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INHIBITION OF THROMBIN-INDUCED PLATELET AGGREGATION BY UTEROGLOBIN

RAMANATHAPURAM MANJUNATH, SONDRA W. LEVIN, KUZHIYILETHU K. KUMAROO*, JEAN DEB. BUTLER, JEROME A. DONLON†, MCDONALD HORNE†, RYOJIRO FUJITA, UWE K. SCHUMACHER and ANIL B. MUKHERJEE‡

Section on Developmental Genetics, Human Genetics Branch, National Institute of Child Health and Human Development, NIH, Bethesda, MD 20892; *Naval Research Institute, Bethesda Naval Hospital, Bethesda, MD; and †Hematology Service, Clinical Pathology Department, The Clinical Center, NIH, Bethesda, MD 20892, U.S.A.

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Abstract—Uteroglobin, a steroid-dependent, small molecular weight (15 K) protein in the rabbit, inhibited thrombin-induced aggregation of both rabbit and human gel-filtered platelets (GFP). GFP aggregation by arachidonic acid was not affected by uteroglobin. There were no effects of uteroglobin on thrombin-induced clotting of plasma or purified fibrinogen, or inhibition of thrombin by antithrombin III. Additionally, preliminary results suggest that uteroglobin does not interfere with binding of thrombin to platelets. We suggest that inhibition of platelet aggregation by uteroglobin may function in preventing thrombosis and ensuring free flow of blood through the microvasculature of the uterus and the placenta and may induce some of the antimotility effects of progesterone on the uterus.

Successful mammalian pregnancy requires an intimate association between the fetus and the mother. During nidation, the embryonic cells make direct contact with the endometrium [1]. Since the developing embryo, for all practical purposes, is an allograft to the mother, antigenic stimulation of the maternal immunological system may occur. The formation of platelet activating factor is induced by immunological and nonimmunological stimulation [2–4], thereby predisposing the maternal organism to develop thrombosis. In fact, an increased risk of thrombosis during pregnancy [5], as well as during estrogen therapy [6–10], has been well recognized.

Uteroglobin [11] or blastokinin [12] is a steroid-dependent, small molecular weight (15 K) protein synthesized by the rabbit endometrium during preimplantation stages of embryonic development and nidation. During early gestation, this protein represents approximately 30–40% of total secreted uterine proteins [13–17]. Using *in vitro* systems we have shown previously that this protein in conjuction with transglutaminase (EC 2.3.2.13) masks embryonic [18] and sperm antigenicity [19] and, thus, prevents maternal immunological response. We have also demonstrated that uteroglobin may be cross-linked by transglutaminase [20]. Recently, Bischof *et al.* [21] reported inhibition of thrombin-induced coagulation of plasma by a human pregnancy associated protein, but no specific effects of this protein on platelet aggregation have been shown.

The present investigation was designed to test the hypothesis that uteroglobin may inhibit thrombin-induced platelet aggregation, thus allowing free flow of blood through the microvasculature of the feto-placental unit.

‡ To whom all correspondence should be addressed: Bldg. 10, Room 10N314, National Institutes of Health, Bethesda, MD 20892.

MATERIALS AND METHODS

Progesterone, human chorionic gonadotrophin (HCG), arachidonic acid, hirudin and lactoperoxidase were obtained from the Sigma Chemical Co., St. Louis, MO. Bovine thrombin was obtained from Parke Davis & Co., Detroit, MI. Purified human thrombin was the gift of Dr. John Fenton, Albany, NY. Sepharose CL2B, G-100 (Superfine) and G-50 Sephadex (Superfine) were obtained from Pharmacia Chemicals, Piscataway, NJ. Antiuteroglobin antisera raised in the goat was a gift from Dr. David Bullock, Baylor University School of Medicine, Houston, TX. This antisera has been characterized [22]. CM52 cellulose was obtained from Whatman Inc., Clifton, NJ. Purified human fibrinogen was purchased from Kabi Diagnostica, Stockholm, Sweden. Na-^{125}I was from the Amersham Corp., Arlington Heights, IL.

Purification of uteroglobin from uterine flushings

HCG induction. Adult virgin New Zealand rabbits, weighing approximately 3 kg, were primed with HCG. The contents of each vial of HCG were reconstituted in 10 ml of sterile deionized water to give a final concentration of 500 I.U./ml. On day 1 a dose of 0.5 ml of a stock solution of HCG was administered intramuscularly to each animal. A second dose was administered 48 hr following the first injection.

Collection of uterine flushings. Two days after the last injection of HCG, rabbits were killed by administering intravenous sodium pentobarbital (150–200 mg). Uterine horns were dissected out immediately and were flushed with 8–10 ml of cold 50 mM Tris–HCl buffer, pH 7.5, containing 1.5 mM EDTA, 100 mM NaCl, and 0.5 mM phenylmethylsulfonylfluoride (PMSF). The flushings were stored at −20° until needed for further processing.

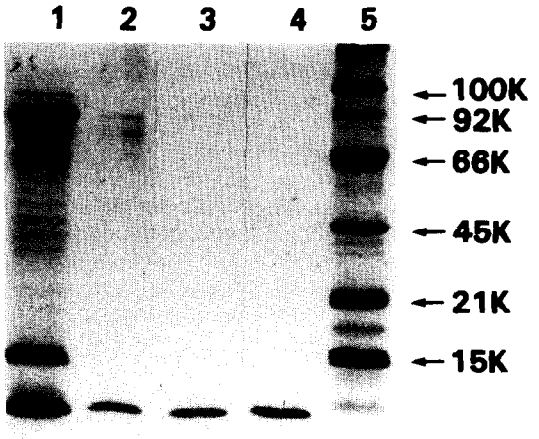

Fig. 1. SDS–PAGE of protein peaks obtained from column chromatography. Lane 1: crude uterine flushing. Lane 2: peak IV from Sephadex G-100 column which was immunoreactive to UTG-antibody. Lane 3: immunoreactive peak from CM-52 column. Lane 4: single immunoreactive peak from Sephadex G-50 column. Lane 5: molecular weight standard. Note that only the UTG monomers (mol. wt 7.5 K) are visualized in these gels because of the presence of mercaptoethanol in the sample buffer which reduces the S—S bonds.

Chromatographic purification. The isolation and purification of uteroglobin were carried out by the method of Nieto and Beato [16], with minor modifications [18]. Briefly, uterine flushings were centrifuged at 5000 *g* for 30 min, and the supernatant fraction was lyophilized after dialysis overnight against three changes of deionized water. The uterine proteins were reconstituted in 5–8 ml of Tris–saline buffer, pH 7.5 (20 mM Tris, 100 mM NaCl) and were applied to a 2.5 × 100 cm Sephadex G-100 column (Superfine, particle size 10–40 μm). Fractions in peak IV were pooled and dialyzed extensively since peak IV had the ability to inhibit platelet aggregation. The major protein present in this fraction was uteroglobin as tested on Ouchterlony double-diffusion plates against specific anti-uteroglobin antisera and radioimmunoassay. Dialyzed peak IV fractions were applied to a 1.5 × 3 cm CM-cellulose column for ion-exchange separation. The uteroglobin-containing fractions from the ion-exchange column were then dialyzed and lyophilized. This protein was reconstituted in 1.5 ml Tris–NaCl buffer, pH 7.5, and loaded on a 1.5 × 72 cm G-50 Sephadex column (Superfine). A single peak eluted from this column was dialyzed against water, lyophilized, and stored at −20°. Purification of uteroglobin was also verified by polyacrylamide gel electrophoresis (Fig. 1).

Platelet aggregation. Blood was collected from healthy human volunteers by venipuncture or from the marginal ear vein of the female rabbit and anticoagulated with 0.38% trisodium citrate. The platelet-rich plasma (PRP) was obtained by centrifuging the blood at approximately 200 *g* for 10 min in a Beckman clinical centrifuge. The platelets were then isolated by gel-filtration of the PRP on a Sepharose CL2B column equilibrated with Tyrode's solution without Ca^{2+} [23]. The platelets eluting at the void volume were isolated and resuspended in Tyrode's solution without Ca^{2+}. The platelet suspensions were reconstituted with Ca^{2+} (1.8 mM) before use in aggregation studies.

All aggregations were performed in siliconized glass cuvettes containing 0.5 ml of platelet suspension (2–5 × 10^8 cells/ml Tyrode's solution). Crude uterine flushings or purified uteroglobin was added in saline and preincubated with platelets for 2 min at 37°. Thrombin (0.25 units/ml or arachidonic acid, 0.1 mM) was added, and the aggregation was measured at 37° in a dual channel aggregometer (Chronolog Corp.). Control aggregation curves were obtained in the absence of uteroglobin with an equivalent amount of buffer to make up for the decrease in assay volume. All aggregation studies were repeated at least ten times and the interassay variations were <5%. The platelet aggregation curves were continuous recordings, and no point to point measurements and/or comparisons were performed. Triplicate runs on the same sample of platelets were superimposable and little variation was noted.

Assays of thrombin function

[^{125}I]-*Thrombin binding to platelets.* Highly purified human thrombin was labeled with Na-^{125}I by solid state lactoperoxidase without loss of fibrinogen-clotting activity [24, 25]. It was diluted to ~0.3 NIH units/ with Tyrode's solution including 1.8 mM Ca^{2+} with or without 5% bovine serum albumin. Various concentrations of uteroglobin were added to aliquots of thrombin before mixing 1:1 with GFP suspended in the same buffer (~2.5 × 10^8/ml). The concentrations of thrombin and uteroglobin were adjusted to approximate the concentrations used in the aggregation studies. The final thrombin concentration was determined by measuring the radioactivity (cpm) of the entire sample in a gamma counter (Tracor Analytic, Des Plains, IL). The platelets were then separated from the suspension by centrifugation at 2000 *g* for 8 min and the supernatant fraction was discarded. The radioactivity of the platelet pellet was remeasured. Nonspecific binding and trapping of the [^{125}I]-thrombin was measured by performing the binding study in the presence of 20 units of hirudin/ml [26].

Additional studies. The ability of thrombin to clot plasma and purified fibrinogen in the presence and absence of uteroglobin was quantitated by measuring the time for a clot to appear in a stirred sample following the addition of ~2 units/ml of thrombin [27, 28]. The ability of antithrombin III to inhibit thrombin in the presence and absence of uteroglobin was measured using a synthetic substrate [29].

RESULTS

Since crude uterine flushing prevented thrombin-induced platelet aggregation, we tested various protein peaks obtained from fractionation of these fluids in G-100 column chromatography. Figure 2 shows the effects of various protein peaks and pure uteroglobin on thrombin-induced platelet aggregation. Although crude uterine proteins inhibited platelet aggregation, Peaks I (90–100 kD), II (65 kD) and III (45 kD) which contained no uteroglobin did not

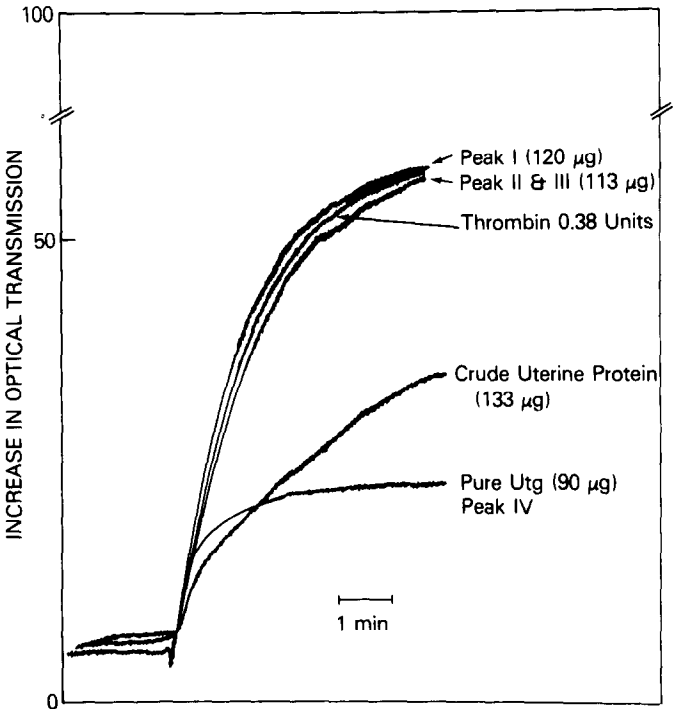

Fig. 2. Effects of various protein peaks obtained from G-100 column chromatography of crude uterine flushings of HCG-primed rabbits on thrombin-induced platelet aggregation.

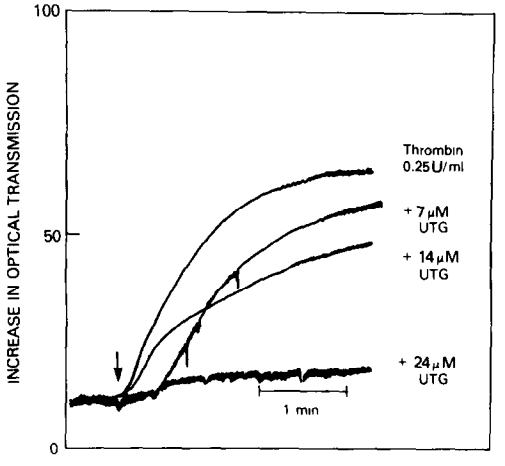

Fig. 3. Dose–response curve for uteroglobin-mediated inhibition of thrombin-induced platelet aggregation.

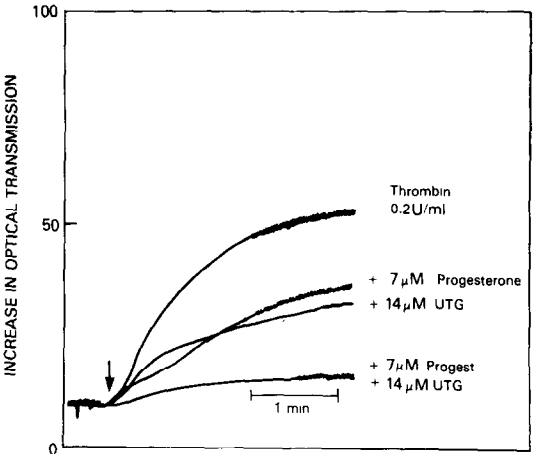

Fig. 4. Additive effect of progesterone on the inhibition by UTG of thrombin-induced platelet aggregation.

inhibit platelet aggregation. The most dramatic effect was obtained with 90 μg pure uteroglobin. Peak IV, like purified uteroglobin, dramatically inhibited thrombin-induced platelet aggregation. Aggregation of gel-filtered platelets by uteroglobin was inhibited in a dose-dependent manner. Complete inhibition was obtained at a final concentration of 24 μM (Fig. 3). There was an additive effect of uteroglobin when used in conjunction with 7 μM progesterone (Fig. 4). The concentration of uteroglobin which inhibited 50% of thrombin-induced platelet aggregation (14 μM) when used in conjunction with 7 μM progesterone completely blocked aggregation of gel-filtered platelets (GFP). It should be noted that 14 μM UTG alone could not produce such complete inhibition. Platelet aggregation by arachidonic acid was not inhibited by uteroglobin (Fig. 5). Albumin as a nonspecific protein control at a concentration of 125 μg/ml did not inhibit thrombin-induced platelet aggregation. Uteroglobin was as effective in inhibiting thrombin-induced aggregation of human platelets as it was for rabbit platelets.

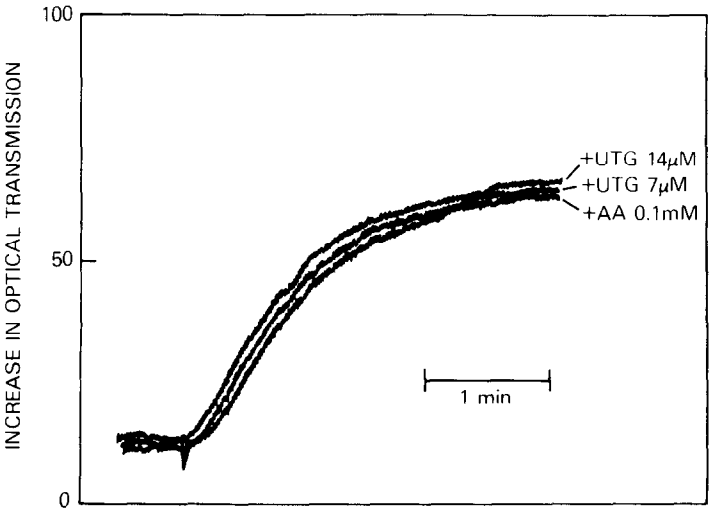

Fig. 5. Inhibition of arachidonic acid induced platelet aggregation by UTG.

Table 1. Effect of uteroglobin on [^{125}I]-thrombin binding to platelets

Uteroglobin (μmoles/liter)	Bound thrombin* (milliunits/10^8 platelets)
33	1.4 ± 0.035 (3)
17	1.6 ± 0.012 (3)
6.7	1.8 ± 0.11 (3)
0	1.4 ± 0.25 (7)

* Total thrombin concentration, 240 milliunits/ml (1 milliunit = 0.001 NIH units). Results are the means of at least three measurements (±SEM) of bound thrombin at each uteroglobin concentration. The number of replicates (N) at each uteroglobin concentration is shown in parentheses. Non-specifically bound thrombin (0.6 milliunits/ml) has been subtracted.

Table 2. Effect of uteroglobin on thrombin time

Additions	Thrombin time (sec) using purified fibrinogen*
Uteroglobin	48.2 ± 2.4
No uteroglobin	44.2 ± 1.4

*Concentration of uteroglobin, 24 μM; concentration of thrombin, 2 units/ml. Results are the means of at least three determinations ± SEM, N = 9.

Uteroglobin even at a concentration (33 μM) in excess of that which produced complete inhibition of platelet aggregation (24 μM) failed to inhibit the binding of thrombin to platelets in the presence or absence of albumin (Table 1).

To delineate the mechanisms of inhibition of platelet aggregation by uteroglobin, we investigated the possibility that uteroglobin directly interferes with thrombin function. As shown in Table 2, uteroglobin at a concentration of 24 μM had no effect on thrombin clotting of purified fibrinogen. The thrombin-induced clotting of plasma and thrombin inhibition by antithrombin III were also unaffected by uteroglobin.

DISCUSSION

Uteroglobin is a progesterone-induced pregnancy-specific protein synthesized by endometrial cells in the rabbit. Besides its function in protecting early developing embryos [18] and male gametes from immunological destruction by the female organism [19], it also inhibits neutrophil and monocyte chemotaxis at micromolar concentrations [30]. The data presented here assigns another related function, namely, inhibition of thrombin-induced platelet aggregation by uteroglobin.

Inflammation and immunological reactions are closely linked [31]. During implantation in the rabbit, the trophoblast juxtaposed with uterine epithelial cells penetrates the basal lamina and blood vessels [32, 33]. As the syncytiotrophoblast advances into the endometrium, it becomes vacuolated, creating a system of small cavities into which the maternal blood eventually flows [1]. The injury to the endometrium by the invading trophoblast may trigger release of thrombin which can activate platelets. The microvasculature as well as the small cavities described above may slow down the flow of blood and initiate thrombosis [34–36].

Thrombin is a unique serine protease which causes platelet aggregation by affecting a step proximal to arachidonic acid metabolism [37,38]. More specifically, thrombin by activating phospholipases causes the release of arachidonic acid from the phospholipids of platelet membranes which then acts as substrate for prostaglandin synthesis as well as the synthesis of chemotactic leukotrienes. Thus, uteroglobin, by inhibiting thrombin-induced platelet aggregation, may not only prevent thrombosis in the microvasculature during early pregnancy but also may prevent uterine contraction induced by prosta-

glandins which may otherwise lead to early abortion. The apparent lack of uteroglobin effect on the ability of thrombin to clot fibrinogen (Table 2) and to be inhibited by antithrombin III suggests that uteroglobin does not interact directly with the thrombin molecule. To evaluate the possibility that uteroglobin may affect the binding sites for thrombin on the platelet surface, we measured the binding of [^{125}I]-thrombin to platelets in the presence and absence of uteroglobin. Thrombin binding to platelets is saturable and is characterized by multiple classes of sites [26, 39, 40]. Because of the limited availability of uteroglobin, we restricted our inhibition studies to the thrombin concentration which yielded 100% platelet aggregation. Under these conditions, no effect of uteroglobin on thrombin binding to platelets was detected (Table 1). On the other hand, data from our laboratory suggest that uteroglobin is a potent inhibitor of phospholipase A_2 [41] and, hence, an indirect inhibitor of prostaglandin synthesis. This may be one of the mechanisms by which uteroglobin inhibits thrombin-induced platelet aggregation rather than by inhibiting binding of thrombin to platelets.

In all mammals, progesterone is essential for the maintenance of pregnancy and in most species it is a prerequisite for implantation [42]. Additionally, it has been firmly established that in both the rabbit and the rat the essential role of progesterone in pregnancy is to inhibit uterine contractility, thereby effecting quiescence and physiological accommodation of the conceptus [42]. Progesterone has been shown to have a direct effect on smooth muscle contraction *in vitro* [43]. Progesterone may also influence contractility indirectly by influencing prostaglandin production. In this respect, we have investigated the effects of uteroglobin on phospholipase A_2 using the assay method described by Cloix *et al.* [44]. Our data suggest that uteroglobin is a potent inhibitor of phospholipase A_2 [41] and hence, indirectly, an inhibitor of prostaglandin synthesis. In the present study, we found an additive inhibitory effect on thrombin-induced platelet aggregation when uteroglobin and progesterone were used together. Uteroglobin is known to bind progesterone [45], and it is tempting to speculate that the uteroglobin–progesterone complex may be a better inhibitor of phospholipase A_2 than any one of these components alone, probably because there is a better conformity of binding of this complex with the phospholipase molecule. Confirmation of these results may delineate the mechanism of observed immuno-modulation [18, 19, 30] as well as platelet aggregation-inhibitory effects of uteroglobin. Alternatively, this may also explain the mechanism by which progesterone exerts its antimotility effects on the pregnant uterus.

In conclusion, we believe that our data support the hypotheses that uteroglobin specifically inhibits thrombin-induced platelet aggregation and, thus, may be instrumental in preventing thrombosis in the feto-placental unit. Although the precise mechanism of inhibition is not entirely clear at this time, experimental evidence [41] suggests that this effect may be due to, at least in part, its inhibitory action on phospholipases.

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