and in baboon arteries (McGill and Sheridan, 1981). However, using rat endothelial cells, Nakhla and co-workers (1984) were unable to find estrogen receptors. In humans the presence of estrogen receptors was demonstrated in cell cultures from umbilical vein (Taggart and Stant, 1980) and in fresh aortic tissue obtained from patients undergoing heart surgery (Campisi et al., 1987). One can therefore speculate that endothelial cell specific receptors are the target protein mediating estrogen effect on uremic bleeding.

Several lines of evidence have indicated recently that besides platelets white blood cells may also play a role in blood coagulation. Thus, white cells have the property to express different types of clot-promoting activities, the so-called procoagulant activity (Niemetz, 1972), whereby blood coagulation can be initiated and propagated. The most commonly described procoagulant activity has been shown to be tissue thromboplastin
(tissue factor) the initiating cofactor of the extrinsic coagulation pathway (Semeraro et al., 1983). On this basis, findings of specific estrogen receptors in human blood mononuclear cells (Weusten et al., 1986; Ranalletti et al., 1988) together with changes in the neutrophil count during the menstrual cycle of women (Bain and England, 1975; Mathur et al., 1979), would suggest as an alternative possibility that estrogens are hormonally effective in uremia through a white cell-dependent receptor mechanism.

In summary, the present data show that 1) 17β-estradiol and estrone sulfate are the active components of conjugated estrogen mixture in shortening the prolonged bleeding time in uremic rats, 17β-estradiol having a longer duration of action and 2) the activity is mediated by estrogen receptors in as much as specific antagonists neutralize the effect.

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References


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