Haemostatic mechanism in the endometrium: role of cyclo-oxygenase products and coagulation factors

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- 1 The primary mechanism of haemostasis in the endometrium of rat was studied and results were compared to that in the mesenteric artery.
- 2 The bleeding time of the rat endometrium as assessed by haemoglobin output was significantly decreased after pretreatment of the animals with either indomethacin (5 mg kg⁻¹, i.v.) or meclofenamate (3 mg kg⁻¹, i.v.) whereas the bleeding time was significantly increased in the rat mesenteric artery.
- 3 The bleeding time of the rat endometrium was unchanged from control values following treatment with prostacyclin (0.5 µg kg⁻¹ min⁻¹, i.v.) or 1-benzylimidazole (50 mg kg⁻¹, i.v.) whereas the bleeding times were increased in the rat mesenteric artery.
- 4 Administration of heparin (100 units kg⁻¹) increased the bleeding time in the rat mesenteric artery but had no effect on the bleeding time of the endometrium.
- 5 Superfusion of the endometrium with 16, 16-dimethyl PGE₂ (1 µg ml⁻¹), a vasodilator, increased the bleeding time of the endometrium but superfusion of PGE₂ over the mesenteric artery did not affect the bleeding time from this site.
- 6 Histological studies of the mesenteric artery and the endometrium following haemostasis revealed that the haemostatic plug in the mesenteric artery was mainly composed of platelets and fibrin whereas in the endometrium it was mainly composed of fibrin.
- 7 These findings suggest that haemostasis in the endometrium may be mediated by the vascular tone and fibrin whereas formation of the platelet plug may be the primary mechanism for haemostasis in the mesenteric artery.

Keywords: Cyclo-oxygenase inhibitors; prostaglandins; non-steroidal, anti-inflammatory drugs; haemostasis; endometrium; mesenteric artery

Introduction

The primary mechanism of haemostasis in the skin and arterial vessels involves adhesion and aggregation of platelets at the site of injury with subsequent platelet plug formation (Sixma & Wester, 1977; Verstraete & Vermeylen, 1984). Thromboxane A₂ (TxA₂), a cyclo-oxygenase pathway product of arachidonic acid (AA) metabolism formed by platelets, has been implicated as an endogenous mediator of platelet aggregation (Whittle & Moncada, 1983). Administration of non-steroidal, anti-inflammatory drugs (NSAIDs) which inhibit the cyclo-oxygenase enzyme (Vane, 1971) can reduce platelet aggregation *in vitro* (Smith & Willis, 1971) and also prolong cutaneous bleeding time in man (Amezcuca *et al.*, 1979). Conversely, NSAIDs promote haemostasis in the endometrium and have been successfully used to treat idiopathic menorrhagia (Anderson *et al.*, 1976; Fraser, 1983; Muggeridge & Elder, 1983) as well as menorrhagia associated with the use of the intra-uterine contraceptive device (IUD) (Damarawy & Toppazada, 1976; Guillebaud *et al.*, 1978; Ylikorkala *et al.*, 1978; Roy & Shaw, 1981). The mechanism of this paradoxical effect of NSAIDs in modulating haemostasis in the skin and endometrium is not known.

The primary mechanism of haemostasis may vary by site. Platelet aggregation plays a major role in the haemostatic events in the skin or vasculature whereas the arrest of gastric haemorrhage is brought about largely by a process primarily involving the coagulation system (Whittle *et al.*, 1986). We, therefore, investigated the mechanism of haemostasis in the endometrium. To accomplish this, we slightly modified the technique used for studying the haemostatic mechanism in the gastric mucosa (Whittle *et al.*, 1986) and developed a

method for evaluating bleeding from a standard incision in the endometrium of the rat. With this technique, we evaluated the mechanism of haemostasis in the endometrium and compared it with that of the mesenteric artery in the rat. Oophorectomized rats supplemented with oestradiol were used in order to control for the hormonal milieu which may modify the bleeding time.

Methods

Sprague–Dawley rats (220–260 g) were obtained from Bantin and Kingman Farms (Freemont, CA, U.S.A.). They were housed under controlled conditions. Water and commercial chow were allowed *ad libitum*. The rats were anaesthetized with ketamine hydrochloride (100 mg kg⁻¹, i.m.) and xylazine (10 mg kg⁻¹, i.m.) after which they were oophorectomized through a ventral mid-line incision. The abdominal layers were sutured with 2.0 chromic and the animals were allowed to recover. The animals were then injected daily with 17β oestradiol benzoate (1 mg kg⁻¹, s.c.) in oil for 4 days and were used on the 4th day at least 2 h after the injection. This ensured that the hormonal milieu was the same in all the experimental animals used for the study.

Evaluation of bleeding time

The technique to assess bleeding time in the endometrium and the mesenteric artery was similar to the previously described method for determining bleeding time in the gastric mucosa (Whittle *et al.*, 1986). The animals were anaesthetized with ketamine hydrochloride (100 mg kg⁻¹, i.m.) and xylazine (10 mg kg⁻¹, i.m.) after which the femoral vein was can-

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nulated for intravenous drug or vehicle administration. The uterus was exposed by a midline laparotomy. The uterine horn was opened along the anti-mesometrial border and the initial bleeding at the incision site was controlled with microelectrocautery. The exposed endometrium was then placed in a modified plastic chamber ensuring that the vascular supply was intact. The endometrium was gently superfused with isotonic saline solution at 37°C delivered with the aid of an infusion pump at a rate of 2 ml min⁻¹. A standardized 3 mm incision was made with microscissors perpendicular to the rugae of the endometrium to initiate endometrial bleeding. The perfusate was directed away from the site of the lesion so as to minimize any potential disruption of the haemostatic plug and collected in 1 min fractions for determination of haemoglobin output. This technique was found to be highly reproducible for studying bleeding time in the endometrium.

Terminal ileum mesenteric arteriolar vessel bleeding time was also determined in the same animal. A loop of the ileum was placed over the edge of the chamber which provided access to the vascular arcades of the mesentery without disrupting the vascular supply. A binocular dissecting microscope was used to identify a branch of the mesenteric artery close to the ileum and bleeding was produced by puncture with a 25-gauge needle (Whittle *et al.*, 1986). Isotonic saline at 37°C was superfused at a rate of 2 ml min⁻¹, taking care not to disrupt the incision site and the superfusate was collected at 1 min intervals.

Bleeding time was determined as the haemoglobin output (mg min⁻¹) into the superfusate collected at 1 min intervals. Samples were treated with Lys-SII (Coutler, Hialeah FL, U.S.A.) 0.1 ml to lyse erythrocytes and the optical density of haemoglobin in the perfusate was determined spectrophotometrically (540 nm). Haemoglobin output (mg min⁻¹) was calculated with the use of a standard curve constructed with rat heparinized arterial blood diluted with saline. The bleeding time was taken as the time from the incision to the first collection period during which the haemoglobin output was <0.1 mg min⁻¹, corrected for the chamber wash out time.

Effect of cyclo-oxygenase inhibitors on bleeding time

Bleeding time in the mesenteric vessel and the endometrium of one uterine horn was initially determined followed by a repeat bleeding time determination in an equivalent mesenteric vessel and in the endometrium of the contralateral uterine horn performed 30 min after administration of either indomethacin (5 mg kg⁻¹, i.v.) or sodium meclofenamate (3 mg kg⁻¹, i.v.). These doses have been previously demonstrated to inhibit formation of cyclo-oxygenase products (Chaudhuri, 1973; Chaudhuri *et al.*, 1982; Whittle *et al.*, 1986).

Effects of inhibition of platelet aggregation

Bleeding times in the mesenteric artery and the endometrium were determined prior to and 10 min following intravenous infusion of prostacyclin (Epoprostenol; Wellcome) and maintained throughout the observation period at a dose of 0.5 µg kg⁻¹ min⁻¹ by an infusion pump. This concentration of prostacyclin has previously been demonstrated to produce near maximal inhibition of platelet aggregation (Whittle *et al.*, 1986).

Effects of thromboxane synthase inhibitor

The bleeding time in the mesenteric artery and the endometrium were determined after intravenous administration of 1-benzylimidazole as the fumarate salt (50 mg kg⁻¹, i.v.). This dose was selected as it has been previously demonstrated to inhibit platelet thromboxane synthesis by over 90% (Whittle *et al.*, 1986).

Effects of heparin on bleeding time

In order to assess the effect of inhibition of the coagulation cascade on the haemostatic mechanism in the endometrium, bleeding time was determined in the mesenteric vessel and endometrium after intravenous injection of heparin (100 units kg⁻¹). This dose does not interfere with platelet function in this species (Whittle *et al.*, 1986).

Effect of prostaglandin E₂ (PGE₂) on bleeding time

In order to assess whether the vasodilator effect of PGE₂ modulates the haemostatic mechanism in the endometrium, bleeding time was assessed in the mesenteric artery and the endometrium 10 min after the initiation of superfusion of 16, 16-dimethyl PGE₂ (1 µg ml⁻¹) and which was continued for the duration of the observation period.

Histology

Histological studies were performed on the mesenteric artery and the endometrium after initiation of bleeding and fixation of the tissues following haemostasis. The tissues were embedded in paraffin, sectioned and stained with haematoxylin and eosin, Giemsa stain and Trichrome stain for evaluation by light microscopy.

Chemicals

Indomethacin and sodium meclofenamate were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Prostacyclin (Epoprostenol) and 1-benzylimidazole were gifts from Wellcome Research Laboratories (Beckenham, Kent, U.K.). The stable analogue of prostaglandin E₂, 16, 16-dimethyl PGE₂ was obtained from Cayman Chemical Co. (Ann Arbor, MI, U.S.A.). Heparin was obtained from UCLA Pharmaceutical Services (Los Angeles, CA, U.S.A.).

Statistical analysis

Results are expressed as the mean ± s.e.mean for each point. Differences between groups were assessed by ANOVA with repeated measures and Students' *t* test where appropriate. *P* values <0.05 were considered as significant.

Results

The bleeding time studies were performed by a single individual in order to control for variation related to incisional length and depth that may arise. This also ensured that the haemoglobin output during the initial collection period was similar in the respective blood vessels under study. The initial haemoglobin output was found to vary between vessels. Initial haemoglobin output in the mesenteric artery was 6.82 ± 0.61 mg min⁻¹ whereas in the endometrium the initial haemoglobin output averaged 2.07 ± 0.15 mg min⁻¹.

Effects of cyclo-oxygenase inhibition on bleeding time

Haemoglobin output from the mesenteric artery (*n* = 6) and the endometrium (*n* = 6) before and after treatment with indomethacin is shown in Figure 1. Prior to treatment with indomethacin, haemoglobin output diminished rapidly from the time of the incision. Bleeding time in the mesenteric artery was 4.80 ± 0.15 min whereas that of the endometrium was 6.10 ± 0.11 min. The haemoglobin output from the mesenteric artery was prolonged following administration of indomethacin (bleeding time 11.22 ± 0.34 min; *P* < 0.001) whereas it was significantly shortened in the endometrium (bleeding time 4.43 ± 0.20 min; *P* < 0.001). Following administration of meclofenamate, the bleeding time of the mesenteric artery (*n* = 5) was prolonged (12.06 ± 0.68 min;

$P < 0.001$) whereas that of the endometrium ($n = 5$) was significantly reduced (3.80 ± 0.19 min; $P < 0.001$).

Effects of inhibition of platelet aggregation

Intravenous infusion of prostacyclin prolonged the bleeding time (10.96 ± 0.63 min; $P < 0.005$) of the mesenteric artery ($n = 5$), whereas in the endometrium there was no significant difference from control values (6.18 ± 0.30 min) ($n = 5$). A similar prolongation of the bleeding time (10.08 ± 0.52 min; $P < 0.001$) of the mesenteric artery ($n = 4$) was observed following administration of BZi as the fumarate salt whereas there was no change in the bleeding time (5.8 ± 0.25 min) of the endometrium ($n = 4$). The haemoglobin output from the mesenteric artery and the endometrium before and after treatment with either prostacyclin or BZi are shown in Figure 2.

Effects of heparin

Intravenous infusion of heparin had no effect on the bleeding time (5.53 ± 0.38 min) of the mesenteric artery ($n = 6$), whereas it significantly prolonged the bleeding time (> 12.0 min; $P < 0.001$) of the endometrium ($n = 6$). The haemoglobin output from the mesenteric artery and the endometrium before and after treatment with heparin are shown in Figure 3.

Effect of PGE_2 on bleeding time

Superfusion of the stable analogue of PGE_2 over the mesenteric artery ($n = 6$) had no effect on the bleeding time of that vessel (4.88 ± 0.12 min) whereas superfusion of the same analogue over the endometrium ($n = 6$) significantly

prolonged the bleeding time (9.42 ± 0.94 min; $P < 0.001$) of that tissue. The haemoglobin output from the mesenteric artery and the endometrium before and after the stable analogue of PGE_2 had been added to the superfusion medium is shown in Figure 4.

Histology

Platelets were the main component in the haemostatic plug of the mesenteric artery as had been demonstrated by others (Whittle *et al.*, 1986) whereas a fibrinous coagulated mass was the main component of the haemostatic plug of the endometrium (Figure 5).

Discussion

It is now well accepted that the primary arrest of bleeding from both the skin and small blood vessels is dependent on platelet adhesion to the cut surface and consequent aggregation. Following this, there is participation of the coagulation system and fibrin strand formation in the stabilization of the initial platelet plug (Jorgensen & Borchgrevink, 1964; Hovig & Stormorken, 1974; Sixma & Wester, 1977; Wester *et al.*, 1978). On the other hand, the mechanism of haemostasis from the endometrium is not known (Aparicio *et al.*, 1979; Christiaens *et al.*, 1980), although vasospasm of endometrial vessels is thought to play a role (Markee, 1940; 1948; Shaw *et al.*, 1972). In the present study, we investigated the role of AA metabolites of the cyclo-oxygenase pathway in controlling haemostasis in the endometrium. We assessed this indirectly by observing the effects of parenteral administra-

Figure 1 Bleeding from the mesenteric artery following a standard puncture with a 25 g needle (a) and that from the endometrium (b) following a standardized 3 mm incision in the endometrium, when they were encased in a plastic chamber and superfused with isotonic saline (37°C) at 2 ml min^{-1} . Bleeding is expressed as the haemoglobin output (mg min^{-1}) determined spectrophotometrically and shown as mean $\pm$ s.e.mean of 6 experiments in each group. Bleeding is shown under control conditions (●) and 30 min following indomethacin (5 mg kg^{-1} , i.v.) administration (○); *significant ($P < 0.05$) difference from control values.

Figure 2 Bleeding time from the mesenteric artery following a standard puncture with a 25 g needle (a) and that from the endometrium (b) following a standardized 3 mm incision in the endometrium, when they were encased in a plastic chamber and superfused with isotonic saline (37°C) at 2 ml min^{-1} . Bleeding time is expressed as the haemoglobin output (mg min^{-1}) determined spectrophotometrically and shown as mean $\pm$ s.e.mean of 4–5 experiments in each group. Bleeding is shown under control conditions (●); during prostacyclin infusion ($0.5\text{ }\mu\text{g kg}^{-1}\text{ min}^{-1}$, ○) or 10 min after 1-benzylimidazole (50 mg kg^{-1} , i.v.; ■); *significant ($P < 0.05$) prolongation.

tion of prostacyclin, which directly inhibits platelet aggregation (Whittle *et al.*, 1980); of the thromboxane synthetase inhibitor, BZi (Whittle *et al.*, 1986), as well as indomethacin and meclofenamate, both of which inhibit the enzyme cyclo-oxygenase (Flower *et al.*, 1972) and thereby reduces all AA products formed by this pathway. In order to assess the role of vasospasm in modulating haemostasis in the endometrium, we superfused the vasodilator prostaglandin E_2 (PGE_2) over the endometrium and the mesenteric artery and assessed the bleeding time. In addition, the effects of low doses of heparin, which interfere with the clotting process (Whittle *et al.*, 1986), were also investigated. These studies, therefore, allowed us to evaluate the relative importance of platelet aggregation, blood coagulation and vasospasm in the haemostatic mechanism of the endometrium.

In the present study, the slightly prolonged bleeding time in the endometrium when compared to the rat mesenteric artery indicated that the mechanism of haemostasis at these two sites may be different. Pretreatment of animals with either indomethacin or meclofenamate at dosages which inhibit cyclo-oxygenase (Flower *et al.*, 1972) and, more specifically, endometrial prostaglandin production (Chaudhuri, 1973; Chaudhuri *et al.*, 1982), increased the bleeding time from the rat mesenteric artery, but shortened the bleeding time in the endometrium. In this respect, the results on bleeding time of the endometrium were different from similar studies on gastric mucosa, where no changes in bleeding time from control values were observed after the administration of indomethacin (Whittle *et al.*, 1986). This observation suggests that AA products of the cyclo-oxygenase pathway modulate the bleeding time of rat mesenteric artery and endometrium by different mechanisms.

In order to elucidate further the role of platelet aggrega-

tion in the haemostatic process of the endometrium, we assessed the effects of inhibition of TxA_2 and administration of prostacyclin on this process. TxA_2 formed by platelets has been implicated as an endogenous proaggregatory mediator and inhibition of its synthesis can reduce platelet aggregation *in vitro* (Whittle & Moncada, 1983) and also prolong cutaneous bleeding time in man (Fitzgerald *et al.*, 1983; Dale *et al.*, 1983). When BZi was administered intravenously at a

Figure 3 Bleeding from the mesenteric artery following a standard puncture with a 25 g needle (a) and that from the endometrium (b) following a standardized 3 mm incision in the endometrium, when they were encased in a plastic chamber and superfused with isotonic saline (37°C) at 2 ml min^{-1} . Bleeding time is expressed as the haemoglobin output (mg min^{-1}) determined spectrophotometrically and shown as the mean $\pm$ s.e.mean of 6 experiments in each group. Bleeding is shown under control conditions (●) and following intravenous heparin (100 units kg^{-1} ; ○); *significant ($P < 0.05$) difference from control values.

Figure 4 Bleeding from the mesenteric artery following a standard puncture with a 25 g needle (a) and that from the endometrium (b) following a standardized 3 mm incision in the endometrium, when they were encased in a plastic chamber and superfused with isotonic saline (37°C) at 2 ml min^{-1} . Bleeding time is expressed as the haemoglobin output (mg min^{-1}) determined spectrophotometrically and shown as mean $\pm$ s.e.mean of 6 experiments in each group. Bleeding is shown under control conditions (●) and 10 min following addition of 16, 16-dimethyl PGE_2 ($1\text{ }\mu\text{g ml}^{-1}$; ○) to the superfusate; *significant ($P < 0.05$) prolongation.

Figure 5 Histological appearance of the rat endometrium (E) after haemostasis following a standardized incision. The tissue was removed and stored in the fixative solution. (10% formalin buffer). By use of routine procedures, the tissues were embedded in paraffin and $2\text{ }\mu\text{m}$ sections were prepared and stained with trichrome stain. The haemostatic plug at the incision site was mainly composed of fibrin (F) (magnification $\times 199$).

dose which caused near-maximal inhibition of TxA_2 (Whittle *et al.*, 1986), the bleeding time assessed on the rat mesenteric artery was significantly increased and was similar to that reported by other investigators (Whittle *et al.*, 1986). However, BZi did not affect the bleeding time of the endometrium. This indicated that the platelet plug formation at the bleeding site plays a less important role in haemostasis in the endometrium. This concept was further supported by our studies with prostacyclin. Prostacyclin inhibits the aggregation of platelets, both *in vivo* and *in vitro* when aggregation is induced by all endogenous agents (Moncada *et al.*, 1976; Whittle *et al.*, 1980). In our studies, intravenous administration of prostacyclin at a dose that has been demonstrated to inhibit, near-maximally, the *ex vivo* platelet aggregation in rats (Whittle *et al.*, 1986), failed to prolong bleeding time in the endometrium, but successfully prolonged bleeding time of the rat mesenteric artery.

Vasoconstriction of the endometrial vessels may play a role in haemostasis of the endometrium (Markee, 1940; 1948). It is therefore conceivable that the presence of vasodilator substances in the endometrium could prolong the bleeding. PGE_2 is a vasodilator and is synthesized by the endometrium (Chaudhuri, 1973) and the concentration of E prostaglandins in the endometrium is increased just prior to menstruation (Downie *et al.*, 1974). In ovulating women with excessively heavy measured menstrual blood loss, there appeared to be a shift in the endometrial synthetic capacity in favour of PGE_2 over $\text{PGF}_{2\alpha}$ (Smith *et al.*, 1981; Cameron *et al.*, 1987). It has been suggested that there is a relationship between the total prostaglandin content of the endometrium and measured menstrual blood loss (Cameron *et al.*, 1987). Similarly, there is also an increase in endometrial prostaglandins released by the IUD (Chaudhuri, 1973; Hillier & Kasonde, 1976), and this increased release of prostaglandins could therefore be responsible for the menorrhagia associated with use of the IUD. We, therefore, decided to assess the role of PGE_2 in modulating haemostasis in the endometrium. Superfusion of the endometrium with a stable analogue of PGE_2 led to an increase in endometrial bleeding time, whereas bleeding time was unchanged when similar experiments were performed on the rat mesenteric artery. This indicated that bleeding time in the endometrium may be partly modulated by the vascular tone of the endometrial vessels and that in the presence of excessive amounts of the vasodilator PGE_2 in the endometrium, increased bleeding may be observed. The inhibition of synthesis of PGE_2 by the endometrium following administration of NSAIDs may partly explain the decrease in endometrial bleeding time after the administration of meclofenamate or indomethacin.

Fibrin has been identified in the first day menstrual endometrium obtained by curettage (Salvatore, 1969;

Aparicio *et al.*, 1979; Christiaens *et al.*, 1980), suggesting the importance of coagulation factors (Rybo, 1966) in controlling menstrual bleeding. Furthermore, the haemostatic platelet plugs in the menstruating uterus are present only for a very limited time period in early menstruation. The predominance of fibrin in the early menstruating endometrium, therefore, prompted us to assess the role of the clotting factors in endometrial haemostasis. Intravenous infusion of heparin ($100 \text{ units kg}^{-1}$) substantially prolonged the bleeding time from the endometrium, whereas no change in bleeding time of the rat mesenteric artery was observed. This indicated that interference of clotting mechanisms with this dose of heparin selectively prolonged endometrial bleeding without affecting mesenteric artery bleeding. This was corroborated by histological findings that fibrin played an important role in endometrial haemostasis, whereas platelets played an important role in controlling bleeding from the mesenteric artery.

The correlation of our findings on the mechanism of haemostasis in the rat endometrium to the human endometrium is not clear at present as rats do not menstruate and shed endometrium. On the other hand, results from our studies, suggesting that vascular tone and coagulation factors may play an important role in endometrial haemostasis in rats may explain many findings in women. The ability of NSAIDs to inhibit the synthesis of the vasodilator PGE_2 may therefore be the explanation for the beneficial effect of this group of drugs in the treatment of idiopathic menorrhagia (Anderson *et al.*, 1976; Fraser, 1983; Muggeridge & Elder, 1983) as well as that associated with the use of the IUD (Ylikorkala *et al.*, 1978; Damarawy & Toppazada, 1981; Roy & Shaw, 1981). Similarly, the importance of the fibrin plug in endometrial haemostasis is supported by the observations that fibrinolytic inhibitors are also highly effective for the treatment of idiopathic menorrhagia and that associated with the use of the IUD (Nilsson & Rybo, 1967; Kasonde & Bonnar, 1975; Bonnar *et al.*, 1976; Ylikorkala & Vinikka, 1983; Milson *et al.*, 1991).

In conclusion, the mechanism of haemostasis can vary in different tissues and more than one mechanism may be involved. In the endometrium, the vascular tone mediated by endometrial prostaglandins and the coagulation system especially fibrin may be more important than platelets, whereas at peripheral sites such as the skin, formation of the platelet plug is the primary mechanism for haemostasis. Results of our study, therefore, explain the paradox as to why NSAIDs increase bleeding time at some peripheral sites, whereas bleeding from the endometrium is diminished.

This work was supported in part by USPHS grant no. HL-46843, and Mr Brian & Phyllis Harvey.

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(Received September 20, 1994

Accepted November 9, 1994)