ELSEVIER

European Journal of Pharmacology 315 (1996) 203–212

ejp

The role of ADP in endotoxin-induced equine platelet activation

Gavin E. Jarvis, Richard J. Evans *, M. Fred Heath

Department of Clinical Veterinary Medicine, University of Cambridge, Madingley Road, Cambridge CB3 0ES, UK

Received 14 March 1996; revised 19 June 1996; accepted 9 August 1996

Abstract

We have shown previously that endotoxin induces platelet aggregation in equine heparinised whole blood in a platelet-activating factor (PAF; 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) dependent manner. ADP is an agonist of platelets and is present in platelet dense granules with ATP in high concentrations. An investigation was carried out to establish whether endotoxin-induced platelet activation was associated with release of platelet ATP and ADP. ADP-scavenging enzyme systems significantly inhibited endotoxin-induced aggregation. Plasma levels of adenine nucleotides were measured using a luminometric assay following incubation of heparinised equine whole blood with endotoxin (300 ng/ml). After addition of endotoxin, ATP and ADP were released from the platelets and then subsequently degraded to AMP. WEB2086 (4-{3-[4-(*o*-chlorophenyl)-9-methyl-6*H*-thieno[3,2-*f*]-*s*-triazolo[4,3-*a*][1,4]diazepin-2-yl]proprionyl}-morpholine) (100 nM), a competitive PAF receptor antagonist, inhibited endotoxin-induced aggregation and also inhibited the release of adenine nucleotides from the platelets. It is concluded that endotoxin-induced aggregation is dependent upon ADP released from platelet dense granules.

Keywords: Endotoxin; Platelet aggregation; Adenine nucleotide; PAF (platelet-activating factor)

1. Introduction

It has been previously shown that endotoxin activates equine platelets in heparinised whole blood (Jarvis and Evans, 1994). This activation was shown to be dependent on platelet-activating factor (PAF; 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) but not prostanoids including thromboxane A_2 (Jarvis and Evans, 1996). In common with platelets from all species investigated, equine platelets are activated by ADP (Siess, 1989), which induces a prompt aggregation response ($EC_{50} \approx 2 \times 10^{-7}$ M; Poole et al., 1993). The dense granules of equine platelets contain high concentrations of both ATP and ADP which can be released on activation of the platelets (Meyers et al., 1982).

PAF has been implicated in the pathophysiology of endotoxaemia (Anderson et al., 1991) and endotoxin-induced disease states by investigators studying a variety of species (Carrick et al., 1993; Emanuelli et al., 1994; Kuipers et al., 1994; Terashita et al., 1985). However, despite the well characterised ability of adenine nucleotides to significantly influence blood vessels and platelets (Burnstock, 1978; Born, 1962), there has been very little suggestion of a role for these mediators in endotoxin-induced pathology. Rowe et al. (1978) demonstrated a reduction in platelet aggregation induced by ADP in piglets with thrombocytopenia induced by gram-negative septicaemia, and Parker and Adams (1993) showed that endotoxaemia resulted in a significant loss of vasodilator responses of guinea pig aortic rings to ADP. Hence, there is evidence to suggest that the purine-induced responses of vessels and platelets can be influenced by endotoxin, although it is less clear whether ADP or other purines actually mediate the effects of endotoxin.

It was the purpose of this study to establish whether ADP played a significant role in the activation of equine platelets by endotoxin. Both the platelets and red cells were potential sources of ADP.

2. Materials and methods

2.1. Reagents

General: ADP; apyrase (EC 3.6.1.5); bovine serum albumin; dimethyl sulphoxide; endotoxin from *Escherichia coli* O55:B5 (phenol extraction); phosphocrea-

* Corresponding author. Tel.: (44-1223) 337-638; Fax: (44-1223) 337-610.

PII S0014-2999(96)00637-1

tine; platelet-activating factor (PAF; 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine); Triton X-100 were from Sigma (Poole, UK). WEB2086 (4-{3-[4-(*o*-chlorophenyl)-9-methyl-6*H*-thieno[3,2-*f*]-*s*-triazolo[4,3-*a*][1,4]diazepin-2-yl]proprionyl}-morpholine) was a kind gift of Dr. C. Justus (Boehringer-Ingelheim Vetmedica, Ingelheim, Germany). Creatine kinase (EC 2.7.3.2) was from Boehringer-Mannheim (Mannheim, Germany). All inorganic salts including calcium chloride solution (1 M); trisodium citrate; ethanol were from BDH Merck (Poole, UK). Heparin sodium (Multiparin) was from CP Pharmaceuticals (Wrexham, UK).

Reagents for luminometry: ADP; AMP; CTP; EDTA (*N*,*N*,*N*′,*N*′-ethylenediamine tetra-acetic acid); phosphoenol pyruvate were from Sigma. Tris acetate buffer (0.1 M, pH 7.75; +2 mM EDTA); Bio-Orbit ATP-monitoring reagent and ATP standard were from LabTech International (East Sussex, UK). Potassium acetate was from BDH Merck. Pyruvate kinase (EC 2.7.1.40); adenylate kinase (EC 2.7.4.3) were from Boehringer-Mannheim.

Reagents for spectrophotometry: Drabkin's reagent was from Sigma. Cyanmethaemoglobin standard solution (57.2 mg/100 ml) was from BDH Merck.

2.2. Collection of samples

For each experiment blood was freshly collected by jugular venepuncture from one of seven clinically healthy barren Welsh mountain pony mares aged 4–9 years, which had not received any medication during the previous 10 days, directly into one part anticoagulant per nine parts blood. The anticoagulants used were trisodium citrate, which was dissolved in deionised distilled water so that the final concentration in the blood was 11 mM, or heparin sodium which was added to sodium chloride for injection (0.9% w/v) so that its final concentration in the blood was 7.5 U/ml. In some experiments, as noted below, these two anticoagulants were used together, their final concentration in the blood being the same as described above. This blood was subsequently recalcified by the addition of calcium chloride (1 M) solution, such that the molar ratio of calcium to citrate was 3:2.

2.3. Aggregometry

Aggregometry was performed using a Chronolog 540-VS dual channel whole blood aggregometer (Labmedics, Romiley, UK).

Turbidimetric aggregometry was conducted as originally described by Born and Cross (1963): platelet rich plasma was prepared by centrifugation of whole blood for 15 min at 250 × *g*, and platelet poor plasma by centrifugation of 2 ml of platelet rich plasma for 5–6 min at 10 000 × *g*. Briefly, on addition of a pro-aggregatory agent to the platelet rich plasma, stirred at 1000 revolutions per minute at 37°C, the platelets are activated and start to aggregate. A beam of light is directed through the platelet suspension and the emerging light intensity detected. As the platelets aggregate, the proportion of the light passing through the plasma increases, owing to the reduction in the scatter of light caused by the platelets. This change in optical density of the platelet rich plasma is measured as a deflection of a trace recording. The greater the rate and degree of aggregation, the greater the rate and degree of deflection of the recording. The platelet poor plasma serves as a reference, providing a theoretical measure of light absorbance after complete aggregation of all platelets in the platelet rich plasma. The rate of aggregation was calculated from the gradient of a straight line drawn through the steepest part of the trace as the percentage of the maximum possible aggregation in 1 min.

Impedance aggregometry was conducted as originally described by Cardinal and Flower (1980). Whole blood for impedance aggregometry was diluted 1:1 with sterile filtered phosphate buffered saline prior to analysis. Briefly, a pair of palladium alloy electrodes separated by a small gap were secured in the cuvette such that they were entirely covered by blood. On addition of a platelet agonist to the whole blood, stirred at 1000 revolutions per minute at 37°C, the platelets aggregated around the electrodes thus increasing the electrical impedance between the electrodes. This change was recorded as a deflection in a trace recording in a similar manner to the output from turbidimetric aggregometry. Calibration was achieved by inducing a fixed increase of 20 Ω between the electrodes prior to addition of any aggregating agent. The rate of aggregation was measured therefore in Ω/min. After each individual aggregation, the electrodes were carefully wiped clean and washed in water and phosphate buffered saline prior to the next aggregation.

2.4. Luminometric assay of adenine nucleotides

Levels of adenine nucleotides (ATP, ADP and AMP) were measured in the plasma using a luminometric assay. The method used was a modified version of that described by Lundin et al. (1986). ADP and AMP were detected by conversion to ATP and measuring the resultant increase in light intensity. ADP was converted to ATP using the enzyme pyruvate kinase and phosphoenol pyruvate. AMP was converted to ADP using the enzyme adenylate kinase with a large excess of CTP. This ADP was then converted to ATP by the pyruvate kinase thereby increasing the light intensity.

The preparation of samples for luminometric assay was as follows. At defined times after the initiation of each aggregation determination, 100 μl EDTA (0.1 M) was added to the the whole blood which was then centrifuged and 500 μl of the plasma supernatant was added to 500 μl absolute ethanol. For the luminometric assay itself, this prepared sample was centrifuged to sediment out the precipitated protein and 100 μl of the supernatant was added to 800 μl of the Tris acetate buffer (containing 10 μl of

potassium acetate (2 M)). 100 μl of ATP-monitoring reagent was then added and the emission of light was detected and recorded with a Bio-Orbit 1250 luminometer and display unit (Bio-Orbit, Turku, Finland). ADP and AMP were measured following conversion reactions as described above.

The results are measured in pmol and represent the quantities of adenine nucleotides present in the 100 μl sample added to the buffer. As all the samples were prepared in an identical fashion, the results are directly comparable.

2.5. Spectrophotometry

The levels of free haemoglobin in cell-free plasma were measured spectrophotometrically to provide an indication of the degree of red cell haemolysis. Haemoglobin was converted to cyanmethaemoglobin using Drabkin's reagent as described by Dacie and Lewis (1975). The levels of cyanmethaemoglobin in these samples were determined by measuring the absorbance of light at 540 nm using a Cecil 5000 series spectrophotometer standardised with the cyanmethaemoglobin solution.

2.6. ADP-scavenging systems

In the absence of a selective competitive antagonist at the platelet ADP receptor, two ADP-scavenging enzyme systems were used to artificially lower the free concentration of ADP. Apyrase derived from potatoes converts ATP to ADP and ADP to AMP. The enzyme, creatine kinase together with phosphocreatine catalyses the conversion of ADP to ATP. Spontaneous and endotoxin-induced aggregation were measured using impedance aggregometry in heparinised whole blood as previously described (Jarvis and Evans, 1994) in the absence and presence of each of these scavenging systems. All of these reagents were freely soluble in phosphate buffered saline: apyrase at a final concentration of 40 μg/ml and creatine kinase and phosphocreatine at 45 U/ml and 10 mM, respectively, were used.

2.7. Statistical analysis

Statistical analysis was performed using SPSS for Macintosh (version 4.0). Multiple linear regression was used to determine significant factors responsible for the levels of adenine nucleotides following incubation with or without endotoxin. Non-linear regression analysis was employed to determine the significance of the effects of the ADP-scavenging systems on spontaneous and endotoxin-induced aggregation. Non-linear regression was used to fit PAF and ADP concentration-response curves to the logistic Hill equation. A full account of these statistical methods used is given in Jarvis (1996).

3. Results

3.1. ADP-scavenging systems

The effects of apyrase (40 μg/ml), and creatine kinase (45 U/ml)/phosphocreatine (10 mM) on spontaneous and endotoxin-induced aggregation in heparinised whole blood are summarised in Fig. 1. The rates of aggregation in the absence of endotoxin (300 ng/ml) and the enzyme system, in the presence of each separately, and of both together were measured. Five or six replicate determinations were obtained for each of these four groups. This experiment was conducted using blood from three different ponies for both enzyme systems. The figure illustrates the expected increase in the rate of aggregation caused by endotoxin (300 ng/ml). Apyrase inhibited the endotoxin-induced aggregation significantly by 92% ($P < 0.001$) and the creatine kinase/phosphocreatine system inhibited it by 83% ($P < 0.001$). It can be seen that the creatine kinase/phosphocreatine system also inhibited spontaneous aggregation (i.e., aggregation in the absence of endotoxin) by 81% ($P < 0.001$) whereas apyrase had no overall effect on spontaneous aggregation.

3.2. Concentrations of nucleotides following aggregation

The levels of adenine nucleotides in plasma were measured following completed paired aggregation determinations in the absence and presence of endotoxin (300 ng/ml) in heparinised whole blood. Aggregation was terminated

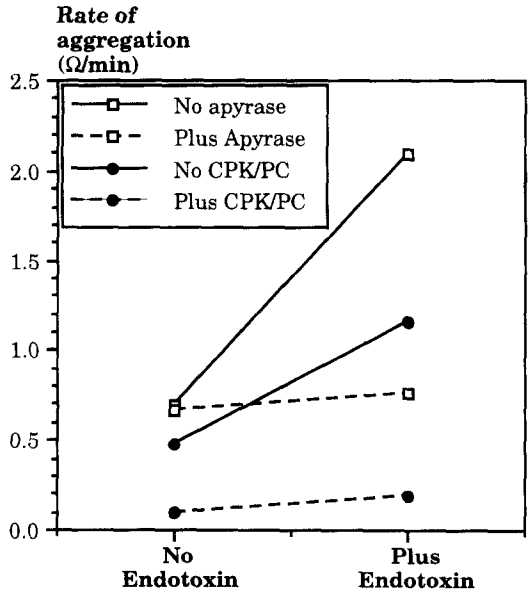

Fig. 1. The effect of apyrase (40 μg/ml), and creatine phosphokinase (CPK: 45 U/ml) and phosphocreatine (PC: 10 mM) on the rate of platelet aggregation in the absence and presence of endotoxin (300 ng/ml). Points represent the combined results from three separate experiments each using heparinised whole blood from different ponies.

25 min after the addition of endotoxin by the addition of EDTA. Fig. 2. illustrates the results from six separate experiments on four ponies. The endotoxin-induced increase in rate of aggregation and levels of nucleotides are illustrated. It can be appreciated from the graph that there is a large increase in the total levels of adenine nucleotides following incubation in the presence of endotoxin and that this increase is due almost entirely to an increase in the levels of AMP. Multiple linear regression analysis confirmed the significance of the increase in the rate of aggregation ($P < 0.001$), the total level of nucleotides ($P < 0.001$) (the total was the sum of the levels of ATP, ADP and AMP) and the levels of AMP ($P < 0.001$). At this late time point following addition of endotoxin, there was no significant endotoxin-induced change in the level of ATP, although there was a small increase in the amount of ADP ($P < 0.01$).

A time-course experiment was conducted to establish the pattern of change in the levels of ATP, ADP and AMP over 25 min induced by endotoxin (300 ng/ml). The results are summarised in Fig. 3. The results shown are from three experiments carried out on separate occasions, each performed using blood from the same donor pony. Times were measured from the addition of endotoxin. Levels of nucleotides were determined in the absence and presence of endotoxin (300 ng/ml), and multiple linear regression was used to analyse the changes in nucleotide levels and to evaluate the endotoxin-induced component.

Between 5 and 10 min, the endotoxin induced a small increase of 1.0 pmol in the level of ATP ($P < 0.01$). Between 10 and 15 min there was a fall in the endotoxin-induced ATP level of 0.7 pmol ($P < 0.10$). The levels of ADP followed a similar pattern to ATP. Endotoxin induced a significant rise in ADP between 5 and 10 min of 2.3 pmol ($P < 0.001$) and between 10 and 15 min the levels of ADP fell by 1.1 pmol ($P < 0.05$). For AMP, the pattern of change was different. Endotoxin caused levels of AMP to rise steadily from between 5 and 25 min. There was a significant rise of 2.1 pmol between 5 and 10 min ($P < 0.01$) and of 2.4 pmol between 10 and 25 min ($P < 0.05$). Overall, there was an endotoxin-induced increase in total nucleotide levels of 5.1 pmol between 5 and 10 min ($P < 0.001$); there was no subsequent significant change in the total levels of nucleotides after 10 min.

3.3. Effect of aggregometry electrodes

It was considered that the presence of the electrodes in the whole blood sample might be responsible for some of the increase in the levels of adenine nucleotides. In order to test this hypothesis, control experiments were conducted in the absence and presence of the electrodes and the levels of nucleotides subsequently measured. As it was not possible to measure aggregation in the absence of the electrodes, platelet counts were performed at the end of each aggregation as an alternative means of measuring the effect of the endotoxin. These experiments were conducted using heparinised whole blood.

In those samples with the electrodes, the endotoxin caused a significant increase in the rate of aggregation ($P < 0.001$). The platelet count at the end of the aggregation determinations was significantly lower in the blood containing endotoxin (300 ng/ml) than in the control ($P < 0.001$). In the absence of the electrodes, the platelet counts were [mean(S.E.M.)] $70(4) \times 10^9$/l (control) and $33(18) \times 10^9$/l (plus endotoxin). In the presence of the electrodes the figures were $56(12) \times 10^9$/l (control) and $19(5) \times 10^9$/l (plus endotoxin). These figures were calcu-

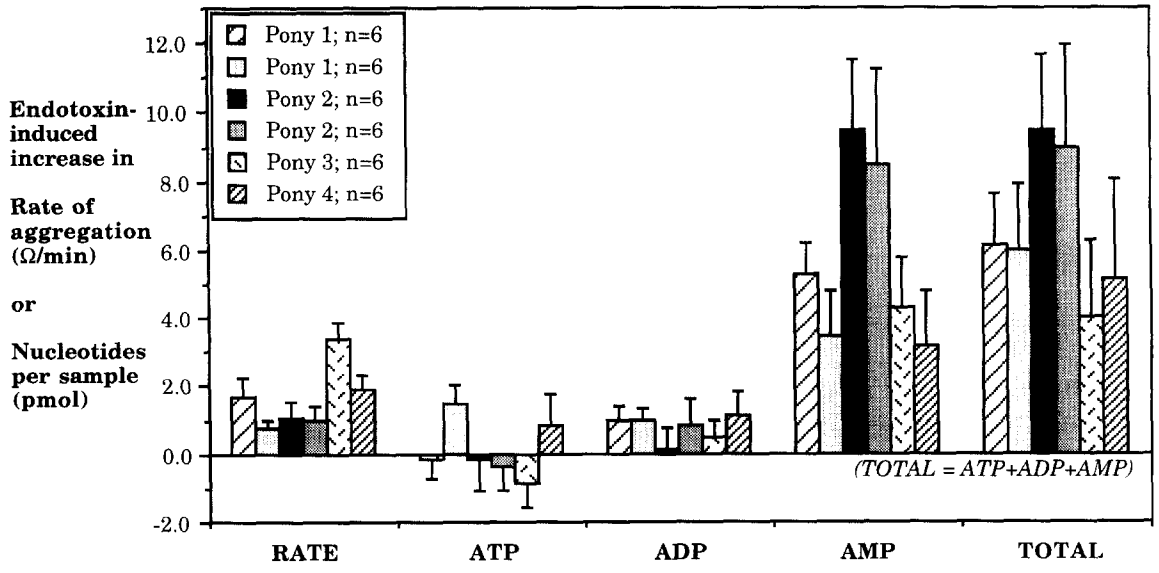

Fig. 2. The endotoxin-induced increase in either the rate of aggregation (RATE) or levels of adenine nucleotides (ATP, ADP, AMP, TOTAL) in heparinised whole blood. Bars represent the mean ± S.E.M. of n replicate determinations. Concentration of endotoxin = 300 ng/ml. Results from six separate experiments.

lated using the mean platelet counts from three separate experiments.

Multiple linear regression showed that the endotoxin induced an increase in the total levels of nucleotides by 8.5 pmol ($P < 0.001$). The electrodes had no effect on the levels of any of the nucleotides, either in the absence or presence of endotoxin.

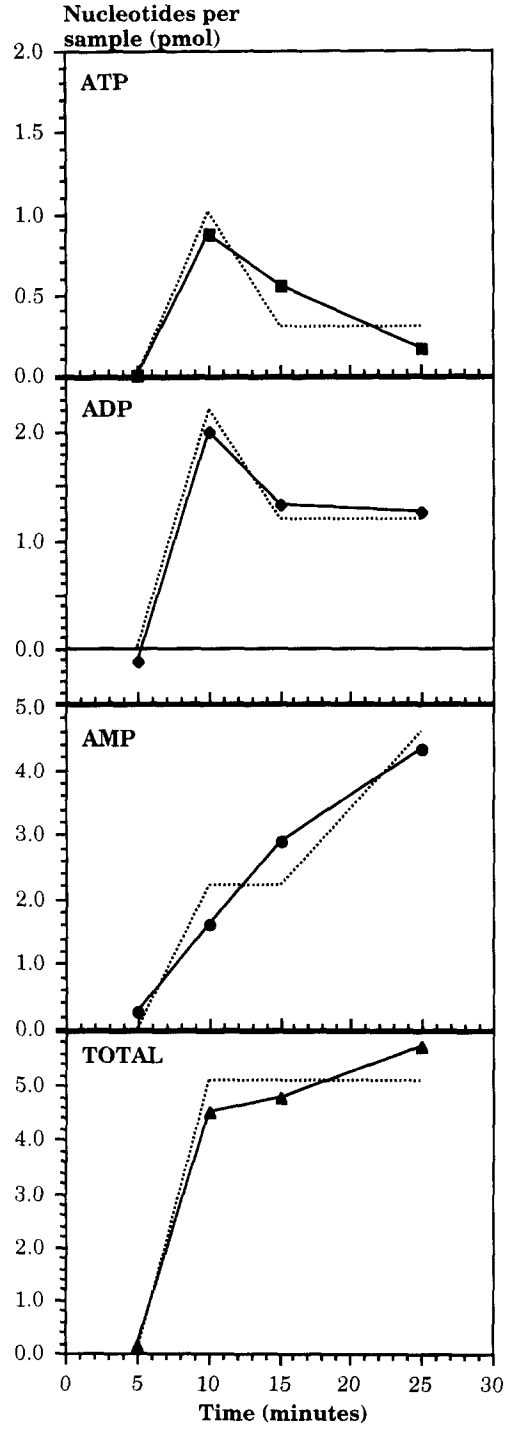

Fig. 3. Changes in levels of adenine nucleotides over time (ATP = ■; ADP = ◆; AMP = •; TOTAL = ▲) induced by endotoxin (300 ng/ml) added at time zero to heparinised whole blood. The best fit estimates as determined by multiple linear regression analysis are also shown (· · · · ·).

3.4. Effect of WEB2086 on levels of adenine nucleotides

WEB2086, a competitive antagonist at the PAF receptor, has previously been shown to inhibit both spontaneous and endotoxin-induced aggregation in a concentration-dependent fashion (Jarvis and Evans, 1996). Three experiments using different ponies were conducted to determine the effect of 100 nM WEB2086 on the endotoxin-induced increase of adenine nucleotides. These experiments were performed using heparinised whole blood, and levels of nucleotides were determined following 25 min incubation with endotoxin as previously described. The results are illustrated in Fig. 4.

In accordance with previous results, this concentration of WEB2086 reduced the level of spontaneous aggregation by 73% and endotoxin-induced aggregation by 86%. In the absence of WEB2086, endotoxin (300 ng/ml) induced an increase of 5.3 pmol in the total levels of nucleotides ($P < 0.001$). In the presence of WEB2086, there was a significant reduction by 3.7 pmol ($P < 0.05$) of the endotoxin-induced rise in total nucleotides. This represents a 70% reduction in the endotoxin-induced release of nucleotides from the platelets.

3.5. Effect of platelet numbers on levels of adenine nucleotides

As a means to determine the source of the increase in the levels of nucleotides, three separate experiments were conducted in order to compare the changes in levels of nucleotides in platelet 'rich' and platelet 'depleted' blood. The blood for these experiments was collected into citrate and heparin as described in the Materials and methods section. This blood was then centrifuged and platelet rich plasma taken off. Half of the platelet rich plasma was centrifuged further to produce platelet depleted plasma. Red and white cells were then resuspended in the platelet rich and platelet depleted plasma in order to produce two comparable blood samples, but with differing platelet counts. The combination of heparin and citrate as an anticoagulant was employed because the manipulation required to produce the platelet rich and platelet poor preparations was not tolerated well in whole blood anticoagulated with heparin alone. These preparations were subsequently recalcified (thereby eliminating the effect of the citrate and facilitating the response to endotoxin) immediately prior to aggregation determinations. The platelet counts for the two preparations were [mean(S.E.M.)] $128(10) \times 10^9/l$ for the platelet rich blood and $38(8) \times 10^9/l$ for the platelet depleted blood. These values are calculated from three separate experiments.

There was a significant reduction by 64% in the endotoxin-induced rate of aggregation in the platelet depleted blood ($P < 0.001$). Fig. 5 summarises the effects of the differing platelet counts on the endotoxin-induced rate of

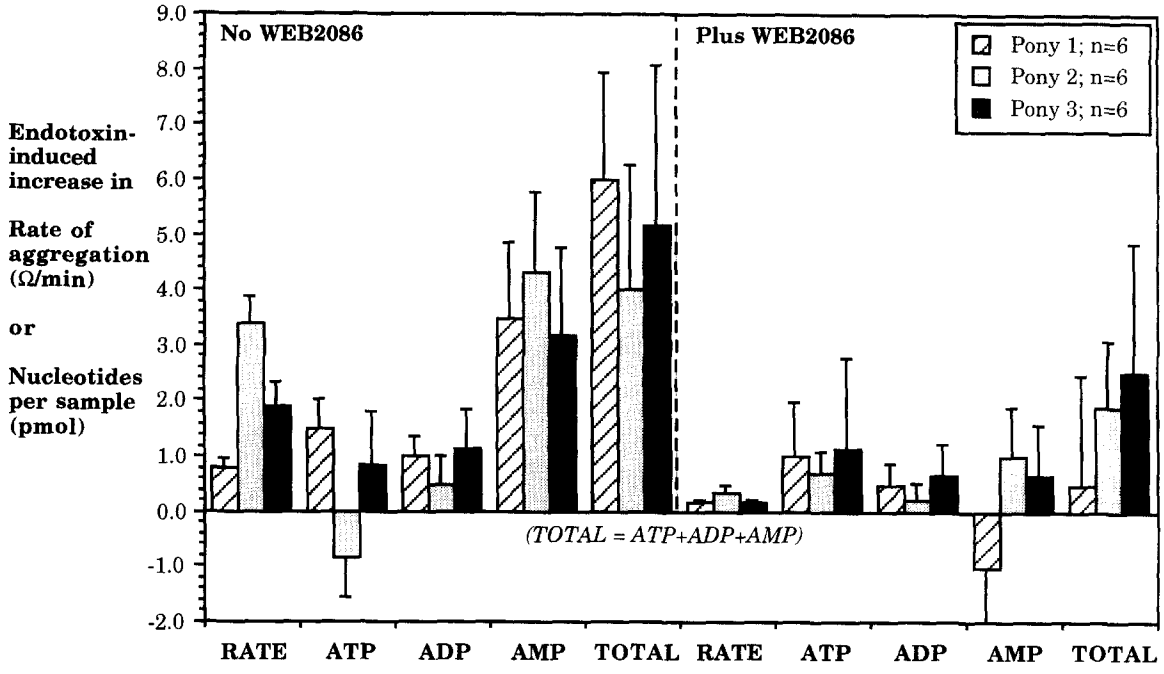

Fig. 4. The effect of WEB2086 (100 nM) on the rate of aggregation (RATE) and the increase in levels of adenine nucleotides (ATP, ADP, AMP, TOTAL) induced by endotoxin in heparinised whole blood. Bars represent the mean ± S.E.M. of n replicate determinations. Concentration of endotoxin = 300 ng/ml. Results from three separate experiments.

aggregation and change in levels of total nucleotides in the three experiments. Multiple linear regression analysis showed that in the absence of endotoxin, the total level of nucleotides was lower in the platelet depleted blood ($P < 0.001$). In platelet rich blood, endotoxin induced a large increase in the total nucleotide level of 10.5 pmol ($P < 0.001$). In platelet depleted blood, however, this increase was significantly reduced by 74% ($P < 0.001$). It is notable that the platelet count in the platelet depleted blood was 70% lower than that of the platelet rich blood.

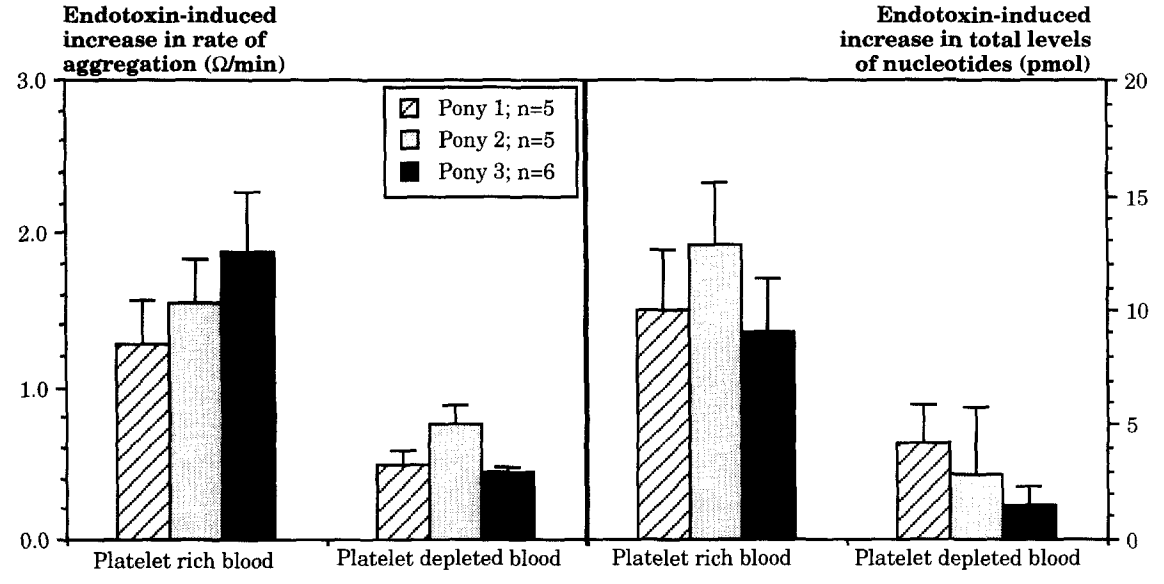

Fig. 5. The endotoxin-induced increase in the rate of aggregation (left hand panel) and total levels of adenine nucleotides (right hand panel) in platelet rich and platelet depleted blood. The bars represent the mean ± S.E.M. of n individual determinations. Platelet counts [mean(S.E.M.)] were: platelet rich blood = 128(10) × 10^9/l and platelet depleted blood 38(8) × 10^9/l. Concentration of endotoxin = 300 ng/ml. Blood coagulated with heparin and citrate, followed by recalcification, was used, as described in the text. Results from three separate experiments.

3.6. Effect of endotoxin on levels of free haemoglobin

Levels of haemoglobin in the plasma following spontaneous and endotoxin-induced aggregation in heparinised whole blood were determined in order to give a measure of red cell haemolysis. Spectrophotometry was employed as described above. Fig. 6 illustrates the results from three separate experiments. It can be clearly seen that the endotoxin induces aggregation whilst having no effect upon the levels of haemoglobin. In a further experiment, the effect of the presence of the electrodes was examined. It was shown that the electrodes increased the levels of haemoglobin in the plasma sample by 5 mg/100 ml ($P < 0.01$), although the presence of the electrodes had no influence upon the effect of the endotoxin.

3.7. Effects of apyrase on ADP- and PAF-induced platelet aggregation

The effects of a large concentration of apyrase (400 μg/ml) on both ADP- and PAF-induced aggregation in citrated platelet rich plasma were investigated. The results are shown in Fig. 7 and Fig. 8. The scales have been normalised in order to facilitate comparison of the curves; however, this results in the maximum for each curve appearing the same, although this was not necessarily the case. Non-linear regression was used to fit the results to the logistic Hill equation. Simultaneous multiple curve fitting was employed in order to facilitate comparison between different curves as described by De Lean et al. (1978). The following results are expressed as mean [95% confidence interval]. Separate experiments were carried out on two ponies.

In the presence of apyrase, there was a shift to the right of the concentration-response relationship for ADP-induced rate of aggregation. In the absence of apyrase, the EC_{50} was 2.01×10^{-7} M [1.65, 2.44] and in the presence of apyrase it was 2.34×10^{-6} M [1.19, 4.59]. These values are significantly different ($P < 0.001$). The Hill constant in the absence of apyrase was 1.54 [1.22, 1.85] and in the presence of apyrase was 1.07 [0.08, 1.34]. These values were also significantly different ($P < 0.05$). Apyrase did not affect the maximum response induced by ADP.

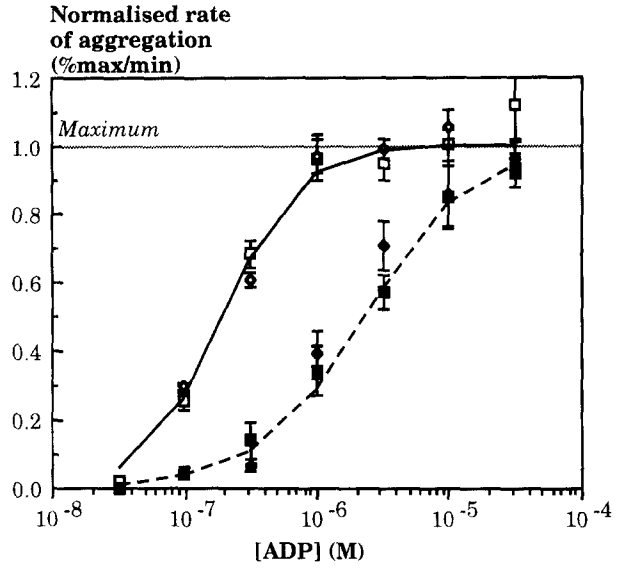

Fig. 7. The effect of apyrase (400 μg/ml) on the rate of ADP-induced platelet aggregation in citrated platelet rich plasma. The responses have been normalised such that the maximum = 1.0 for each of the four sets of data. Each point represents the mean ± S.E.M. of four replicate determinations. (□ = pony 1, control; ■ = pony 1, plus apyrase; ◇ = pony 2, control; ◆ = pony 2, plus apyrase; ——— = best fit curve, control; – – – = best fit curve, plus apyrase).

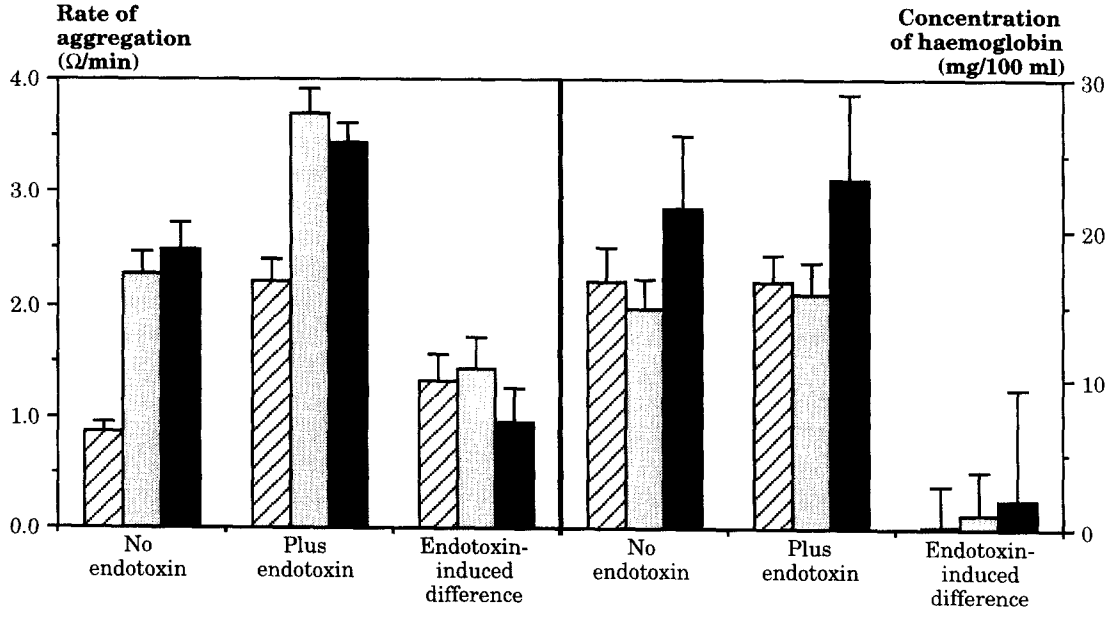

Fig. 6. The rate of aggregation (left hand panel) and the plasma concentration of haemoglobin (right hand panel) in heparinised whole blood following incubation in the presence or absence of endotoxin (300 ng/ml). The endotoxin-induced difference is also shown. Each bar represents the mean ± S.E.M. of 30 (hatched bars), 18 (shaded bars), and 12 (black bars) replicate determinations from different ponies.

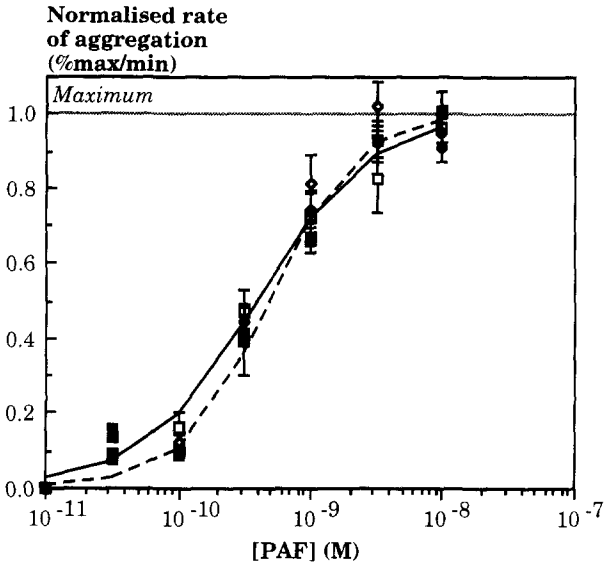

Fig. 8. The effect of apyrase (400 μg/ml) on the rate of PAF-induced platelet aggregation in citrated platelet rich plasma. The responses have been normalised such that the maximum = 1.0 for each of the four sets of data. Each point represents the mean ± S.E.M. of four replicate determinations. (□ = pony 1, control; ■ = pony 1, plus apyrase; ◇ = pony 2, control; ◆ = pony 2, plus apyrase; ——— = best fit curve, control; – – – = best fit curve, plus apyrase).

These results confirmed the ability of apyrase to inhibit ADP-induced aggregation.

For PAF, the control EC_{50} was 4.01×10^{-10} M [1.88, 8.56] and in the presence of apyrase was 5.01×10^{-10} M [2.85, 8.82]. These values were not significantly different. The Hill constants in the absence (1.00 [0.52, 1.49]) and presence of apyrase (1.33 [0.90, 1.76]) were not significantly different either. In one of the two ponies, however, there was a significant ($P < 0.05$) reduction in the maximum response from 123 (% maximal aggregation per minute) [95, 152] to 83 [64, 103]. The maximum was unchanged in the other pony and overall, there was no significant reduction in the maximum response.

4. Discussion

Luminometric assay showed that there was a significant increase in the levels of adenine nucleotides following incubation with endotoxin. This increase was mainly due to an increased level of AMP. AMP exerts no effect on equine platelets (Poole et al., 1993); nevertheless, it is clear that the AMP was derived from ADP (and ATP) which could have activated the platelets before it subsequently degraded.

There are two obvious potential rich sources of ADP in whole blood. Platelets contain both ATP and ADP in high concentrations in their dense granules (Meyers et al., 1982). Red cells also contain large quantities of ADP (Gaarder et al., 1961). In order to determine whether either of these potential sources was responsible for the increase in nucleotides, various experiments were conducted.

Haemoglobin levels were measured in plasma following aggregation determinations as a marker of red cell destruction or leakage. It was reasoned that any release of ADP from red cells sufficient to induce platelet aggregation would be likely to be associated with an increase in free haemoglobin. More obviously, any red cell haemolysis would certainly be expected to be associated with an increase in the levels of ADP. The results indicate that there is no relationship between levels of haemoglobin and presence of endotoxin, or rate of aggregation. Hence, it is concluded that endotoxin does not cause red cell haemolysis and that red cell ADP is probably not responsible for the increase in levels of adenine nucleotides caused by endotoxin.

In order to determine whether the platelets were responsible for the increase in levels of nucleotides caused by endotoxin, experiments using platelet rich and platelet depleted blood were conducted. The results reported here show that the endotoxin-induced rise in the levels of nucleotides is reduced in platelet depleted blood by a similar degree to the reduction in platelet numbers (approximately 70%), suggesting strongly that the platelets are the source for these nucleotides.

Nevertheless, the nucleotide which showed the most pronounced increase following incubation with endotoxin was AMP, not ADP. In order to show that the AMP was increased as a consequence of the degradation of ADP (and ATP), a time-course experiment was conducted. Interpretation of these results is hampered by the lack of any information relating to the potential breakdown of AMP to adenosine. Nevertheless, the results are of interest. The total level of nucleotides rose within 10 min following the addition of and incubation with endotoxin. This was associated with an increase in levels of both ATP and ADP (as well as AMP). By 15 min the levels of ATP and ADP had fallen, whereas the level of AMP continued to rise until the end of the aggregation determination. Such a pattern is consistent with the hypothesis that dense granules release their contents following addition of endotoxin, thereby giving rise to an increase in both ATP and ADP and causing activation of platelets. During the following 15–20 min, the ATP and ADP is degraded to AMP which accumulates as time progresses. The total level of nucleotides did not change greatly after 10 min. This seems to suggest that most of the release of platelet ATP and ADP is complete within 10 min following the addition of endotoxin. This time scale of release is broadly consistent with the pattern of aggregation that is observed following addition of endotoxin, i.e., endotoxin-induced aggregation is not apparent until approximately 10 min following its addition (Jarvis and Evans, 1994).

The results of the experiments with the ADP scavenging systems further support the view that endotoxin-induced aggregation is mediated by ADP. Both systems

abolished endotoxin-induced aggregation. This suggests that by converting ADP to either AMP or ATP, these enzymes are preventing the released ADP from activating the platelets in response to endotoxin.

The possible contribution of the electrodes to aggregation of platelets in this system cannot be ruled out, particularly as the phenomenon of spontaneous aggregation is so persistent. It is obviously not possible to directly measure aggregation in the absence of the electrodes. It was possible, though, to assess whether they were responsible for any increase in the nucleotide or haemoglobin concentration, that might have arisen from damage caused by the collision of the platelets and red cells with the electrodes. The results indicated that levels of adenine nucleotides were not affected by the presence of the electrodes, although there was a small increase in haemoglobin concentration in their presence. This observation was not problematic, however, owing to the lack of any association between these levels of haemoglobin released and either the rates of aggregation, or the presence of endotoxin.

It has previously been shown that endotoxin-induced aggregation can be inhibited by the specific competitive PAF antagonist, WEB2086 (Jarvis and Evans, 1996). An experiment conducted to determine whether it also inhibited release of adenine nucleotides showed that WEB2086 does prevent the endotoxin-induced increase in levels of adenine nucleotides that is seen in the controls. Hence, it appears to be the case that endotoxin causes release of platelet ADP and ATP in a PAF-dependent manner. It is known that PAF is a very potent activator of equine platelets and is also capable of causing the release from the platelets of large quantities of ATP and ADP which degrade to AMP over a period of approximately 25 min (Poole, 1992). In order to establish whether PAF-induced platelet aggregation is dependent on release of platelet ADP, the effect of apyrase on PAF-induced aggregation was investigated. It was first confirmed that apyrase significantly inhibited ADP-induced aggregation. Using the same preparation of apyrase with platelets from the same blood sample, its effect on PAF-induced aggregation were shown to be minimal. The only statistically significant effect was a reduction in the maximum response in 1/2 ponies. These results do not support the view that PAF-induced aggregation is dependent on autoactivation of platelets by released ADP.

The effects of endotoxin beyond approximately 30 min following its addition to whole blood have not been investigated. Nevertheless, it is interesting to speculate about how this initial response to endotoxin, measured as aggregation and release of nucleotides, might subsequently develop in vivo in the horse. A role for PAF and ADP has been demonstrated in endotoxin-induced aggregation. PAF, an inflammatory mediator, is able to induce an increase in vascular permeability (Foster et al., 1992) and ATP and ADP, acting on P_2 receptors can induce either vasodilatation or constriction (Burnstock, 1978). In equine digital vein, it has been shown that ATP can cause vasodilatation which is not dependent on endothelium (Elliott et al., 1994). It was suggested that ATP was exerting its effects following degradation to adenosine, which then caused relaxation of vessels via activation of P_1 receptors of the A_2 subtype (Elliott and Soydan, 1994). The failure of PAF to induce any response in equine digital arteries (Scarlett, 1995) opens up the possibility of a platelet-dependent mechanism of PAF-induced vasodilatation in equine vessels, mediated via the release of ATP and ADP from aggregating platelets stimulated by PAF. These nucleotides would then be degraded to adenosine which could induce vasodilatation. Hence a possible mechanism for endotoxin-induced vasodilatation as a consequence of platelet degranulation presents itself, perhaps also promoting the subsequent development of shock.

It is concluded that platelet ADP mediates endotoxin-induced aggregation. The process of release is PAF dependent, although it is not clear where the PAF is acting. In addition to its familiar role as an intercellular mediator, it has also been reported that PAF can act as either an intracellular messenger or as a cell surface adhesion molecule (Stewart and Phillips, 1989; Zimmerman et al., 1990). It is possible that endotoxin induces expression of cell surface PAF on leucocytes (endotoxin-induced aggregation is leucocyte dependent (Jarvis and Evans, 1994)) which could then bind platelets which would be activated, but unable to aggregate owing to the fact that they were bound to the white cells. However, the bound platelets would release ADP thereby recruiting unbound platelets and promoting aggregation. This model is supported by the observation that the endotoxin-induced platelet aggregates are closely associated with leucocytes (Jarvis and Evans, 1994). Spontaneous aggregation could occur as a consequence of a low level of PAF expression, which is promoted by the physiological levels of Ca^{2+} present in heparinised blood in the absence of any of the anti-haemostatic properties of normal endothelium (Pearson, 1993). Further investigation is warranted in order to elucidate more precisely the manner in which the PAF and ADP interact in mediating endotoxin-induced platelet aggregation.

Acknowledgements

G.E.J. was in receipt of a Biotechnology and Biological Sciences Research Council Veterinary Research Fellowship. The authors also acknowledge support for this work from the Horserace Betting Levy Board.

References

Anderson, B.O., D.D. Bensard and A.H. Harken, 1991, The role of platelet activating factor and its antagonists in shock, sepsis and multiple organ failure, Surg. Gynecol. Obstet. 172, 415.

Born, G.V.R., 1962, Aggregation of blood platelets by adenosine diphosphate and its reversal, Nature 194, 927.

Born, G.V.R. and M.J. Cross, 1963, The aggregation of blood platelets, J. Physiol. 168, 178.

Burnstock, G., 1978, A basis for distinguishing two types of purinergic receptor, in: Cell Membrane Receptors for Drugs and Hormones: A Multidisciplinary Approach, eds. R.W. Straub and L. Bolis (Raven Press, New York, NY) p. 107.

Cardinal, D.C. and R.J. Flower, 1980, The electronic aggregometer: a novel device for assessing platelet behaviour in blood, J. Pharmacol. Methods 3, 135.

Carrick, J.B., D.D. Morris and J.N. Moore, 1993, Administration of a receptor antagonist for platelet-activating factor during equine endotoxaemia, Equine Vet. J. 25, 152.

Dacie, J.V. and S.M. Lewis, 1975, Practical Haematology, 5th edn. (Churchill Livingstone, Edinburgh) p. 30.

De Lean, A., P.J. Munson and D. Rodbard, 1978, Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose-response curves, Am. J. Physiol. 235, E97.

Elliott, J. and J. Soydan, 1994, Characterisation of the responses of equine digital veins to adenosine and its analogues, Br. J. Pharmacol. (Proc. Suppl.) 110, 146P.

Elliott, J., C.E. Bryant and J. Soydan, 1994, The role of nitric oxide in the responses of equine digital veins to vasodilator and vasoconstrictor agents, Equine Vet. J. 26, 378.

Emanuelli, G., G. Montrucchio, L. Dughera, E. Gaia, E. Lupia, E. Battaglia, A. De Martino, P. De Giuli, L. Gubetta and G. Camussi, 1994, Role of platelet activating factor in acute pancreatitis induced by lipopolysaccharides in rabbits, Eur. J. Pharmacol. 261, 265.

Foster, A.P., F.M. Cunningham and P. Lees, 1992, Inflammatory effects of platelet activating factor (PAF) in equine skin, Equine Vet. J. 24, 208.

Gaarder, A., J. Jonsen, S. Laland, A. Hellem and P.A. Owren, 1961, Adenosine diphosphate in red cells as a factor in the adhesiveness of human blood platelets, Nature 192, 531.

Jarvis, G.E., 1996, Endotoxaemia: the in vitro activation of equine platelets by endotoxin, PhD Thesis (University of Cambridge, Cambridge).

Jarvis, G.E. and R.J. Evans, 1994, Endotoxin-induced platelet aggregation in heparinised equine whole blood in vitro, Res. Vet. Sci. 57, 317.

Jarvis, G.E. and R.J. Evans, 1996, Platelet-activating factor and not thromboxane A_2 is an important mediator of endotoxin-induced platelet aggregation in equine heparinised whole blood in vitro, Blood Coag. Fibrinol. 7, 194.

Kuipers, B., T. Van der Poll, M. Levi, S.J.H. Van Deventer, H. Ten Cate, Y. Imai, E. Hack and J.W. Ten Cate, 1994, Platelet-activating factor antagonist TCV-309 attenuates the induction of the cytokine network in experimental endotoxemia in chimpanzees, J. Immunol. 152, 2438.

Lundin, A., M. Hasenson, J. Persson and Å. Pousette, 1986, Estimation of biomass in growing cell lines by adenosine triphosphate assay, Methods Enzymol. 133, 27.

Meyers, K.M., H. Holmsen and C.L. Seachord, 1982, Comparative study of platelet dense granule constituents, Am. J. Physiol. 243, R454.

Parker, J.L. and H.R. Adams, 1993, Selective inhibition of endothelium-dependent vasodilator capacity by *Escherichia coli* endotoxemia, Circ. Res. 72, 539.

Pearson, J.D., 1993, The control of production and release of haemostatic factors in the endothelial cell, Ballière's Clin. Haematol. 6, 629.

Poole, A.W., 1992, Responses of equine blood platelets induced by adenosine 5′-diphosphate, PhD Thesis (University of Cambridge, Cambridge).

Poole, A.W., M.F. Heath and R.J. Evans, 1993, ADP induces desensitisation of equine platelet aggregation responses: studies using ADPβS, a stable analogue of ADP, Res. Vet. Sci. 54, 235.

Rowe, M.I., M.B. Marchildon, A. Arango, T. Malinin and M.A. Gans, 1978, The mechanisms of thrombocytopenia in experimental gram-negative septicemia, Surgery 84, 87.

Scarlett, J.A., 1995, Platelet-activating factor in the horse, PhD Thesis (University of Cambridge, Cambridge).

Siess, W., 1989, Molecular mechanisms of platelet activation, Physiol. Rev. 69, 58.

Stewart, A.G. and W.A. Phillips, 1989, Intracellular platelet-activating factor regulates eicosanoid generation in guinea-pig resident peritoneal macrophages, Br. J. Pharmacol. 98, 141.

Terashita, Z.-I., Y. Imura, K. Nishikawa and S. Sumida, 1985, Is platelet activating factor (PAF) a mediator of endotoxin shock?, Eur. J. Pharmacol. 109, 257.

Zimmerman, G.A., T.M. McIntyre, M. Mehra and S.M. Prescott, 1990, Endothelial cell-associated platelet-activating factor: a novel mechanism for signaling intercellular adhesion, J. Cell Biol. 110, 529.