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The Influence of Adrenaline on Gender Difference in Adenosine Diphosphate-Induced Aggregation of Platelets in the Rat

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Key words

Intravascular aggregation – Oestradiol – Adrenalectomy – Adrenaline – Fibrinolysis

Summary

The role of adrenaline on the inhibitory effects of physiological levels of oestradiol on ADP-induced intravascular aggregation has been studied. Platelets from pro-oestrous female rats aggregated less than those from dioestrous and male rats. Following adrenalectomy, there was no longer any difference(s) in the aggregability of the platelets to ADP in any of the rats. Adrenaline infusion (20 mg kg^{-1} hr^{-1}) restored platelet aggregation to preadrenalectomy levels in pro-oestrous rate. Measurement of spontaneous fibrinolytic activity of the plasma showed highest value in pro-oestrous rats. Adrenalectomy reduced, while adrenaline infusion increased the fibrinolytic activity. The results suggest that the inhibitory effects of oestradiol on intravascular aggregation are dependent on endogenous adrenaline possibly working through the fibrinolytic pathway.

Introduction

The platelet is recognized as playing a role in thrombus formation and hyperaggregability of platelets is a common feature of various thrombotic states. The gender difference in platelet function as well as in the incidence of cardiovascular diseases in man has been reported widely (1, 2). The lower incidence of cardiovascular diseases in women (and of experimental thrombus formation in animals) as well as a lower sensitivity of platelets in the female sex of humans, rats and hamsters (3, 4, 5) have implicated oestrogens.

Interestingly, oestrogens have been shown to require an adrenal gland secretion in order to exert their effects in this regard. Thus, in a mouse model of thrombosis, Phenhos et al. (6) observed that, following adrenalectomy in male and female mice, the gender difference in mortality to arachidonic acid were abolished. Moreover, it was also demonstrated that the sex-dependent effects of the stress of mock operation on platelet aggregation in rats was abolished in female rats following acute adrenalectomy (7).

The observation that oestrogens stimulate the secretory capacity of the adrenal glands more in females than males (8, 9), is an evidence for the adrenal gland-dependence of oestrogens. This thus means that there is a necessity to examine more critically, the determinant factors in the underlying thrombus formation/platelet function, apart from sex hormones.

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This investigation examined the role played by adrenaline directly or indirectly on the inhibitory effects of endogenous oestradiol on platelet aggregation in vivo. Towards this end, intravascular aggregation induced by ADP was studied in male and female rats. The effects of acute adrenalectomy and fibrinolysis were also examined.

Materials and Methods

Drugs

Adenosine diphosphate (ADP, Sigma) and adrenaline hydrogen tartrate (British Drug House) were prepared fresh everyday using normal saline (0.9% NaCl w/v) and kept on ice during the experiment. Urethane (Sigma), fibrinogen (Sigma), thrombin (Sigma), acetic acid (Sigma) and urokinase (Sigma) were dissolved in distilled water. Fibrinogen, thrombin and urokinase were stored at 0° C until use, prostaglandin E_1 was stored in methanol (1 mg/ml) and kept at 0° C until use. Tyrode's salt (Gibco) and sodium bicarbonate (Gibco) was made up fresh daily with distilled water.

Indium (In-111) oxine solution, 1 mCi/ml at reference data was purchased from Amersham International.

Procedure

Vaginal smears were made from female rats (Wistar strain) and examined daily between 09.00 and 10.00 hours. The vaginal cytology was evaluated (10) and only rats that showed at least two 4-day regular consecutive cycles were used in this study.

Adrenalectomy (acute)

Adult Wistar rats of both sexes were adrenalectomized by the midline bilateral incision technique (under urethane anaesthesia; 1.5 g kg^{-1} intraperitoneally). The adrenal glands were removed and the animals left to stabilise for 1 hour before testing the responsiveness of their platelets. The mock-adrenalectomised rats were similarly treated except that the adrenal glands were located but not removed.

Preparation of Buffer

The buffer used in these investigations is calcium-free Tyrode's fluid containing PGE_1 (CFTPGE). 0.9 g Tyrode's salt (Sigma) and 0.1 g sodium bicarbonate (Gibco) were dissolved in 100 ml distilled water (solution I). Trisodium citrate (Sigma, 2.2 g) and citric acid (Sigma, 0.8 g) were dissolved in 100 ml distilled water (solution II). The buffer was prepared by mixing 18 ml of solution I with 1.8 ml solution II and then adding 6.7 µl of PGE_1 solution (1 mg/1 ml in absolute alcohol).

Preparation, Labelling and Administration of 111-Indium-Labelled Platelets

The method used is that of Oyekan and Botting (5). Briefly, 5 ml citrated arterial blood (1 part citrate [3.5%]:9 parts blood) collected by cardiac puncture or from the abdominal aorta in a lightly ether-anaesthetized rat was spun at 200 × g for 15 minutes. The resultant platelet-rich plasma (PRP) containing 192 ± 18 × 10^3 platelets/µl of plasma was pelleted and made up to 10 ml with calcium-free tyrode buffer containing 6.7 µg prostaglandin E_1 (CFTPGE). This was spun at 640 × g

for 15 minutes and the resultant platelet pellet was washed with the CFTPGE buffer (the supernatant having been discarded) and resuspended in 1.5 ml of the buffer. A measured volume of 111-Indium oxine (equivalent to 2 μCi) was added to the suspension and after incubation at 37° C for 90 seconds, was spun at 640 × g for 10 minutes. (The volume of 111-Indium oxine solution added depends on the activity it possessed.) The supernatant was discarded and the resultant 111-Indium-labelled platelet pellet was washed and resuspended in 1 ml of the buffer. The labelled platelet suspension was then administered intravenously into matched, urethane-anaesthetized (1.5 g/kg i/p) recipient rats (homologous transfer) using indwelling intravenous catheters (Venisystem Infusion Set, Abbott Laboratories) implanted into the tail veins. The tail vein was also used for the injection of ADP. Each injection was followed by a saline flush of 0.5 ml.

Measurement of Aggregation

Following a stabilization period of 1 hour after the injection of the labelled platelets, a dose-response relationship was established to ADP in each rat and the resultant peak increase in 111-In counts (expressed as percentage change over the basal count) was taken as the magnitude of the aggregatory response to the particular dose of ADP. Radioactivity was measured by means of a single 1″ crystal scintillation detector (Mini Instruments Ltd) enclosed in a lead tube with 2.5 cm diameter hole that allows radioactivity over the lungs to be monitored. The detector was connected to a ratemeter (Panax, Model PK RTM-4) and a permanent record of count was obtained on a pen recorder (Servogor) connected to the output of the rate meter. No difference was observed in count when blood was collected from the aorta or by cardiac puncture.

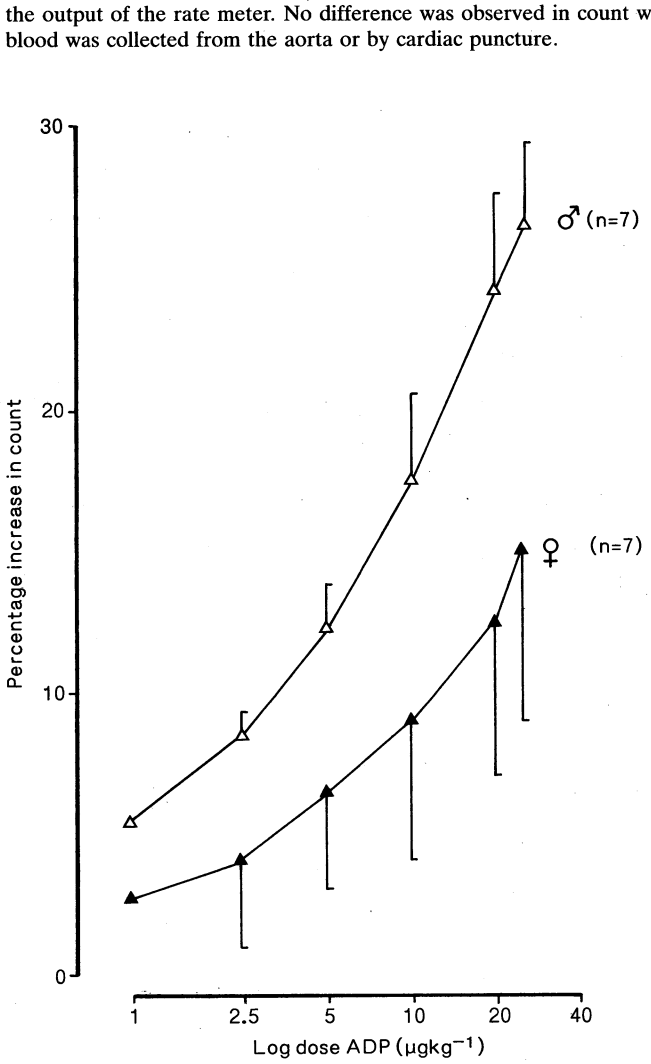

Fig. 1 The aggregatory response to ADP in age-matched male and female rats. Data presented as mean ± S.E.M. The number of rats (n) is annotated. One-way ANOVA and Wilcoxon's signed rank sum test were used to compare mean values between the two groups ($p < 0.05$)

In those experiments involving the infusion of adrenaline, the adrenaline solution was prepared in normal saline and given by constant slow intravenous infusion (2 ml/hr × 120 min) via an indwelling catheter (Abbott Laboratories) implanted in the foot veins. ADP-induced aggregation was evaluated under continuing adrenaline infusion, 15 minutes from the commencement of the infusion. The ADP was injected every 15 minutes starting with the highest dose.

Preparation of Fibrin Plates

A fibrin film was obtained by mixing in the plate 0.3 ml plasminogen-contaminated thrombin (20 NIH/ml) with 25 ml of 0.15% fibrinogen. Disposable petri dishes (Gallenkamp, Ltd), flat and heat-resistant were used. To secure rapid and homogeneous intermixture of the solutions, the thrombin was spread on the plate first and thereafter the fibrinogen solution was added with the aid of a pipette with a large distal opening so that it can be emptied in a few seconds. The plate was afterwards gently tilted to secure proper intermixture of the thrombin and fibrinogen and then left in a perfectly horizontal position for 30 minutes, during which a fibrin film approximately 0.25 cm thick was formed.

Testing Procedure and Reading of the Results

Resuspended euglobulin precipitates prepared from platelet-poor plasma (11) were used as test solutions within at most 30 minutes of collection of the blood sample. The solution to be tested was deposited on the fibrin film in aliquots of 30 μl in triplicate. Thereafter, the plates were incubated for 22 hours at 37° C. The lysis areas were measured in two perpendicular main diameters and multiplied to obtain the lytic areas in mm^2 (averages of triplicates).

The sensitivity of the plates and comparable reproducibility on different days were ascertained with a standard preparation of urokinase.

Analysis of Results

The statistical significance of differences between the mean values for the groups was calculated by a one-way analysis of variance (ANOVA) together with a studentized range test. The Wilcoxon's signed rank sum test was used when comparing data from two groups of rats. For both tests, a p value ≤ 0.05 was considered significant.

Results

Aggregatory Responses to ADP between Age-Matched Male and Female Rats

Male (average weight 410 g; range 345–440 g) and female (average weight 330 g; range 290–370 g) rats aged 4.5 months ± 3 days at the time of investigation were used. (The female rats were randomly selected without regard to their stage of oestrous cycle.) Using platelets from the respective matched donor rats (homologous samples), platelets from male rats were on the average 92.9 ± 5.0% more responsive to ADP ($p < 0.05$) than platelets from age-matched females. The responses in females were attended by very large variation. The mean coefficient of variation ([s.e.m./mean] × 100) was 89.5 ± 12.5% (Fig. 1).

Aggregation of Platelets to ADP between Male, Pro-Oestrous and Dioestrous Female Rats

The variation in hormonal levels during the oestrous cycle shows the level of oestradiol to be highest during pro-oestrous and lowest during the dioestrous phase of the cycle (12). Evaluating the aggregation of 111-Indium-labelled platelets in their homologous "milieu", it was observed that platelets of female rats were most reactive to ADP during the dioestrous stage and least during the pro-oestrous stage. The aggregation of platelets during the dioestrous stage was lower than that of age-matched male rats. The difference was not significant. The dioestrous/pre-oestrous

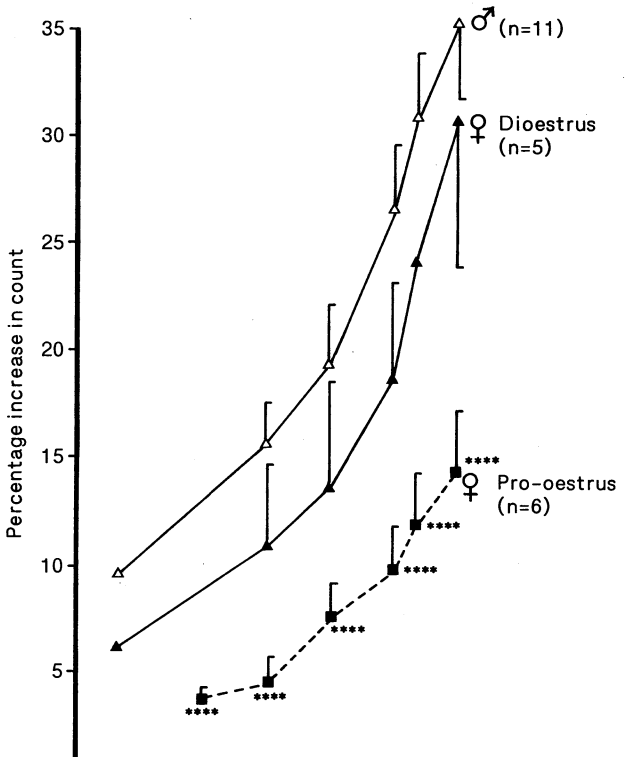

Fig. 2 The reactivity of platelets of male (△), dioestrous (▲) and pro-oestrous (■) female rats to ADP. Data presented as mean ± S.E.M.; number of rats is given in parentheses. A one-way ANOVA and Student's test were used to compare values of dioestrous and pro-oestrous rats with male rats ($p < 0.005$). No significant differences was found between dioestrous and male rats. **** $p < 0.005$

difference was 120.6 ± 10.8% ($p < 0.05$) while the male/pro-oestrous difference was 190.4 ± 12.5% ($p < 0.005$) (Fig. 2).

Effects of Adrenalectomy on the Aggregation of Platelets of Male Rats, Pro- and Dioestrous Rats to ADP

Adrenalectomy increased the aggregability of platelets to ADP-induced aggregation in male rats as well as in pro- and dioestrous female rats. When compared to the respective mock-operated rats, the removal of the adrenal glands caused increased aggregation to ADP, the effects being greatest in pro-oestrous rats ($p = 0.01$–0.005). The increase was also significant in dioestrous ($p < 0.05$) as well as in male rats ($p = 0.05$–0.02). These changes are such that the aggregatory response to ADP in adrenalectomized pro-oestrous rats is not different from those of male or dioestrous rats (intact or adrenalectomized). Mock operation did not significantly alter the aggregation of the platelets in any of the groups of rats (Fig. 3). Infusion of adrenaline (20 μg kg^{-1} hr^{-1} × 120 min) in adrenalectomized pro-oestrous female rats significantly reduced ($p < 0.01$) the aggregability of their platelets to ADP (data not shown) to pre-adrenalectomy levels.

Differences in Fibrin Plate Lysis Area in the Plasma of Male Rats, Pro-Oestrous and Dioestrous Rats – Effects of Adrenalectomy and Infusion of Adrenaline

The lysis area on plasminogen-contaminated plates was highest in pro-oestrous rats (308.6 ± 33.5 mm^2) than in dioestrous (219.3 ± 20.6 mm^2; $p < 0.05$) and age-matched male rats (183.4 ± 12.3 mm^2; $p < 0.01$). Following adrenalectomy, the lysis areas reduced significantly to 144.3 ± 15.1 mm^2 in pro-oestrous ($p < 0.005$), to 120.7 ± 19.8 mm^2 in dioestrous ($p < 0.01$) and to 134.3 ± 9.4 mm^2 male rats ($p < 0.01$). The infusion of adrenaline (20 μg kg^{-1} hr^{-1} × 120 min) increased the effects on fibrin plates to within pre-adrenalectomy levels (Fig. 4).

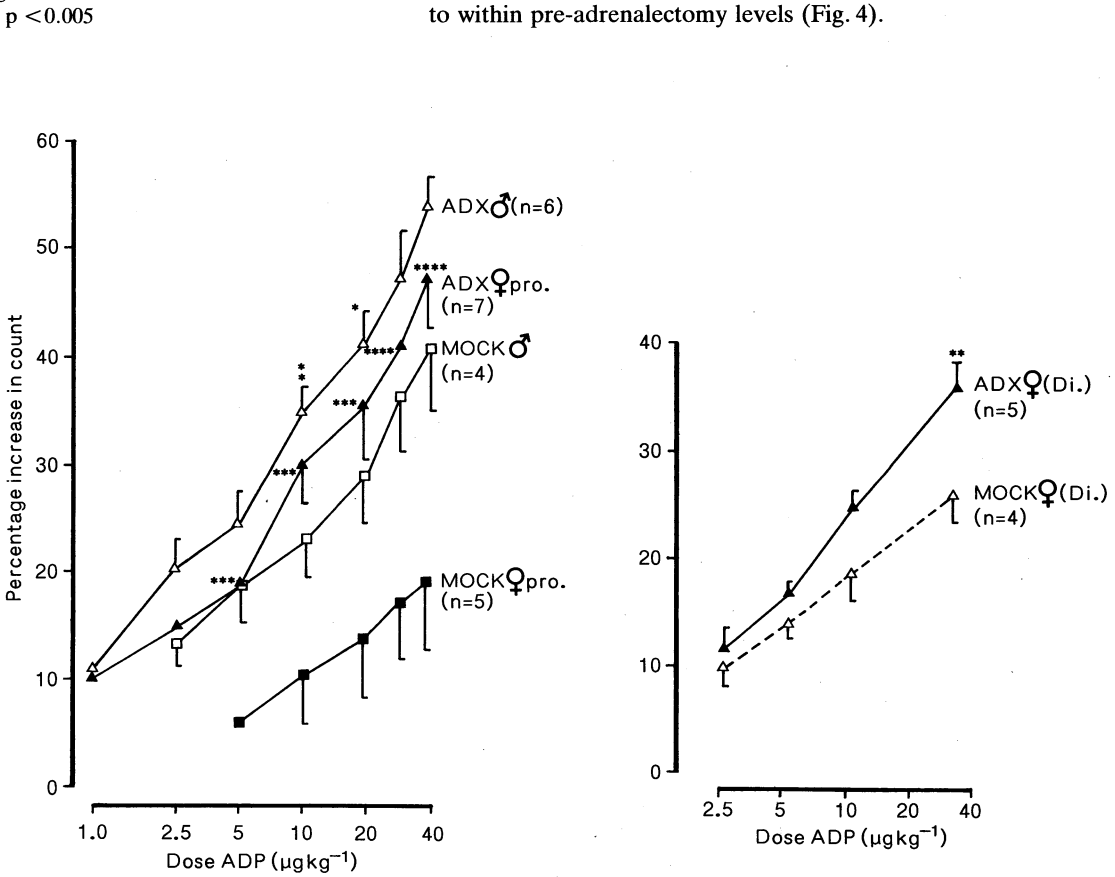

Fig. 3 Effects of acute adrenalectomy (ADX) on the sensitivity of platelets of male (♂), pro-oestrous (♀pro) and dioestrous (♀di) female rats to ADP. Data presented as mean ± S.E.M. with n in parentheses. Values were compared to the respective mock-adrenalectomized control rats (Mock). * $p < 0.05$; ** $p < 0.02$; *** $p < 0.01$; **** $p < 0.005$

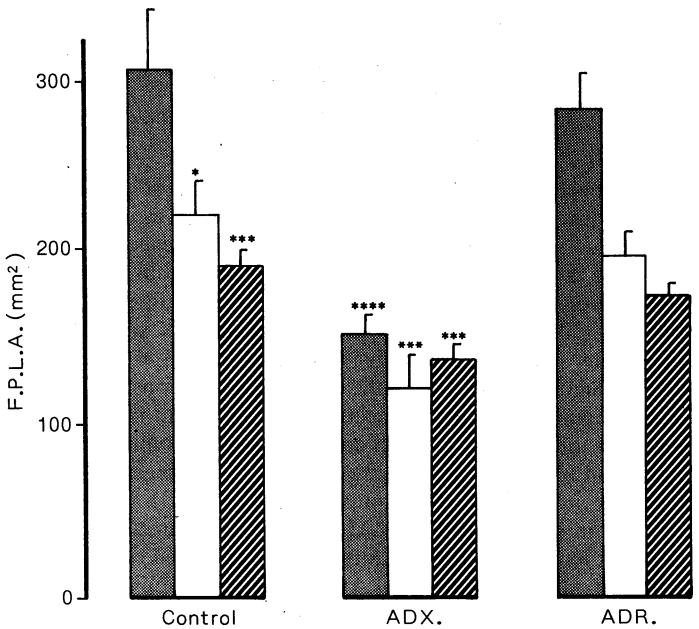

Fig. 4 The spontaneous fibrinolytic activity (expressed as fibrin plate lysis area (F.P.L.A.) in control i.e. mock-adrenalectomized pro-oestrous (▨), dioestrous (□) and male (▨) rats. The fibrinolytic activities following adrenalectomy (ADX) and adrenaline infusion (ADR) in the respective rats are also shown. Data are presented as mean ± S.E.M. (n = 6). Values were compared in control rats to the pro-oestrous data. The effects of adrenalectomy were assessed by comparing the adrenalectomy data of the rats with the respective mock-adrenalectomized rats. * $p < 0.05$; *** $p < 0.01$; **** $p < 0.005$

Discussion

The results of this investigation demonstrate a very important role played by adrenaline (endogenous and exogenous) in platelet function and fibrinolytic activity in rats. Owing to the conflicting findings on platelet sensitivity between male and female rats (3, 4, 7), we examined platelet aggregation in male and female rats without a bias for the oestrous cycle in females. The wide variation observed in female rats led us to studying platelet aggregation in pro- and dioestrous female rats. We observed that platelets from male rats aggregated more to ADP than those from females. This is in agreement with in vitro observation (3, 13). The variation in platelet sensitivity in vitro has been shown in the human during the menstrual cycle (14) though not in rats (4, 7). The variation observed here in platelet aggregation to ADP showed platelets from pro-oestrous rats to be significantly less responsive than those of dioestrous rats. The platelets from dioestrous rats aggregated less than those of male rats. It has been suggested that oestradiol is primarily responsible for such observations (2), since there is an abrogation of such effects following ovariectomy (3, 7).

17B oestradiol was reported to elevate adrenaline content of plasma with female rats having a higher adrenal secretory capacity than male rats (9, 15). Moreover, adrenaline [which was shown to be antiaggregatory in the rat (5)] has been reported to be the mediator of fibrinolytic activity induced by various agents (16). Based on these facts, we examined the effects of adrenalectomy on the aggregation of platelets to ADP as well as the spontaneous fibrinolytic activity of the plasma in pro-, dioestrous and male rats with or without adrenalectomy.

Acute adrenalectomy is known to selectively reduce circulating levels of adrenaline appreciably with little or no effect on corticosteroid levels. When the effect of acute adrenalectomy was examined on platelet aggregation, we observed that the removal of endogenous adrenaline enhanced the reactivity of platelets to ADP. The effect was greatest in pro-oestrous rats and least in male rats. This was such that there was no longer a difference in the aggregation of platelets between female (pro-, dioestrous) and male rats. This observation supports our earlier finding that adrenaline is antiaggregatory in the rat (5). The observation also agrees with the fact that oestrogens enhance the secretory capacity of the adrenal glands (9). It thus appears that during pro-oestrous (when the level of oestradiol is highest), there is a correspondingly high amount of endogenous adrenaline and of course, the reduced aggregatory response. During dioestrous, the reverse obtains. The low level of oestradiol in dioestrous rats is higher than that in male rats, hence, the lower aggregability of their platelets. The removal of the source of adrenaline will abolish its inhibitory effects. Thus, the greatest effect of adrenalectomy on platelet aggregation was observed in pro-oestrous rats. The reversal of the effects of adrenalectomy by adrenaline infusion in pro-oestrous rats supports the role of adrenaline on platelet aggregation.

It is necessary to evaluate whether these effects of adrenaline on platelet aggregation are direct or indirect. Recently, we have shown that the antiaggregatory effect of adrenaline in the rat is indirect (17) – being dependent to some extent on cyclooxygenase products. Similar observation has been shown for adrenaline on the isolated rat uterus (18) and the antiaggregatory and profibrinolytic activities of cyclooxygenase products have also been demonstrated in parallel studies (19). In agreement with the study of Desnoyers (16) that adrenalectomy led to a reduction in the activity of the plasminogen activating system, the results of this study suggests that the antiaggregatory effects of adrenaline in the rat may be mediated via a profibrinolytic activity.

The evaluation of the spontaneous fibrinolytic activity of the plasma as measured by digestion of fibrin by resuspended euglobulin precipitate showed that the lysis area was greatest with plasma from pro-oestrous rats. Following adrenalectomy, there was a reduction in the fibrinolytic activity in pro-, dioestrous and male rats. The infusion of adrenaline reversed the effects of adrenalectomy on fibrinolytic activity. These observations demonstrate an inverse relationship between platelet function and fibrinolysis. The increased fibrin digestion by the plasma from pro-oestrous rats is suggestive of an increased amount of plasminogen activator in their plasma – a possible outcome of the high levels of oestradiol and adrenaline levels at this stage. Adrenaline may possibly act by releasing plasminogen activators from the vascular endothelium. The activators so released may then interfere with platelet-platelet contact following the administration of agonists. A plausibility of this was supported by the fact that slow intravenous infusion of streptokinase and urokinase inhibited intravascular aggregation induced by ADP, collagen and arachidonic acid (17).

The findings from this investigation suggest that in the rat, the inhibitory effects of oestradiol on platelet aggregation is a direct function of adrenaline levels in the circulation. Adrenaline in itself works indirectly by stimulating fibrinolytic activity to inhibit platelet aggregation. In other species where adrenaline is proaggregatory, it is difficult to speculate the exact interrelationship between oestradiol and adrenaline.

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