Comparative Effects of Phenylbutazone, Naproxen and Flunixin Meglumine on Equine Platelet Aggregation and Platelet Factor 3 Availability *in vitro*

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ABSTRACT

Nonsteroidal anti-inflammatory drugs are commonly used in the treatment of inflammatory conditions, and have potential value in the treatment of thrombotic disease in the horse. This study compares the potency of three nonsteroidal anti-inflammatory drugs phenylbutazone, naproxen (equiproxen) and flunixin meglumine (banamine) with respect to their effects on equine platelets. Two functional responses of horse platelets were evaluated *in vitro*: their ability to aggregate and their ability to make available platelet factor 3 procoagulant activity.

Flunixin at a concentration of 10^{-6} M significantly depressed the maximum degree of adenosine diphosphate-induced (10^{-6}M) aggregation while much higher concentrations of phenylbutazone and naproxen (5×10^{-5}M) were required to produce similar effects. None of the nonsteroidal anti-inflammatory drugs significantly affected the duration of the lag phase or the initial velocity of adenosine diphosphate-induced aggregation within the range of drug concentrations used (10^{-6}-10^{-3} M). The lag phase and initial velocity of acid-soluble collagen-induced aggregation were significantly affected by 10^{-6} M flunixin and 10^{-4} M phenylbutazone or naproxen was required to produce equivalent effects. Concentrations of 5×10^{-6} M flunixin and 5×10^{-4} M phenylbutazone or naproxen were required to significantly depress the degree of collagen-induced aggregation of horse platelets.

Although the effects of the nonsteroidal anti-inflammatory drugs were qualitatively similar, flunixin was a much more potent inhibitor of platelet aggregation than either of the other two drugs (which were equipotent). At very high drug concentrations (5×10^{-4} M and greater), all three drugs produced the same degree of inhibition of equine platelet aggregation.

Platelet factor 3 activity was made available by exposing horse platelets to 10^{-5} M adenosine diphosphate or 1:800 acid-soluble collagen; but not by exposure to a suspension of kaolin particles. Only a small portion of the total platelet factor 3 activity was made available on stimulation with either adenosine diphosphate or collagen. Pretreatment of horse platelets with any of the nonsteroidal anti-inflammatory drugs (10^{-4} M concentration) had no significant effect on adenosine diphosphate or collagen-induced platelet factor 3 availability.

Key Words: Nonsteroidal anti-inflammatory drugs, equine platelets, platelet aggregation, platelet factor 3 availability.

RÉSUMÉ

On utilise fréquemment des drogues anti-inflammatoires autres que des stéroïdes pour traiter des conditions inflammatoires; ces drogues offrent aussi une valeur potentielle pour le traitement de la maladie thrombotique du cheval. Cette expérience visait à comparer les possibilités de trois de ces drogues, à savoir: la phénylbutazone, le naproxen, ou équiproxen, et la méglumine de flunixine, ou banamine, relativement à leurs effets sur les plaquettes du cheval. On étudia *in vitro* les deux réponses fonctionnelles suivantes de ces plaquettes: leur habilité à former des agrégats et à rendre disponible l'activité procoagulante du facteur #3 plaquettaire.

Une concentration de 10^{-6} M de flunixine déprima de façon appréciable le degré maximum de l'agrégation provoquée par 10^{-6} M d'adénosine-diphosphate, tandis qu'il fallut des concentrations beaucoup plus élevées de phénylbutazone et de naproxen (5×10^{-5} M) pour produire des effets similaires. Ni l'une ni l'autre des trois drogues expérimentales n'affecta de façon appréciable la durée de la phase de délai ou la vélocité initiale de l'agrégation provoquée par l'adénosine-diphosphate, à l'intérieur des concentrations qu'on utilisa, i.e. 10^{-3}-10^{-6} M. La phase de délai et la vélocité initiale de l'agréga-

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tion provoquée par le collagène soluble dans l'acide s'avérèrent affectées de façon significative par 10^{-6} M de flunixine. Il fallut 10^{-6} M de phénylbutazone ou de naproxen pour produire des effets équivalents. Il fallut utiliser 5×10^{-6} M de flunixine et 5×10^{-4} M de phénylbutazone ou de naproxen pour déprimer de façon significative le degré d'agrégation des plaquettes équines provoqué par le collagène.

Même si les effets des trois drogues expérimentales se révélèrent qualitativement similaires, la flunixine s'avéra un inhibiteur de l'agrégation plaquettaire beaucoup plus puissant que l'une ou l'autre des deux autres drogues, lesquelles affichèrent par ailleurs une puissance égale. À des concentrations aussi élevées que 5×10^{-4} M ou plus, les trois drogues produisirent le même degré d'inhibition de l'agrégation plaquettaire équine.

L'activité du facteur #3 plaquettaire devint disponible en exposant les plaquettes équines à 10^{-5} M d'adénosine-diphosphate ou à 1:800 de collagène soluble dans l'acide, mais non en les exposant à une suspension de particules de kaolin. Seulement une petite partie de l'activité totale du facteur #3 plaquettaire devint disponible, à la suite d'une stimulation par l'adénosine-diphosphate ou le collagène. Un traitement préalable des plaquettes équines avec l'une ou l'autre des trois drogues expérimentales, à la concentration de 10^{-4} M, n'exerça aucun effet appréciable sur la disponibilité du facteur #3 plaquettaire provoquée par l'adénosine-diphosphate ou le collagène.

Mots clés: drogues anti-inflammatoires non stéroïdes, plaquettes équines, agrégation plaquettaire, disponibilité du facteur #3 plaquettaire.

INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAID) inhibit prostaglandin synthesis in many biological systems, including blood platelets (1, 2, 3). The production of specific prostaglandins, particularly endoperoxides and thromboxane A_2 (TXA_2), plays an important role in platelet aggregation (4, 5). The NSAID impair the production of these prostaglandins by interfering with the arachidonate pathway (6). Drugs which markedly affect platelet function predispose to impairment of the hemostatic response.

Nonsteroidal anti-inflammatory drugs are widely used in veterinary medicine particularly in the treatment of inflammatory disease in the equine species. Unlike the platelets of some species (man and dog), horse platelets appear to have a much more poorly developed arachidonate pathway (7). Meyers *et al* (8) have shown that the aggregation of horse platelets is partially depressed by phenylbutazone although the skin bleeding time was not significantly increased. Aspirin, another NSAID, has been shown to prolong the skin bleeding time in horses (9).

The purpose of this study was to compare the relative potency of the most commonly used NSAID, phenylbutazone, to that of two newer NSAID, naproxen and flunixin meglumine (banamine), with respect to their effects on horse platelet reactivity *in vitro*. Although therapeutic concentrations of NSAID may not significantly impair hemostasis in healthy animals, they may cause a significant clinical bleeding tendency in animals with an already compromised hemostatic response (due to other disease). Certain NSAID, because of their antiplatelet activity, may be useful in the treatment of thrombotic conditions which are frequently encountered in the equine species (9). Our results could prove useful in selecting an NSAID with high/low platelet-inhibitory activity.

Two different functional responses of platelets were studied: the ability of horse platelets to aggregate and the ability of horse platelets to make available platelet factor 3 (PF 3) procoagulant activity. Platelet aggregation responses to adenosine diphosphate (ADP) and collagen were studied since ADP and collagen are generally considered to be important stimulants of platelet clumping *in vivo*. This ability of platelets to aggregate *in vivo* is critical in the response of platelets at sites of vascular injury and in the subsequent formation of the primary hemostatic plug (10). Platelets must also be capable of making available, when stimulated, PF 3 activity which acts as a catalyst in fibrin production thus contributing to the stabilization of the primary platelet plug (11). Impaired PF 3 availability could also contribute to defective hemostasis.

MATERIALS AND METHODS

The animals used in this study were clinically normal adult Standardbred horses housed at the University of Guelph. They had received no drugs for at least two weeks prior to use.

Blood was collected by jugular puncture using 16 gauge needles and plastic syringes. Blood was immediately dispensed into siliconized glass tubes containing 1/9 volume of 3.8% trisodium citrate. Each tube was sealed with Parafilm (American Can Co., Neenah, Wisconsin) and gently tilted several times to ensure complete mixing of the blood and anticoagulant. All glassware used in the handling of blood or plasma was siliconized and all procedures were conducted at room temperature unless otherwise indicated.

PREPARATION OF PLATELET-RICH PLASMA (PRP), PLATELET-POOR PLASMA (PPP), AND PLATELET-FREE PLASMA (PFP)

Citrated PRP was obtained by centrifuging the blood at $70 \times g$ for 10 min. The upper 2/3 of the PRP was carefully removed, and the remainder of the blood centrifuged at $2510 \times g$ for 15 min to obtain PPP. The PFP was obtained by centrifuging PPP for a further 20 min at $12350 \times g$. No platelets

were detected in samples of PFP examined with phase-contrast microscopy.

Platelet counts of the PRP were adjusted with PPP to 200×10^9/L for aggregometry and with PFP to 100×10^9/L for PF 3 availability studies. The PRP was stored in sealed aliquots at room temperature and was used within four hours of collection.

REAGENTS AND DRUGS

Adenosine diphosphate (Sigma Chemical Co., St. Louis, Missouri) was prepared as a 10^{-2} M stock solution in tris-buffered saline (TBS), pH 7.4. Acid-soluble bovine collagen (ASC) was prepared according to Cazenave *et al* (12) as a 0.25% stock solution. Kaolin (Baker Chemical Co., Phillipsburg, New Jersey) was suspended in TBS at a concentration of 50 mg/mL and calcium chloride (Fisher Scientific, Fair Lawn, New Jersey); was prepared as an M/40 stock solution. Russell's viper venom (Wellcome Research Lab., Beckenham, England) was used at a concentration of 10 μg/mL in 0.85% sodium chloride. Phenylbutazone (Sigma Chemical Co., St. Louis, Missouri), naproxen (Syntex, Palo Alto, California) and flunixin (Schering Corp., Pointe Claire, Quebec) were prepared as 10^{-2} M stock solutions and either used fresh or stored up to two weeks at -60°C. Dilutions of the NSAID stock solutions were in TBS.

PLATELET AGGREGOMETRY

Platelet aggregometry was studied using a dual channel aggregometer module model 300 BD and chart recorder model SPHSV (Payton Associates, Scarborough, Ontario). The aggregometer was calibrated for minimal and maximal light transmission using PRP and PPP respectively (13).

The method for studying platelet aggregation responses was as follows: 0.20 mL of PRP was stirred for 1 min at 900 RPM and 37°C in the aggregometer cuvette to establish a base line tracing; 0.025 mL of a NSAID or its diluent was then added. After a 5 min incubation period with stirring, 0.025 mL of the appropriate aggregating agent was added. The final concentrations of the aggregating agents were ADP 10^{-6} M and ASC (stock) 1:1000. The final concentrations of the NSAID ranged from 10^{-3} M to 10^{-6} M.

Platelet aggregation responses were quantitated using three parameters: the duration of the lag phase, the initial velocity of aggregation and the maximum degree of aggregation (14, 15). The lag phase (LP) represented the time in seconds from the addition of the aggregating agent until the initial onset of platelet aggregation as indicated by the first evidence of an increase in light transmission following the addition of the aggregating agent. The initial velocity of aggregation (A_v) was determined by drawing a tangent through the steepest linear part of the aggregation tracing. The slope of this tangent was expressed in chart units/min. If a biphasic aggregation response was observed, only the slope of the primary wave was determined. The maximum degree of aggregation (A_m) was determined by measuring the maximum height of the aggregation wave over a 3 min period beginning at the onset of platelet aggregation (end of the lag phase). The maximum degree of aggregation was expressed in chart units and as a percent of the maximum possible change in light transmission. The degree of inhibition of A_v and A_m at each drug concentration was expressed as a percentage of the untreated control value.

PLATELET FACTOR 3 AVAILABILITY

Platelet factor 3 availability was determined according to Spaet and Cintron (16). Mixtures consisting of 9 vol PRP and 1 vol of kaolin (5 mg/mL final concentration) suspension, ADP (10^{-5} M final concentration), ASC (1:800 final concentration), or their diluent were stirred in aggregometer cuvettes at 300 RPM for 5 min. At the end of this incubation period, 0.1 mL of the mixture was pipetted into each of two tubes containing 0.1 mL Russell's viper venom (RVV) and 0.1 mL calcium chloride already warmed to 37°C. The clotting times were recorded using a manual "tilt tube" technique. The clotting times for 100% PF 3 availability were determined using PRP and PFP that had been freeze-thawed five times (17, 18).

In order to determine the possible effects of ADP, collagen, kaolin, and 5× freeze-thawing on the RVV clotting time in the absence of platelets, the procedures were repeated using PFP (shown to be essentially free of platelets) instead of PRP in the incubation mixture.

Platelet factor 3 availability was expressed as a ratio of the clotting time in the PRP to the clotting time of the corresponding PFP treated in the same way. The use of PF 3 availability ratios for expressing PF 3 availability eliminated the effects of variations in the clotting times of individual animal PFP (18). A PF 3 ratio of 1 indicates that there is no shortening of the RVV clotting time in PRP compared to the PFP; in other words PF 3 activity is not made available by the platelets. The PF 3 ratio obtained with freeze-thawed PRP/PFP gives an indication of the anticipated ratio for 100% PF 3 availability.

The effects of the NSAID on PF 3 availability were studied using an identical protocol except that the PRP and PFP were incubated with one of the drugs for 5 min at 37°C prior to the addition of the ADP or collagen. Each of the NSAID drugs was used at a final concentration of 10^{-4}M.

STATISTICAL ANALYSIS

The results of this study were expressed as the mean ± standard deviation of the mean. Statistical comparisons between untreated and a drug-treated group were performed by Student's t test. A p value of < 0.05 was considered to be statistically significant.

RESULTS

ADP AND COLLAGEN-INDUCED AGGREGATION OF UNTREATED HORSE PLATELETS

Typical aggregation responses of horse platelets to 10^{-6}M ADP are shown in Fig. 1 (upper tracing of each set). The aggregation responses to 10^{-6}M ADP were irreversible and on occasion, distinctly biphasic. The lag phase was short and there was a transient decrease in light transmission following the addition of the ADP. This transient change is generally considered to reflect a change in platelet shape (13, 20).

TABLE I. Quantitative Parameters for ADP and Collagen-Induced Aggregation of Untreated Horse Platelets

	ADP 10^{-6} M (21)[a]	ASC 1:1000 (21)
Lag phase(s)	8 ± 1[b]	46 ± 5
Initial velocity of aggregation (chart units/min)	21.8 ± 4.0	23.3 ± 3.7
Maximum aggregation in 3 min (chart units)	23.3 ± 3.7	26.7 ± 4.4
(% of maximum possible change in optical density)	64.3 ± 10.6	70.3 ± 12.5

[a](n)
[b]Mean ± 1 SD

Typical aggregation responses to collagen (1:1000) are shown in Fig. 2 (upper tracing of each set). Collagen-induced aggregation was characterized by a much longer lag phase but, as with ADP, irreversible aggregation.

The calculated values for LP, A_v and A_m are shown in Table I.

EFFECTS OF THE NSAID ON ADP-INDUCED PLATELET AGGREGATION

Typical effects of the NSAID on ADP-induced aggregation of horse platelets are shown in Figs. 1 and 3. None of the drugs, at the concentrations used had any significant effect on the LP or A_v of ADP-induced aggregation (i.e. the first phase of platelet aggregation).

The A_m of ADP-induced aggregation was significantly depressed by all three NSAID and high concentrations of these drugs produced reversible platelet aggregation (Figs. 1 and 3). Flunixin was very potent in this respect, producing a significant and nearly max-

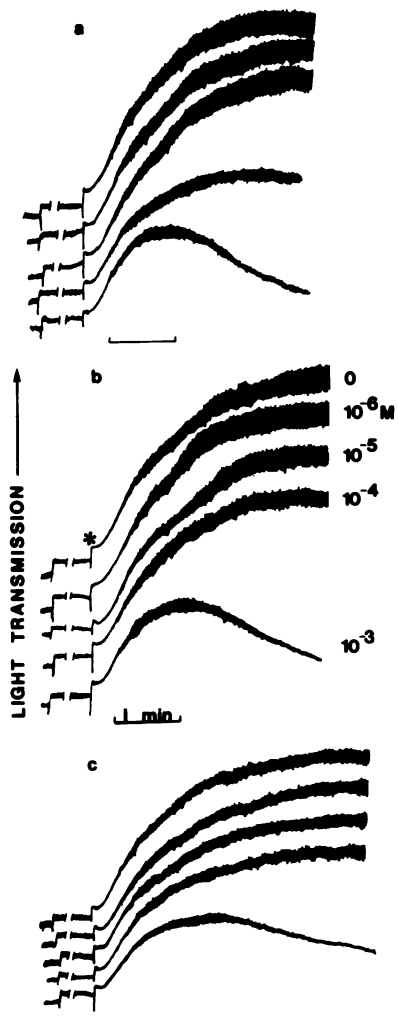

Fig. 1. Typical tracings illustrating the effect of a) phenylbutazone, b) naproxen and c) flunixin on ADP-induced aggregation of horse platelets. The asterix indicates the addition of the aggregating agent (10^{-6} M ADP) after a 5 min incubation period with the appropriate NSAID. The final drug concentrations given in b) also apply to a) and c).

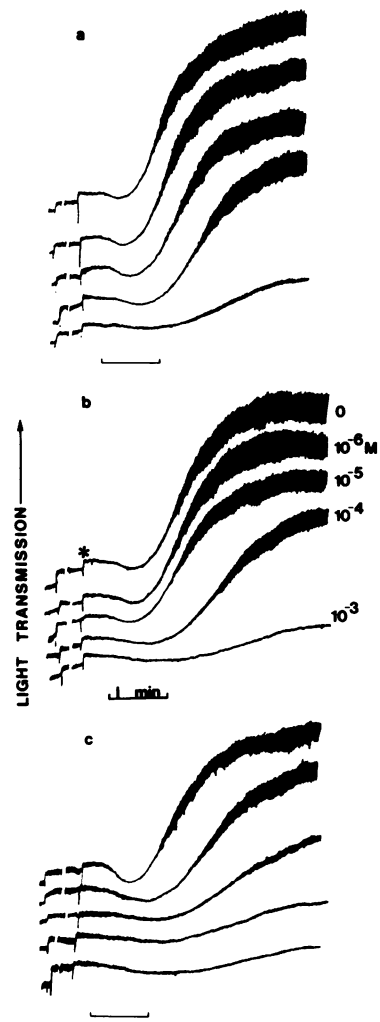

Fig. 2. Typical tracings illustrating the effect of a) phenylbutazone, b) naproxen and c) flunixin on collagen-induced aggregation of horse platelets. The asterix indicates the addition of the aggregating agent (1:1000 ASC) after a 5 min incubation period with the appropriate NSAID. The final drug concentrations given in b) also apply to a) and c).

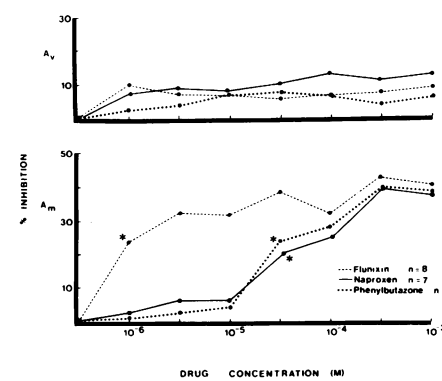

Fig. 3. Percent inhibition of ADP-induced aggregation of horse platelets by phenylbutazone, naproxen and flunixin (Mean values). A_v = initial velocity of aggregation; A_m = maximum degree of aggregation in 3 min. Asterix signifies the *lowest* drug concentration at which there is significant inhibition.

imal depression in A_m at the lowest concentration of the drug used (10^{-6} M). Naproxen and phenylbutazone were equipotent producing significant depression of ADP-induced A_m at concentrations of 5×10^{-5} M. At higher concentrations, the degrees of depression of A_m induced by naproxen and phenylbutazone were equal to that produced by flunixin.

EFFECTS OF THE NSAID ON COLLAGEN-INDUCED PLATELET AGGREGATION

Each of the NSAID studied had significant effects on collagen-induced aggregation of horse platelets; the drugs however were not all equipotent.

Flunixin markedly prolonged the LP of collagen-induced aggregation by an average of 20 s at the lowest concentration used (10^{-6} M). Higher concentrations of phenylbutazone and naproxen (10^{-5} and 5×10^{-5} M respectively) were necessary to significantly prolong the LP of the aggregation response induced by collagen. At concentrations of 5×10^{-4} and higher, all three NSAID prolonged the lag phase by an average of 35 s.

Flunixin significantly reduced A_v of the aggregation response induced by collagen at even the lowest drug concentration used (Fig. 4). There was a further dose-related reduction in A_v as the concentrations of flunixin were increased to 5×10^{-5} M; at this concentration, the degree of inhibition of A_v appeared to be maximal (Fig. 4). Phenylbutazone and naproxen produced significant inhibition of collagen-induced A_v at drug concentrations of 10^{-5} M and 5×10^{-5} M respectively; however, the degrees of inhibition were significantly less than those observed with flunixin. With increased concentrations of naproxen and phenylbutazone, the degree of inhibition of A_v increased to a degree comparable with flunixin.

The A_m of collagen-induced aggregation was significantly inhibited by flunixin at a 5×10^{-6} M concentration of the drug. Maximal inhibition of A_m was attained with a flunixin concentration of 10^{-4} M. Phenylbutazone and naproxen were equipotent with respect to their effects on collagen-induced A_m. Only at high concentrations (5×10^{-4} M and higher) did they significantly depress A_m.

In summarizing the effects of the NSAID drugs on ADP and collagen-induced aggregation, flunixin is considerably more potent than the other drugs with respect to its inhibitory effects on equine platelet aggregation. Phenylbutazone and naproxen are essentially equipotent in this respect.

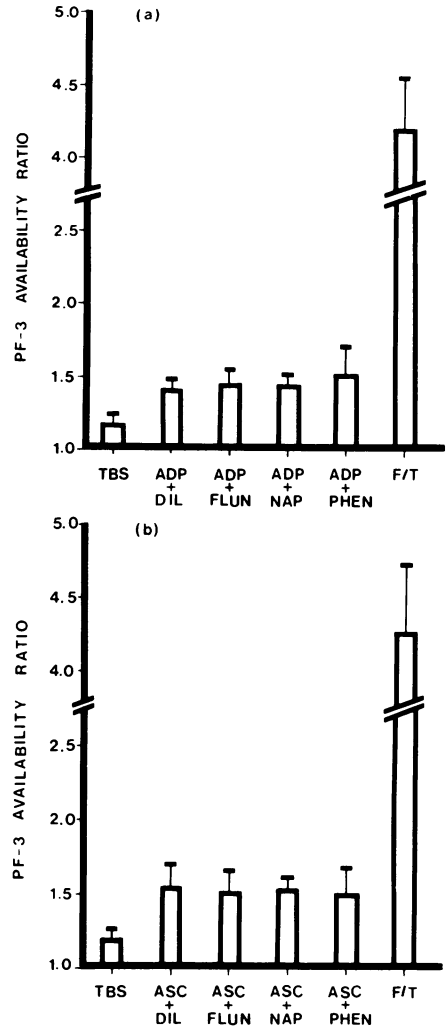

Fig. 4. Percent inhibition of collagen-induced aggregation of horse platelets by phenylbutazone, naproxen and flunixin (Mean values). A_v = initial velocity of aggregation; A_m = maximum degree of aggregation in 3 min. Asterix signifies the *lowest* drug concentration at which there is significant inhibition.

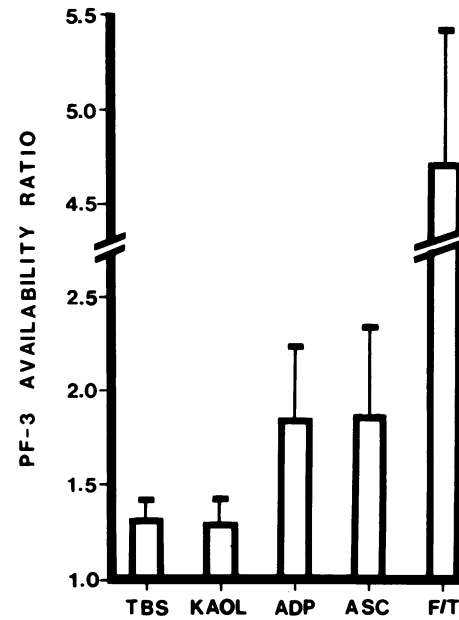

Fig. 5. Platelet factor 3 availability ratios for horse platelets exposed to kaolin (Kaol), adenosine diphosphate (ADP) acid-soluble collagen (ASC) or their diluent (TBS). Ratio for freeze-thawed platelets (F/T) represents 100% PF 3 availability (Mean ± 1 SD; n = 5).

PF 3 AVAILABILITY IN UNTREATED HORSE PLATELETS

Platelet factor 3 availability was determined by the ability of "stimulated" platelets to shorten the RVV time of plasma.

The PF 3 availability ratios shown in Fig. 5 indicate that some PF 3 activity (PF 3 ratio = 1.317) is made available from control horse platelets, suggesting that the preparation, handling and/or the stirring of the platelets is sufficient to cause some PF 3 availability. Compared to this "basal release" of PF 3 activity however, exposure of platelets to ADP or collagen increased the availability of PF 3 as indicated by the significant increase in the PF 3 availability ratio ($p<0.02$ and $p<0.05$ respectively). Kaolin particles did not increase PF 3 availability compared to control (TBS exposed) platelets. One hundred percent PF 3 availability was represented by a PF 3 availability ratio of 4.725.

It was determined that under the conditions of this technique for determining PF 3 availability, neither ADP, collagen, kaolin or freeze-thawing produced any sig-

nificant changes in the clotting times of individual PFP.

EFFECTS OF NSAID ON PF 3 AVAILABILITY

The PF 3 availability ratios for PRP/PFP exposed to various NSAID then stimulated with either ADP (10^{-5} M) or ASC (1:800) are shown in Figure 6. The results indicate that none of the drugs, at concentrations of 10^{-4} M, had any significant effect on PF 3 availability from horse platelets as induced by stimulation with ADP or collagen.

DISCUSSION

Our aggregometric studies on untreated horse platelets are in general agreement with similar studies on normal horse platelets conducted by Sinakos and Caen (21), Calkins *et al* (22), and more recently Meyers et al (23). Equine platelets, like those of many mammals, aggregate in response to either ADP or collagen, producing typical platelet aggregation profiles (24). Like Meyers *et al* (23), we observed a biphasic irreversible aggregation response in PRP from some horses when stimulated with 10^{-6} M ADP. However, in most platelet preparations, a monophasic irreversible aggregation response was observed. Low concentrations of collagen consistently produced irreversible aggregation following a long delay period. This long lag phase is typical of collagen-induced aggregation in many species (24).

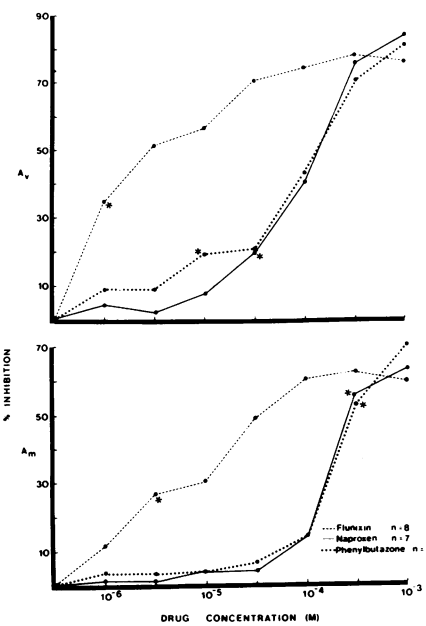

Fig. 6. Platelet factor 3 availability ratios for horse platelets stimulated with a) ADP 10^{-5} M, or b) acid-soluble collagen (ASC) 1:800, after exposure to flunixin, naproxen or phenylbutazone (final concentrations 10^{-4} M) or their diluent. Control platelets were stimulated with buffer (TBS) or were repeatedly freeze-thawed (F/T) (Mean ± 1 SD; n = 5).

All of the NSAID studied produced qualitatively similar effects on the aggregation of horse platelets. The drugs differed in their relative potency. The range of NSAID concentrations used in this study was selected in order to include concentrations that might be anticipated in plasma following therapy with these drugs. It has been shown that following therapeutic doses of phenylbutazone, naproxen and flunixin, peak plasma levels in the horse are likely to reach approximately 30 μg/mL (10^{-4} M), 25 μg/mL (10^{-4} M) and 1.5 - 3.0 μg/mL (about 5 x 10^{-6} M) respectively (8, 25).

Adenosine diphosphate is thought to initiate platelet aggregation through a direct interaction with specific receptors on the platelet surface to induce intracellular calcium flux and subsequent platelet contraction (5, 26). The aggregation response is then enhanced by secretion of products of arachidonic acid metabolism and by ADP release (11, 13, 20, 27). Nonsteroidal anti-inflammatory drugs such as phenylbutazone and naproxen interfere with the arachidonate pathway and platelet secretion (2, 28, 29).

Our studies using horse platelets indicated that the LP and A_v of ADP-induced aggregation were not affected by any of the NSAID over the wide range of concentrations used. This suggested that the primary response to ADP was normal in the treated horse platelets. The maximum degree of ADP-induced aggregation (A_m) was significantly depressed by all three NSAID suggesting that the secondary response had been inhibited. This lack of a secondary response was further substantiated by the reversible aggregation response observed with the higher concentrations of all these drugs. Our observations with phenylbutazone are in agreement with results published by Meyers *et al* (8) in which they described the effects of 33 μg/mL phenylbutazone (approximately 10^{-4} M) on the ADP-induced aggregation response of horse platelets. Naproxen and phenylbutazone were equipotent with respect to their effects on ADP-induced aggregation of equine platelets; flunixin was much more potent.

In this study all of the NSAID markedly affected collagen-induced aggregation of horse platelets; their potency however varied. Flunixin produced significant effects at relatively lower concentrations than either phenylbutazone or naproxen. The latter drugs were nearly equipotent.

The long lag phase seen with collagen-induced aggregation is thought to reflect the time required for structural changes in the collagen to occur and for platelets to adhere to the collagen (11, 14). According to Henry (20), platelets will adhere to the primary structure of collagen monomers but polymer collagen must form if aggregation is to occur. In human platelets, the generation of TXA_2 through the arachidonate pathway plays an important role in collagen-induced aggregation and release, and it is likely that the release phenomenon is necessary for maximal aggregation (20, 27, 28). In fact, with low concentrations of collagen not all platelets will react with collagen directly and a large part of the aggregation response will depend on the second order effect of substances released from the collagen-stimulated platelets (27).

Our results indicate that it is the LP and A_v that are first affected by NSAID and that higher concentrations of the drugs are necessary to reduce A_m. Our results are in agreement with observations by Meyers *et al* (8) who studied the

effects of 33 μg/mL phenylbutazone on the collagen-induced aggregation of horse platelets, and suggest that the three NSAID may interfere with the structural changes of the collagen and/or the adhesion of the horse platelets to collagen. Naproxen and phenylbutazone have both been shown to inhibit the adhesion of human platelets to collagen (5, 30).

The progressive reduction in A_v (and to a lesser extent A_m) when horse platelets are treated with increasing concentrations of these NSAID may reflect a progressive suppression of prostaglandin synthesis and platelet secretion. We, like others (8) observed that, even with high concentrations of the NSAID, collagen-induced aggregation in equine PRP was never totally inhibited. There is now evidence that collagen-induced aggregation may be mediated, at least in part, by nonthromboxane dependent mechanisms (8, 26). Such mechanisms would not likely be inhibited by NSAID. They may be particularly important in horse platelets where the arachidonate pathway is not well developed (7).

Platelet factor 3 activity is a property of platelets which represents their contribution to the interaction of factors X, V, and IV in the activation of prothrombin. This procoagulant activity of platelets only becomes available when platelets are stimulated and this activation induces structural changes which can provide a physiological surface for optimal interaction and binding of clotting factors with enzymatic activity necessary for coagulation (18, 31). Platelet factor 3 activity can be measured by its ability to shorten the RVV (Stypven) time of plasma (13, 16, 32). A small amount of PF 3 activity was made available from horse platelets stirred with TBS. Similar observations have been made in studies using human platelets (33). We attributed the "basal release" to some activation of platelets due to the processing, handling, and possibly mechanical stirring of platelets in the aggregometer cuvette.

Adenosine diphosphate, kaolin and collagen have been shown to induce PF 3 availability in PRP from man and some animal species (17, 32, 33, 34, 35). Stirring the platelets with ADP or collagen resulted in a significant increase in the availability of PF 3 from the horse platelets. However, compared to the total PF 3 content of the platelets (as measured by repeatedly freeze-thawing the platelets), only a relatively small proportion of the total PF 3 activity was made available. This is consistent with studies on human platelets in which it was determined that usually less than 20% of the total PF 3 content of platelets was made available during activation (17, 36).

In this study, kaolin did not significantly increase the availability of PF 3 from horse platelets. The kaolin concentration was comparable to that used in studies in man, although a shorter incubation period was used (17). The shorter contact time (5 min) was not an important factor in these observations since other studies in our laboratory, using incubation periods of up to 20 min, still failed to produce a significant shortening of the RVV time of equine PRP. Physical adhesion of platelets to kaolin particles appears to be important in initiating platelet activation and PF 3 availability (16). It is possible that the failure of kaolin particles to induce PF 3 activity availability from horse platelets was somehow related to a failure of horse platelets to adhere to kaolin particles.

The results of our study of the effects of phenylbutazone, naproxen and flunixin on PF 3 availability indicate that none of these NSAID, at a concentration of 10^{-4} M, have any significant effect on the ability of horse platelets to provide this essential procoagulant activity when stimulated with either ADP or collagen. It is of particular interest that flunixin at this concentration, produced marked depression of collagen-induced platelet aggregation (compared to either naproxen or phenylbutazone) yet no inhibition of PF 3 availability.

The results of this *in vitro* study suggest that flunixin is a much more potent platelet-inhibitory drug than either naproxen or phenylbutazone, significantly depressing the ADP-and collagen-induced aggregation responses of horse platelets. None of these NSAID affect the ability of horse platelets to make available PF 3 activity when stimulated *in vitro* by either ADP or collagen.

Although the effects of drugs on platelets *in vitro* may not always be indicative of their effect *in vivo*, often there is a good correlation between the therapeutic level of the drug and the effectiveness in inhibiting platelet function *in vitro* (37). There is always the possibility of further effects from metabolites of the drug; these effects cannot be readily evaluated *in vitro*.

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REFERENCES

1. **SMITH JB, INGERMAN, C, KOCSIS JJ, SILVER MJ.** Prostaglandins in platelet function. Thromb Res 1974; 4: 49-55.
2. **ALI M, McDONALD JWD.** Reversible and irreversible inhibition of platelet cyclooxygenase and secretion release by nonsteroidal anti-inflammatory drugs. Thromb Res 1978; 13: 1057-1065.
3. **DAWSON, W.** Mechanisms of action of anti-inflammatory drugs. Adv Prostaglandin Thromboxane Res 1980; 8: 1741-1745.
4. **MONCADO S, VANE JR.** Prostaglandin, platelet aggregation and thrombosis. In: de Gaetano G, Garattini S, eds. Platelets: a multi-disciplinary approach. New York: Raven Press,

1978: 239-258.
5. **PHILP RB.** Methods of testing proposed antithrombotic drugs. Boca Raton: CRC Press Inc., 1981.
6. **FUSTER V, CHESBRO JH.** Antithrombotic therapy: Role of platelet-inhibitor drugs. II Pharmacologic effects of platelet-inhibitor drugs. Mayo Clin Proc 1981; 185-195.
7. **MEYERS KM, KATZ JB, CLEMONS RM, SMITH JB, HOLMSEN H.** An evaluation of the arachidonate pathway of platelets from companion and food producing animals, mink and man. Thromb Res 1980; 20: 13-24.
8. **MEYERS KM, LINDNER C, KATZ J, GRANT B.** Phenylbutazone inhibition of equine platelet function. Am J Vet Res 1979; 40: 265-270.
9. **JUDSON DG, BARTON M.** Effect of aspirin on haemostasis in the horse. Adv Vet Sci Comp Med 1981; 30: 241-242.
10. **McCOY LE.** Vascular function in hemostasis. In: Murano G, Bick RL, eds. Basic concepts of hemostasis and thrombosis. Boca Raton: CRC Press Inc., 1980: 1-15.
11. **SCHAFER AI, HANDIN RI.** The role of platelets in thrombotic and vascular disease. Prog Cardiovasc Dis 1979; 22: 31-52.
12. **CAZENAVE JP, PACKMAN MA, MUSTARD JF.** Adherence of platelets to a collagen-coated surface: development of a quantitative method. J Lab Clin Med 1973; 82: 978-981.
13. **DAY HJ.** Laboratory tests in platelet function. In: Seligson D, ed. Handbook series in clinical laboratory science. Section I: Hematology vol. 1. Boca Raton: CRC Press Inc, 1979: 329-347.
14. **COLLER BS.** Platelet aggregation by ADP, collagen and ristocetin: A critical review of methodology and analysis. In: Seligson, D., ed. Handbook series in clinical laboratory science. Section I: Hematology vol. 1. Boca Raton: CRC Press Inc, 1979: 381-396.
15. **JOHNSTONE IB, LOTZ F.** Ristocetin-Willebrand factor activity in dog plasma. Can J Physiol Pharmacol 1980; 58: 1128-1134.
16. **SPAET TH, CINTRON J.** Studies in platelet factor-3 activity. Br J Haemat 1965; 11: 269-275.
17. **HARDISTRY RM, HUTTON RA.** The kaolin clotting time of platelet-rich plasma: a test of platelet factor-3 availability. Br J Haemat 1965; 11: 258-268.
18. **SALEEM A, KRIEG AF, CIESLICA R.** Platelet activity ratio: a new approach for quantitation of platelet factor 3. Am J Clin Pathol 1975; 63: 68-73.
19. **ADLER HL, ROESSLER EB.** Introduction to probability and statistics. 5th ed. San Francisco: WH Freeman and Co, 1968.
20. **HENRY RL.** Platelet function in hemostasis. In: Murano G, Bick RL, eds. Basic concepts of hemostasis and thrombosis. Boca Raton: CRC Press Inc, 1980: 17-41.
21. **SINAKOS A, CAEN JP.** Platelet aggregation in the mammalian (human, rat, rabbit, guinea pig, horse, dog) — a comparative study. Thromb Diath haemorrh 1967; 17: 99-111.
22. **CALKINS J, LANE KP, LO SASSO B, THURBER LE.** Comparative study of platelet aggregation in various species. J Med 1974; 5: 292-296.
23. **MEYERS KM, LINDNER C, GRANT B.** Characterization of the equine platelet aggregation response. Am J Vet Res 1979; 40: 260-264.
24. **DODDS WJ.** Platelet function in animals: species specificities. In: de Gaetano G, Garattini S, eds. Platelets: a multidisciplinary approach. New York: Raven Press, 1978: 45-59.
25. **TOBIN T.** Pharmacology review: the nonsteroidal anti-inflammatory drugs. II Equiproxen, meclofenamic acid, flunixin and others. J Equine Med Surg 1979; 3:298-302.
26. **FUSTER V, CHESEBRO JH.** Antithrombotic therapy: Role of platelet-inhibitor drugs. I. Current concepts of thrombogenesis: role of platelets. Mayo Clin Proc 1981; 56: 102-112.
27. **CHARO IF, FEINMAN RD, DETWILER TC.** Interrelations of platelet aggregation and secretion. J Clin Invest 1977; 60: 866-873.
28. **PACKHAM MA, MUSTARD JF.** Clinical pharmacology of platelets. Blood 1977; 50: 555-573.
29. **McINTYRE BA, PHILP RB.** Effect of three nonsteroidal anti-inflammatory agents on platelet function and prostaglandin synthesis *in vitro*. Thromb Res 1977; 12: 67-76.
30. **CAZENAVE JP, PACKHAM MA, GUCCIONE MA, MUSTARD JF.** Inhibitors of platelet adherence to a collagen-coated surface by nonsteroidal anti-inflammatory drugs, pyrimidopyrimidine and tricyclic compounds, and lidocaine. J Lab Clin Med 1974; 83: 797-806.
31. **WALSH PN.** Laboratory evaluation of platelet coagulant activities. In: Seligson D, ed. Handbook series in clinical laboratory science. Section I: Hematology vol. 1. Boca Raton: CRC Press Inc., 1979: 403-407.
32. **WALSH PN.** Contributions of platelets to intrinsic coagulation. In: Seligson D, ed. Handbook series in clinical laboratory science. Section 1: Hematology vol. 1. Boca Raton: CRC Press Inc., 1979: 351-359.
33. **HOROWITZ HI, COHEN BD, MARTINEZ P, PAPAYOANOU MF.** Defective ADP-induced platelet factor 3 activation in uremia. Blood 1967; 30: 331-340.
34. **CORNELL CN, KAHN RA, COOPER RG, MUHRER ME.** Platelet factor 3 availability in bleeder swine. Comp Biochem Physiol 1972; 42: 817-822.
35. **DODDS WJ.** Canine von Willebrand's disease. J Lab Clin Med 1970; 76: 713-721.
36. **PEREZ-REQUEJO JL.** A standardized bioassay for platelet factor 3 released by kaolin. Br J Haemat 1976; 33: 39-51.
37. **GARATTINI S.** Platelet-drug interactions: an open field. In: de Gaetano G, Garattini S, eds. Platelets: a multidisciplinary approach. New York: Raven Press, 1978: 61-73.