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Effect of Thromboxane Synthetase Inhibition on Platelet Function and Morphology During Ovine Pregnancy-Induced Hypertension

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Key words

Thromboxane – Platelet aggregation – Pregnancy-induced hypertension – Platelet ultrastructure – Ovine pregnancy toxemia

Summary

Arterial blood pressure, serum fibrin/fibrinogen degratory products, plasma thromboxane B_2, in vitro platelet aggregation, and platelet ultrastructure were studied in ten gravid ewes during fast-triggered ovine pregnancy-induced hypertension and subsequent administration of the thromboxane synthetase inhibitors CGS13080 and CGS12970. During the hypertensive period, blood pressure ($p < 0.005$) and plasma thromboxane B_2 levels ($p < 0.005$) were significantly altered. Collagen-induced in vitro platelet aggregation lag times increased ($p < 0.01$), and percent aggregation ($p < 0.05$), primary ($p < 0.01$), and secondary ($p < 0.005$) aggregatory slopes decreased. Collagen also failed to induce aggregation in some ewes. Primary slopes of ADP-induced in vitro platelet aggregation decreased ($p < 0.01$) during hypertension. Degranulation and open canalicular tubule system swelling were observed in platelets which produced abnormal or no aggregation response. However, these ultrastructural abnormalities did not necessarily correspond to hypertensive periods.

Thromboxane synthetase inhibitor administration lowered blood pressure ($p < 0.005$) and plasma thromboxane B_2 levels ($p < 0.005$). Abnormalities in collagen and ADP-induced platelet aggregation curves were also corrected, and ultrastructural abnormalities were not detected. Marked elevations in plasma thromboxane levels during ovine pregnancy-induced hypertension may have had an "exhaustive" effect on thrombocytes which was reversed by thromboxane synthetase inhibition.

Introduction

Preeclampsia (pregnancy-induced hypertension or PIH), a hypertensive disorder of pregnancy which usually occurs in late gestation, is a major cause of fetal and maternal mortality and morbidity (1). Changes in thrombocyte function are characteristic complications of severe preeclampsia, and some believe these changes may lead to disseminated intravascular coagulation. Often, changes in function are accompanied by alterations in thrombocyte ultrastructure (2). However, the significance of preeclamptic coagulopathy is much debated. Therefore, the study of platelet function and ultrastructure in preeclampsia is important in determining the role of coagulopathy in this disease.

No attempt has been made to characterize the response of thrombocytes in an animal model of pregnancy-induced hypertension. The ovine pregnancy toxemia syndrome is generally triggered by an acute stress (long distance shipping) or a starvation episode in the last 3–4 weeks of pregnancy. Historically, once the syndrome has been triggered, the only effective treatment has been termination of pregnancy (3). Therefore, after establishment, the disease is pregnancy dependent. We have reproduced the spontaneously occurring veterinary field disease in the laboratory in chronically instrumented ewes and have documented the development of hypertension, decreased uterine blood flow, proteinuria, decreased glomerular filtration rate, and neurological deficits which eventually progress to seizure activity (4, 5). The ovine condition is not a disease like that of human pregnancy-induced hypertension which may begin early after conception. Rather, the fast-triggered ovine pregnancy-induced hypertension syndrome is a rapidly developing vasospastic disease of late pregnancy which closely mimics the clinical manifestations present in women who develop preeclampsia in the last trimester of pregnancy.

We have recently reported beneficial effects of thromboxane synthetase inhibition in our ovine model of pregnancy-induced hypertension (5). The purpose of this investigation was to characterize thrombocyte function and morphology in an ovine model of pregnancy-induced hypertension during normal pregnancy, during fast-triggered hypertension, and after thromboxane synthetase inhibition.

Subjects, Materials and Methods

Thirteen gravid ewes were obtained from a commercial breeder. Ewes were taken into the laboratory in pairs and placed in metabolism crates around the 120th day of gestation. Mixed hay, grain, water, and mineralized salt were offered ad lib. Each ewe was allowed an acclimation period of approximately 3–7 days. The laboratory environment was kept at constant temperature and photoperiod. During gestational days 127 through 134, measurements of maternal parameters were taken to establish baseline values for each animal. Platelet aggregation tests were performed, and aggregated and unaggregated thrombocytes were collected for ultrastructural examination.

On the 134th day of gestation, grain and hay were removed to trigger ovine pregnancy-induced hypertension (ovine pregnancy toxemia or OPT). Water and salt were provided ad lib throughout the fast. Induction fasting periods varied for each ewe from 24 to 84 hrs (the majority were fasted approximately 72 hrs, days 134–137).

The onset of ovine pregnancy toxemia varies from ewe to ewe in response to acute food deprivation. Each ewe was fasted until sustained hypertension was documented during two subsequent recording sessions 12 hrs apart. One ewe required only 24 hrs of fasting to develop hypertension while 84 hrs was required in one ewe. The others which developed hypertension required 72 hrs of fasting.

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Of the thirteen animals fasted, ten became hypertensive. An animal was considered to have OPT when: 1) sustained hypertension was demonstrated by hypertension being manifested during two sequential recording sessions 12 hrs apart; 2) serum chemistries indicated hypoglycemia and renal dysfunction (proteinuria and ketonuria were also noted); and 3) neurologic disturbances (i.e. depression or muscle tremors) were observed.

At the onset of hypertension, the ewes were allotted randomly into three groups control-no treatment (n = 3); CGS13080 treatment (n = 4); or CGS12970 treatment (n = 3). Thromboxane synthetase inhibitors were obtained from Ciba Geigy Corporation, Summit, NJ. CGS13080 (N[1-carboxyheptyl] imidazole, imidazo 1,5-a pyridine-5-hexanoic acid) was dissolved in normal saline and administered intravenously at a rate of 0.1 mg $kg^{-1}hr^{-1}$ for 6 hrs. CGS13080 is a relatively short-acting inhibitor at this dosage. It's half-life is less than 1 hr (6).

CGS12970 (3-methyl-2-[3-pyridyl]-1-idolectanoic acid) was dissolved in a 5% sodium bicarbonate solution and administered as a single intravenous bolus injection of 1 mg/kg. In rabbits 1 mg/kg orally produces thromboxane synthetase inhibition for at least 12 hrs (7).

No previous sheep studies have been attempted and thus no pharmacokinetic data of either CGS13080 or CGS12970 exists for this species.

The acute effects of inhibitor administration were monitored in each ewe and then at 24 hrs after treatment each ewe was refed. Complete monitoring was accomplished at 24 and 48 hrs after treatment. At 48 hrs after treatment, the ewes were removed from the metabolism crates and allowed to complete gestation in a small lambing enclosure.

Maternal Blood Pressure

As the ewes stood quietly in the metabolism crates, morning and evening recordings of systolic, mean, and diastolic maternal blood pressures were obtained at 12 hr intervals throughout the experiment (gestational day 127 until approximately day 140), using the oscillometric method (Dinamap research monitor, Critikon). During the 30 min recording periods, indirect blood pressure estimates were determined automatically at 3 min intervals. The blood pressure cuff was placed on a front limb just below the olecranon process. The recording technician observed each determination and noted any random limb movements which might have influenced readings. Maternal head position was also noted and only readings determined during normal head-up posture were recorded. Therefore, during each recording session 4–10 estimates of maternal blood pressure were obtained.

Fibrin/Fibrinogen Degratory Product Analysis and Thromboxane B_2 Determination

10 ml whole blood was obtained on alternate days during the baseline period, daily during fasting, and at 2 hr, 6 hr, 24 hr and 48 hr post treatment by clean jugular venipuncture. 1 ml was placed in a thrombin tube and allowed to clot for fibrin/fibrinogen degratory product (FDP) analysis. After the sample had clotted, the blood was centrifuged at 1,500 rpm for 10 min at room temperature. The thrombowellcotest (Wellcome Diagnostics) was performed on the serum for the detection of FDP's.

The other 9 ml blood was thoroughly mixed with 1 ml of 3.8% sodium citrate solution containing indomethacin (50 μg/ml). The sample was immediately centrifuged at 1,500 rpm for 10 min at room temperature. Plasma was decanted from the cellular fraction, and immediately frozen at −70° C until analyzed. Radioimmunoassays were performed on 300 μl aliquots of the unextracted plasma for thromboxane B_2, the stable metabolite of TxA_2, by commercially available (^3H) RIA kits (Amersham) as previously described (5).

Total Platelet Count and Platelet Aggregation

Whole blood was obtained using the same sampling scheme as described for FDP and thromboxane determination by clean jugular venipuncture. Sodium citrate (3.2%) was used as the anticoagulant (1 part citrate : 9 parts blood). Total platelet counts were performed manually by the hemocytometer method (unopette 5453). The blood was centrifuged twice at 850 rpm for 3 min to harvest platelet rich plasma (PRP). Platelet poor plasma (PPP) was obtained by centrifugation at 1,500 rpm for 10 min.

Aggregation studies in PRP were performed according to the light transmission method of Born (8) with a dual channel platelet aggregometer (Payton Associates, Buffalo, NY). The PRP was incubated at 37.5° C and stirred at 800 rpm. 2 min of baseline recording was taken to test for spontaneous aggregation and baseline variability. Bovine collagen and ADP (Sigma) were added to induce aggregation at final concentrations of 0.10 μg/ml and 8.5 μg/ml, respectively. Aggregation curves were recorded until maximum aggregation had occurred. Aggregated and unaggregated samples of platelet rich plasma (PRP) were fixed in modified McDowell-Trumps fixative (pH 7.4) for at least 24 hrs for transmission electron microscopic examination.

Thrombocyte and Aggregate Ultrastructure

Fixed free platelets, collagen-induced platelet aggregates, and ADP-induced platelet aggregates were centrifuged at 1,500 rpm for 3 min at room temperature. The supernatant was discarded, and the platelets were washed 2× in Tyrode's buffer (pH 7.4). The cells were post fixed in 1% osmium tetroxide for 1 hr, and again washed 2× in Tyrode's buffer. They were then embedded in 2% Nobel agar, and cut into 1 mm cubes. After dehydration in a series of ethanols and then propylene oxide, the agar cubes containing platelets were put in equal volumes of propylene oxide and Polybed 812 resin (Polysciences) for approximately 8–12 hrs. Fresh resin was used to embed the platelet containing agar cubes in molds. These molds were allowed to stand for an additional 8 hrs, and then oven cured at 60° C for 72 hrs.

Sections of approximately 600–900 angstroms were obtained from resin blocks using either a LRB IV or Sorvall MT2B ultramicrotome. Sections were placed on # 200 mesh grids and stained with a 1:1 solution of uranyl acetate and acetone. A second staining was conducted with Reynolds lead citrate stain for 5 min.

Unaggregated and aggregated platelets were examined on a Jeol 100 CX-II scanning-transmission electron microscope.

Statistical Analysis

Each ewe served as its own control and statistical analysis was performed by two tailed t-test comparisons within each ewe. Group means were analyzed by analysis of variance routines. Probability of <0.05 was considered to be significant. Throughout the results section, baseline values refer to the samples taken before fast. Hypertension values refer to samples during the last recording or sample period prior to treatment or endpoint of study (untreated ewes). Post treatment values are those obtained 12 hrs after treatment with either thromboxane synthetase inhibitor. All results are reported as mean ± SE.

Results

Maternal Blood Pressure

Baseline oscillometric arterial pressures from the ten triggered animals were 130 ± 1.11 mmHg (systolic), 90 ± 1.00 mmHg (mean arterial), and 67 ± 0.96 mmHg (diastolic) (n = 415 estimates). Blood pressure rose significantly ($p < 0.005$) during the hypertension period to 150 ± 1.24 mmHg (systolic), 107 ± 1.37 mmHg (mean arterial), and 81 ± 1.49 mmHg (diastolic) (n = 233 estimates). 12 hr after the administration of CGS13080 or CGS12970, systolic (144 ± 2.45 mmHg), mean arterial (98 ± 2.82 mmHg), and diastolic (75 ± 2.80 mmHg) (n = 69 estimates) blood pressures fell to levels that were significantly different from those of OPT ($p < 0.005$), but not of baseline. Blood pressures in the 3 control, untreated ewes were significantly elevated at the last recording session prior to progression to eclampsia (1 ewe) or spontaneous abortion (2 ewes). Since death or spontaneous abortion were considered endpoints of the study, pressures were not measured in these ewes after these events occurred.

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Fibrin/Fibrinogen Degratory Product Analysis and Thromboxane B_2 Determination

FDP's were present in a concentration of at least 10 μg/ml in most animals for at least one of the three time periods. However, no consistent changes in the presence or absence of FDP's were noted at any time during the study, and no trends were able to be established. Therefore, FDP's did not prove to be a reliable indication of abnormal coagulation.

Plasma levels of thromboxane B_2 rose significantly from 57.69 ± 3.1 pg/ml (n = 21) during baseline to 99.87 ± 8.28 pg/ml during OPT (n = 20) ($p < 0.005$). After CGS13080 or CGS12970 administration, thromboxane B_2 levels fell to 42.57 ± 5.21 pg/ml (n = 15), a level significantly lower than either OPT or baseline values ($p < 0.005$). The least detectable concentration of thromboxane B_2 was 5 pg/tube. Interassay variation was ≤10%, and intraassay variation was ≤5%. Fifty percent binding of the standard curves for thromboxane B_2 was 27 pg. Recovery rates for added thromboxane B_2 ranged from 91–134%.

Total Platelet Count and Platelet Aggregation

Total platelet counts, given in Table 1, remained relatively constant throughout baseline, OPT, and after thromboxane synthetase inhibition.

ADP-induced platelet aggregation characteristics are provided in Table 2, and their corresponding aggregation curves are illustrated in Fig. 1. Collagen-induced platelet aggregation characteristics are described in Table 3. Their corresponding aggregation curves are also illustrated in Fig. 1. Fig. 2 presents aggregation curves for ewes whose thrombocytes were nonreactive to collagen during OPT. Standard errors in Table 3 are large due to this occurrence. In Figs. 1 and 2, sharp drops appear in all curves at 2 min. These are artifacts from the addition of aggregating agents.

During OPT, there was a delayed to absent collagen-induced aggregatory response, with increased lag time, and decreased

Table 1 Total platelet counts from 10 nontreated, CGS13080, and CGS12970 treated, initially normotensive, gravid ewes in which pregnancy toxemia was induced (mean ± SE)

	Baseline	Toxemic	Post Rx
Total Count	579,382 ± 23,834 (n = 34)	617,650 ± 55,037 (n = 10)	588,000 ± 92,660 (n = 7)

Table 2 ADP-induced aggregation curve characteristics from 10 nontreated, CGS13080, and CGS12970 treated, initially normotensive, gravid ewes in which pregnancy toxemia was induced (mean ± SE)

	Baseline	Toxemic	Post Rx
% Aggregation	67.21 ± 2.37	71.67 ± 2.23	71.50 ± 1.31
Lag time	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Primary slope	3.05 ± 0.12	2.63 ± 0.22[a]	2.99 ± 0.33
Secondary slope	1.03 ± 0.04	0.99 ± 0.09	1.07 ± 0.21
	(n = 34)	(n = 10)	(n = 7)

[a]Baseline vs. toxemia; $p < 0.05$

Table 3 Collagen-induced aggregation curve characteristics from 10 nontreated, CGS13080, and CGS12970 treated, initially normotensive, gravid ewes in which pregnancy toxemia was induced (mean ± SE)

	Baseline	Toxemic	Post Rx
% Aggregation	61.25 ± 5.96	43.00 ± 13.62[b]	65.67 ± 13.46[d]
Lag time	165.33 ± 16.41	226.67 ± 29.42[a]	166.00 ± 34.04[d]
Primary slope	1.67 ± 0.20	0.88 ± 0.32[a]	1.57 ± 0.42[d]
Secondary slope	0.60 ± 0.07	0.33 ± 0.11[c]	0.60 ± 0.13[d]
	(n = 33)	(n = 10)	(n = 7)

[a]Baseline vs. toxemia; $p < 0.01$.
[b]Baseline vs. toxemia; $p < 0.05$.
[c]Baseline vs. toxemia; $p < 0.005$.
[d]Toxemia vs. treatment; $p < 0.005$.

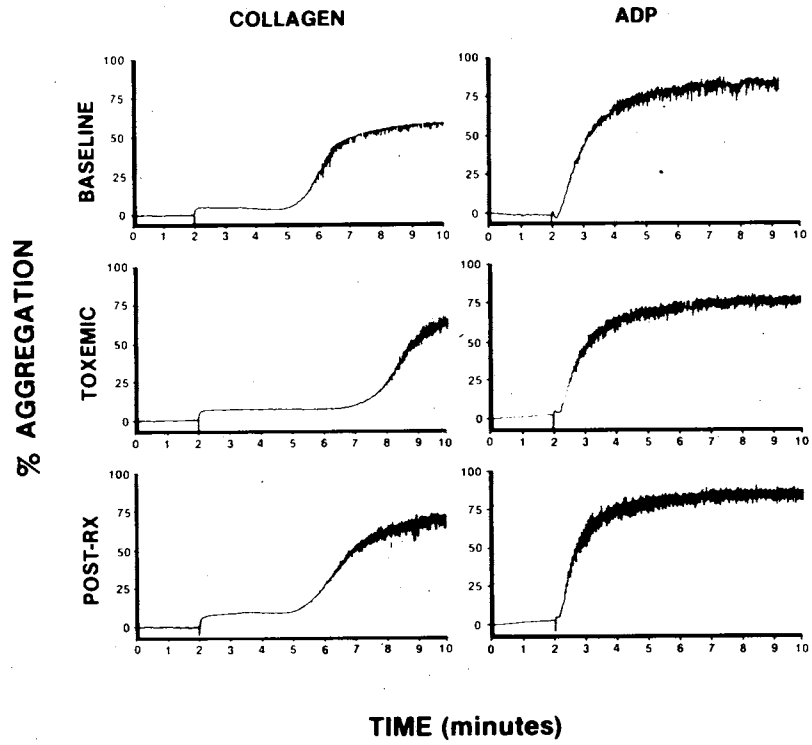

Fig. 1 Representative collagen and ADP-induced aggregation curves from nontreated, CGS13080 treated, or CGS12970 treated multiparous gravid ewes

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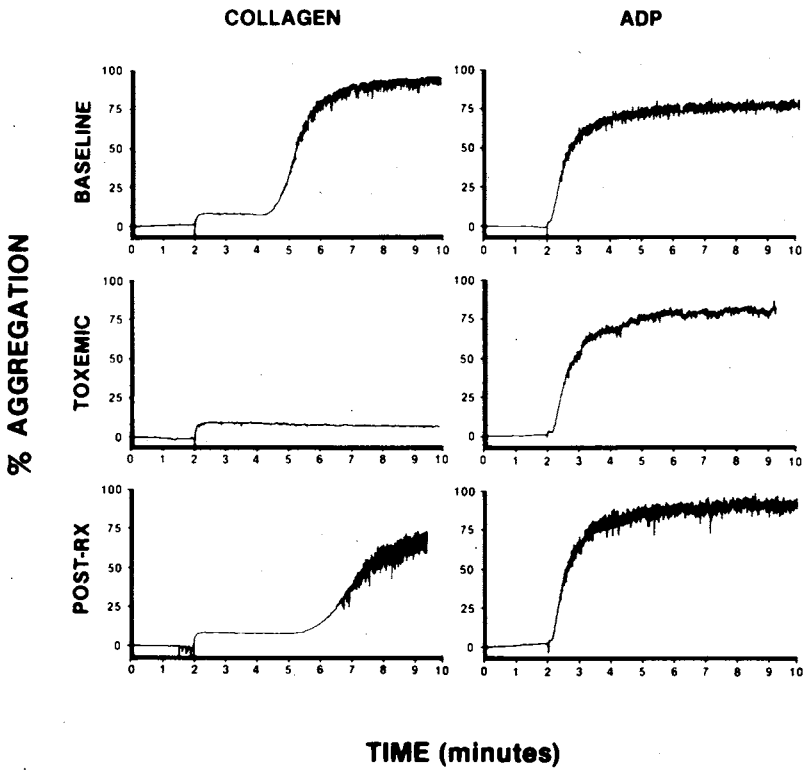

Fig. 2 Representative collagen and ADP-induced aggregation curves from those nontreated, CGS13080 treated, or CGS12970 treated multiparous gravid ewes in which platelets failed to aggregate when challenged with collagen

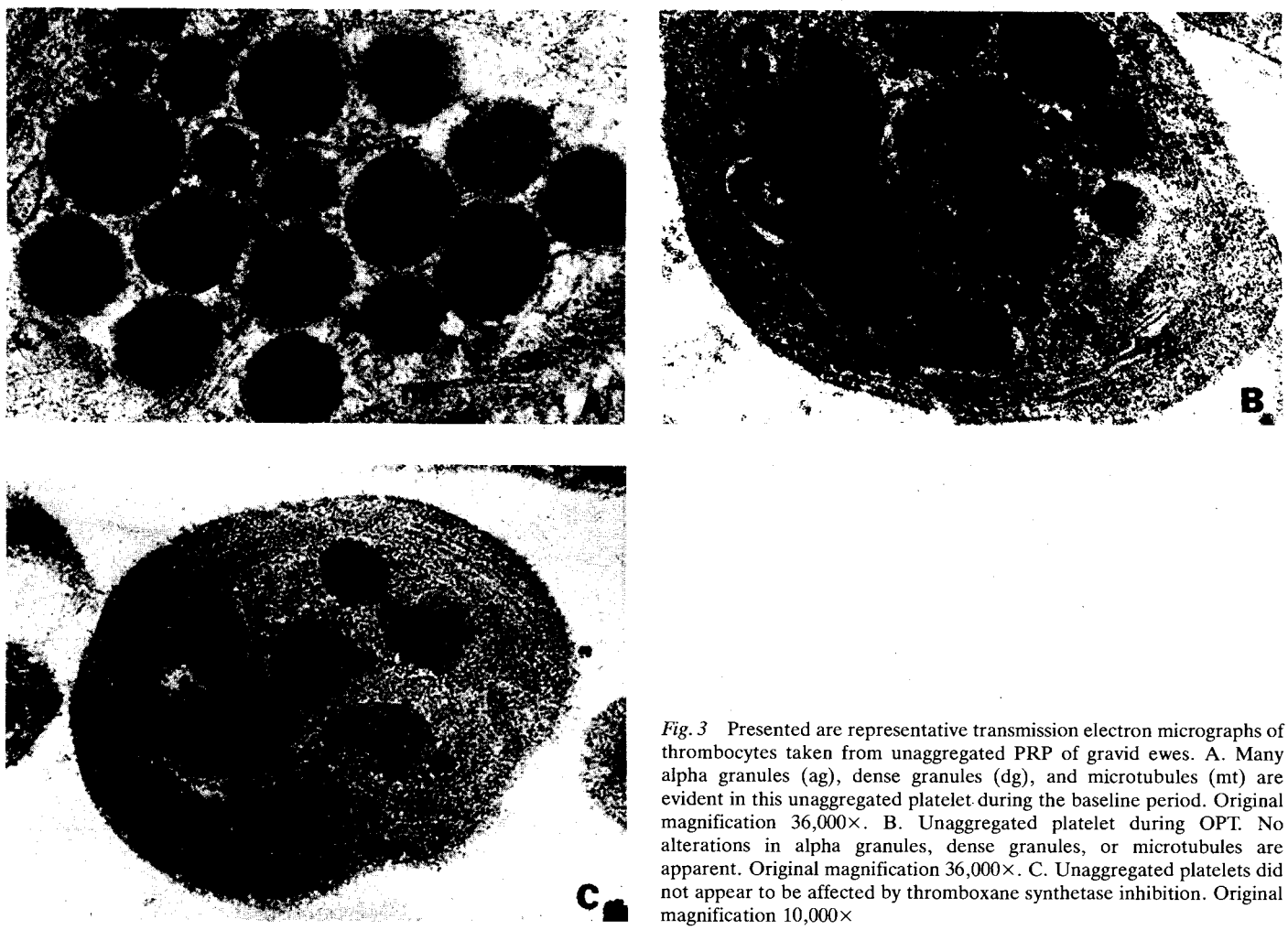

Fig. 3 Presented are representative transmission electron micrographs of thrombocytes taken from unaggregated PRP of gravid ewes. A. Many alpha granules (ag), dense granules (dg), and microtubules (mt) are evident in this unaggregated platelet during the baseline period. Original magnification 36,000×. B. Unaggregated platelet during OPT. No alterations in alpha granules, dense granules, or microtubules are apparent. Original magnification 36,000×. C. Unaggregated platelets did not appear to be affected by thromboxane synthetase inhibition. Original magnification 10,000×

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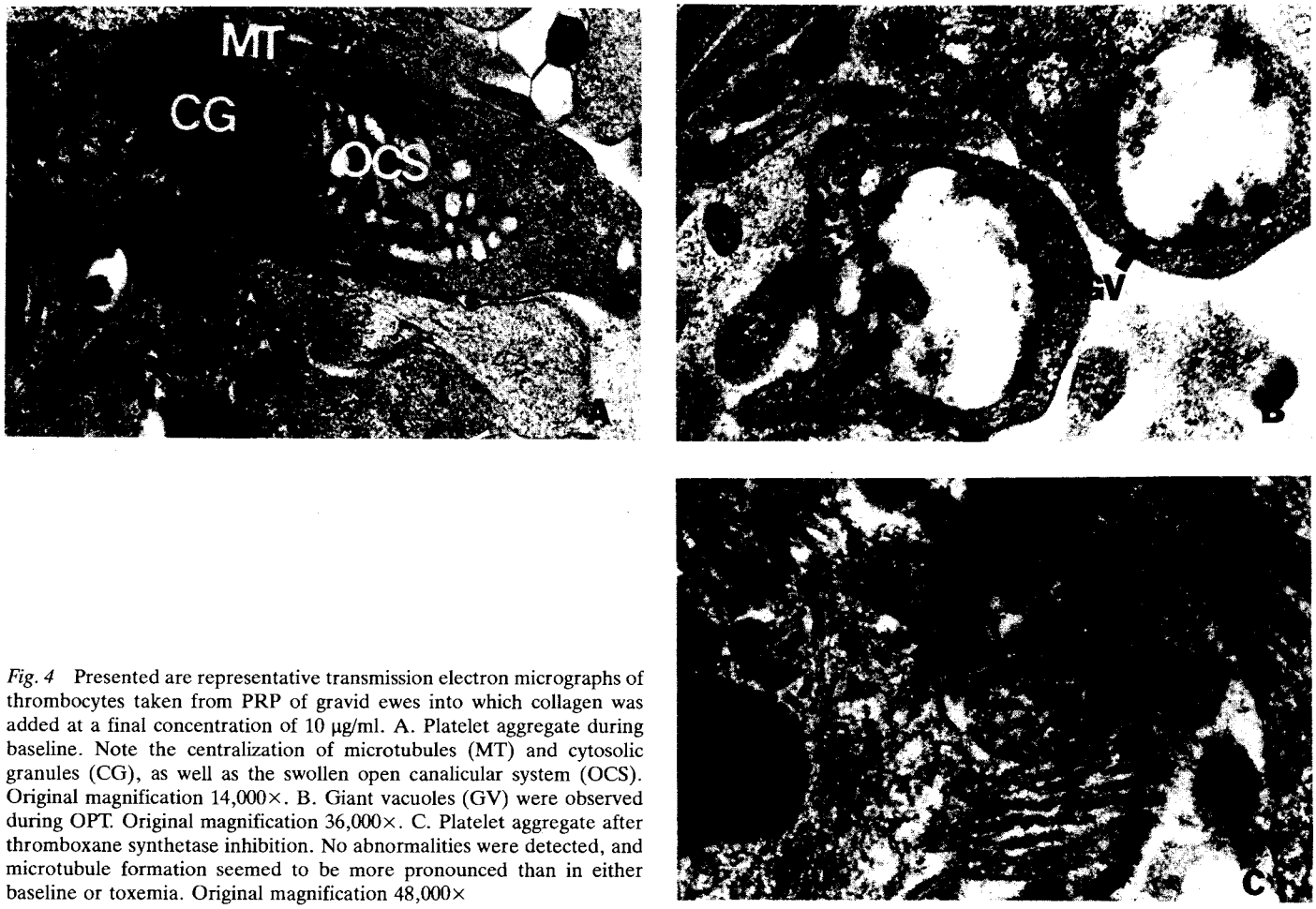

Fig. 4 Presented are representative transmission electron micrographs of thrombocytes taken from PRP of gravid ewes into which collagen was added at a final concentration of 10 μg/ml. A. Platelet aggregate during baseline. Note the centralization of microtubules (MT) and cytosolic granules (CG), as well as the swollen open canalicular system (OCS). Original magnification 14,000×. B. Giant vacuoles (GV) were observed during OPT. Original magnification 36,000×. C. Platelet aggregate after thromboxane synthetase inhibition. No abnormalities were detected, and microtubule formation seemed to be more pronounced than in either baseline or toxemia. Original magnification 48,000×

percent aggregation, and primary and secondary aggregation slopes. An essentially normal ADP-induced aggregation response was noted throughout the hypertensive period, except for a decreased primary slope. After administration of thromboxane synthetase inhibitors, the aggregation slope characteristics of both collagen and ADP-induced curves returned to baseline levels. This hypoaggregatory response is in direct contrast to the hyperaggregation previously reported during ovine pregnancy-induced hypertension (5). Chronic instrumentation of the animals in the previous study, may have promoted the hyperaggregation that was reported.

Thrombocyte and Aggregate Ultrastructure

Figs. 3, 4, and 5 illustrate the ultrastructural changes that occurred in unaggregated and collagen and ADP-induced aggregated platelets throughout baseline and OPT, and after thromboxane synthetase inhibition. Degranulation and swelling of the canalicular tubule system were noted in platelets which produced abnormal or no aggregation response. However, the ultrastructural abnormalities that were observed did not necessarily correspond to hypertensive periods. No abnormalities were seen in platelet ultrastructure after thromboxane synthetase inhibitor administration.

Pregnancy Outcome

The pregnancies in the control group ended prematurely in eclampsia (n = 1) or spontaneous abortion (n = 2) following at least one intrauterine death in each case. All 7 ewes treated with either CGS13080 or CGS12970 completed gestation successfully and were delivered at term.

Discussion

The most significant findings of this investigation were the hypo-aggregatory response of platelets during hypertension, a time when plasma thromboxane B_2 levels were significantly elevated and the reversal of this effect by thromboxane synthetase inhibition. Changes in the chemical environment of circulating blood are more commonly thought of as making platelets hypersensitive (9) presumably due to alterations in the sensitivity of platelet eicosanoid receptors (10). This phenomenon has been seen in thromboembolic disorders. However, a platelet "exhaustive" syndrome, where platelet function is decreased, has been described in preeclampsia (11). The changes in platelet function reported here closely resemble this "platelet exhaustion" phenomenon. A situation where platelets are capable of producing thromboxane, but are unresponsive to the thromboxane that is produced has been described (12). Since preeclampsia may involve the alteration of both vascular and platelet eicosanoid oxygenation pathways, it is of interest to note that there may be a differentiation between vascular and platelet receptors for thromboxane A_2 (13). Potency of thromboxane agonists in different in platelets than in vascular smooth muscle from the same species (14).

Impaired collagen-induced platelet aggregation with normal ADP-induced aggregation has been described in human disease

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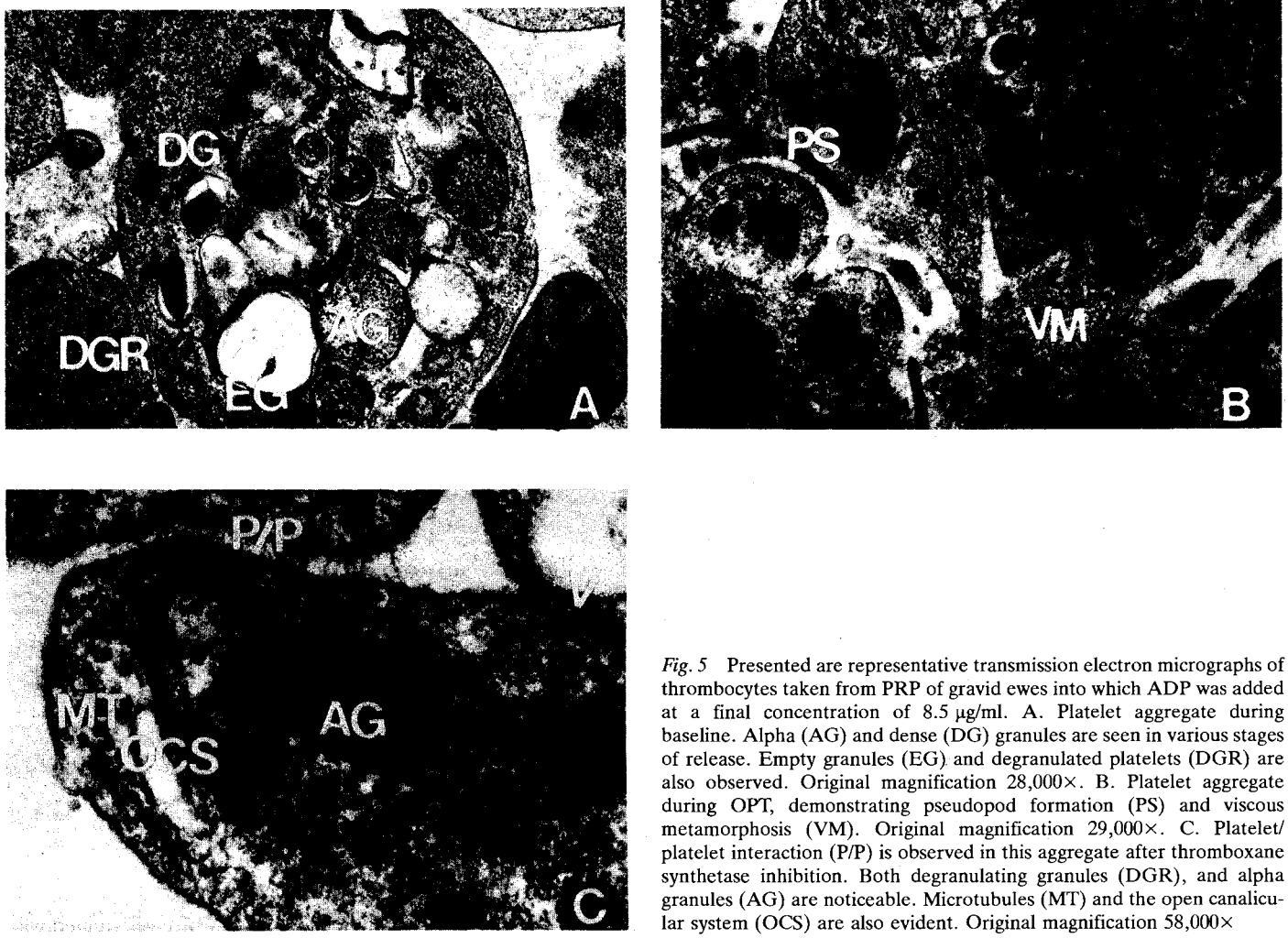

Fig. 5 Presented are representative transmission electron micrographs of thrombocytes taken from PRP of gravid ewes into which ADP was added at a final concentration of 8.5 μg/ml. A. Platelet aggregate during baseline. Alpha (AG) and dense (DG) granules are seen in various stages of release. Empty granules (EG) and degranulated platelets (DGR) are also observed. Original magnification 28,000×. B. Platelet aggregate during OPT, demonstrating pseudopod formation (PS) and viscous metamorphosis (VM). Original magnification 29,000×. C. Platelet/platelet interaction (P/P) is observed in this aggregate after thromboxane synthetase inhibition. Both degranulating granules (DGR), and alpha granules (AG) are noticeable. Microtubules (MT) and the open canalicular system (OCS) are also evident. Original magnification 58,000×

(15, 16), even in the face of normal total platelet counts. Lack of aggregation response to collagen but normal response to ADP, epinephrine, and ristocetin has been reported clinically (17), and defective platelet receptors for collagen have been observed (18). We speculate that the ovine platelet abnormality described here is due to an abnormality of the membrane receptor specific for collagen-platelet interactions, much like that theorized for humans. As the response to collagen was abolished and later returned, it is unlikely that the abnormality was due to the collagen itself. It is possible, but less likely, that these platelets could have disturbances in intracellular calcium mobilization. Down regulation of TxA_2 receptors on these platelets may have occurred in response to increased circulating levels of TxA_2. Changes in platelet TxA_2 receptor sensitivity or populations may have affected collagen receptor sensitivity or populations. However, no receptor studies were accomplished in this investigation.

Platelet survival in preeclamptic patients appears to be reduced even in the absence of thrombocytopenia, and the platelet surface is altered in women with preeclampsia and their neonates, even in the absence of bleeding (19). However, normal total platelet counts during human pregnancy-induced hypertension have been reported (20). Normal platelet counts during hypertension were observed in this study as well. This finding, in noninstrumented ewes, differs from that which has been previously reported in chronically instrumented ewes (5). The artificial surfaces of the instrumentation devices used in previous studies may have been responsible for the hyperaggregability and decreased platelet counts that were observed. Interestingly, thromboxane synthetase inhibition reversed those effects in chronically instrumented ewes (5).

Defects in function may or may not be accompanied by ultrastructural abnormality (2). When ultrastructural anomalies do occur, the most commonly occurring problem is abnormal granulation. In this study, defects in platelet function were generally accompanied by ultrastructural changes. Degranulation was the most striking change noted, with canalicular swelling also being observed. The presence of microtubules during collagen-induced aggregation was particularly prevalent in samples taken after thromboxane synthetase inhibitor administration. No firm correlations can be drawn between OPT and ultrastructural changes, as the most pronounced changes did not always occur during this period.

Mild increases in the serum levels of FDP's are present in preeclamptics (21). We have also observed FDP's in the ovine model, but without any degree of constancy.

It is becoming well accepted that increased activity of the platelet-TxA_2 metabolic pathway (22) and increased rates of platelet consumption or turnover (23) occur in pregnancy-induced hypertension. In fact, preliminary reports (24) indicate that platelet thromboxane synthetase inhibition by low-dose aspirin

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may be beneficial in pregnancy-induced hypertension. If further aspirin studies continue to support this concept, then specific thromboxane synthetase inhibitors such as CGS13080 and CGS12970 may be even more potent drugs in treatment of pregnancy-induced hypertension.

We have previously studied thromboxane synthetase inhibition in our model (5) and have documented beneficial results. In this paper, we report that both of the chemically distinct compounds studied here, which have the same specific thromboxane synthetase inhibitory activity, have the same beneficial effects in this animal model of preeclampsia. No difference in effect was seen in this first study of these two inhibitors in the ovine model of preeclampsia, other than the fact that the onset of action of CGS13080 was slower than CGS12970. However, this could have been dosage dependent. More importantly, both of these compounds allowed pregnancy to continue in the treated ewes, while in the untreated ewes pregnancy terminated by eclampsia or spontaneous abortion. Further studies of these compounds in this ovine model and in human pregnancy-induced hypertension appear warranted.

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