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Dual effects of diclofenac on human platelet adhesion *in vitro*

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The effect of two non-steroidal anti-inflammatory drugs on the adhesion function of human platelets was evaluated. Platelets isolated from healthy human subjects were treated for 10 min with the indicated drugs and then incubated in fibrinogen-coated microwell plates in the absence or in the presence of ADP (10 μM) and thrombin (0.05 U/ml). After 1 h of incubation, adherent platelets were measured using an enzymatic assay. ADP- and thrombin-stimulated adhesion was significantly inhibited by high doses (> 500 μM) of diclofenac, while doses ranging from 50 to 300 μM stimulated adhesion in the absence of agonists (resting platelets). A similar stimulatory effect on platelet adhesion was observed also with 200–500 μM flurbiprofen. Moreover, immunocytofluorimetry demonstrated that diclofenac dose-dependently (100–500 μM) induced the expression of GMP-140 and increased the expression of GPIIb/IIIa on the membrane of unstimulated platelets. High doses (> 500 μM) of this drug inhibited thrombin-stimulated expression of GPIIb/IIIa and GMP-140.

Key words: Diclofenac, flurbiprofen, platelet adhesion, GPIIb/IIIa, GMP-140, cyclo-oxygenase, non-steroidal anti-inflammatory drugs.

Introduction

Most of the non-steroidal anti-inflammatory agents have been reported to inhibit platelet aggregation by blocking cyclo-oxygenase and thereby preventing endogenous release of thromboxane A_2.[1] Less is known, however, on the regulation by these drugs of other platelet functional responses, i.e. adhesion to the vessel wall or fibrinogen-coated surfaces. Adhesion to the vessel wall may be regarded as the first and crucial step of the complex series of events by which platelets perform their functions in the haemostatic process, involving a series of plasma and subendothelial tissue components that specifically bind to several different membrane glycoproteins.[2] Physiological platelet agonists such as adenosine diphosphate (ADP), collagen and thrombin activate stimulus-response coupling pathways that may increase either the number or the ligand affinity of specific adhesion receptors.

During studies designed to investigate the sensitivity to anti-inflammatory drugs of platelet adhesion, we noted an unexpected stimulatory effect of diclofenac and flurbiprofen on this function. This stimulatory effect was associated with upregulation of GPIIb/IIIa and GMP-140, two relevant membrane adhesion glycoproteins.

Materials and methods

Human fibrinogen (type I), p-nitrophenyl phosphate, Triton X-100, diclofenac and flurbiprofen were purchased from the Sigma Chemical Company (St Louis, MO); ADP from Boehringer (Mannheim, Germany); thrombin from Calbiochem (La Jolla, CA); human albumin from Behring Institut (Marburg, Germany); PBS from Gibco Ltd (Paisley, UK). Sterile 96-well microtiter plates with flat-bottom wells (Linbro type) were from Flow Laboratories. Other materials and reagents were of the highest purity available.

Platelets were harvested from human blood by differential centrifugation. A final volume of 10 ml of blood were drawn by venipuncture in 1.66 ml of anticoagulant

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solution (15 g/l citric acid, 20 g/l dextrose, 25 g/l sodium citrate), and platelet-rich plasma was obtained by centrifugation at 300 ***g*** for 10 min. The platelet-rich plasma was re-centrifuged at 700 ***g*** for 15 min and platelets were gently suspended (10^8/ml) in a buffer composed by 145 mM NaCl, 5 mM KCl, 10 mM Hepes, 0.5 mM Na_2HPO_4, 6 mM glucose and 0.2% human serum albumin, pH 7.4 (buffer A). The platelet suspension was kept at room temperature and utilized within 1 h. Ten min before use, platelets were warmed up to 37°C.

A colorimetric procedure measuring the activity of acid phosphatase was used for the determination of the adhesion of human platelets to protein-coated culture microwells.[3] The 96-well microtiter plates were coated overnight with 0.2 mg/ml human fibrinogen in PBS and washed twice with physiological saline. Immediately after coating and washing, the wells were supplemented with 25 μl of the test drug (3 × the final desired concentration, in buffer A containing 2 mM $CaCl_2$ and 2 mM $MgSO_4$). Plates were then brought to 37°C, and 25 μl of the platelet suspension (2.5×10^6 platelets), pre-warmed at 37°C, were added to each well using a multichannel pipette. Platelets were incubated 10 min under static conditions in humidified thermostat (37°C, 5% CO_2), then 25 μl of the indicated stimulatory agents (thrombin or ADP, 3 × the final desired concentration, dissolved in buffer A supplemented with 1 mM $CaCl_2$ and 1 mM $MgSO_4$) were added to each well. The incubation was carried out for a further 60 min, then plates were transferred to the automatic washer (Easy Washer 2, SLT Labs Instruments) and subjected to two washing cycles with PBS at room temperature. After washing, the wells containing adherent platelets were rapidly supplemented with 150 μl of 0.1 M citrate buffer, pH 5.4, containing 5 mM p-nitrophenyl-phosphate and 0.1% Triton X-100. After incubation at room temperature for 60 min, the reaction was stopped and the color was developed by the addition of 100 μl of 2 N NaOH. The p-nitrophenol produced by the reaction was measured with a microplate reader (Reader 400, SLT Labs Instruments) at 405 nm against a platelet-free blank. The percentage of adherent cells was calculated on the basis of a standard curve obtained with defined number of platelets from the same donor.

Measurements of the expression of GPIIb/IIIa (CD41a) and GMP-140 (CD62) on the platelets were done by fluorescence-activated flow cytometry. Platelet suspensions (1.5×10^7/ml) were incubated 60 min at 37°C in buffer A (supplemented with 1 mM $CaCl_2$ and 1 mM $MgSO_4$) with the indicated concentration of drugs, then treated with thrombin 0.05 U/ml (activated platelets) or with buffer (resting platelets) for 10 min. The incubation was stopped with a 5 × excess of ice-cold buffer A and each sample was divided in two 60 μl aliquots, one of which was incubated with 6 μl of anti-human CD41a mAb (FITC) and with 6 μl of anti-human CD62 mAb (phycoerythrin), while the other with a corresponding amount of fluoresceinated and phycoerythrinated control IgG1 mAbs. Treatment with mAbs was done for 20 min at 4°C and the fluorescence was measured with a Becton Dickinson FACScan equipped with a 488 nM argon laser. The analysis was made on 10 000 events in each sample, using the software Lysis-II.

Data were analyzed using Student's *t*-test for unpaired data. Results with a $P < 0.05$ were regarded as significant.

Results

Figure 1A shows adhesion to fibrinogen coated wells of platelets and the effect of increasing doses of diclofenac. In the absence of drug, less than 6% platelets adhered to the microwells, while a marked increase of adhesion was induced by ADP and thrombin. Agonist-stimulated adhesion was significantly inhibited only by high doses

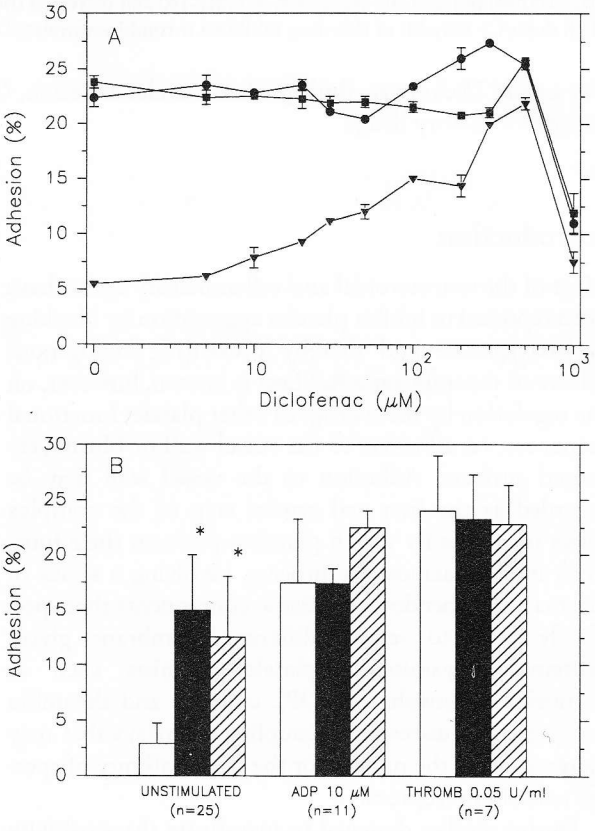

Figure 1. Effects of anti-inflammatory agents of platelet adhesion. (A) platelet adhesion and the effect of increasing doses of diclofenac in the absence of stimulants (▼) and in the presence of 10 μM ADP (●) or 0.05 U/ml thrombin (■). (B) mean results of a series of experiments. Open bars: no stimulants; solid bars: + diclofenac (peak stimulatory doses: 100–200 μM); hatched bars: + flurbiprofen (peak stimulatory doses: 200–500 μM). *$P < 0.001$. Values are expressed as mean ± SEM.

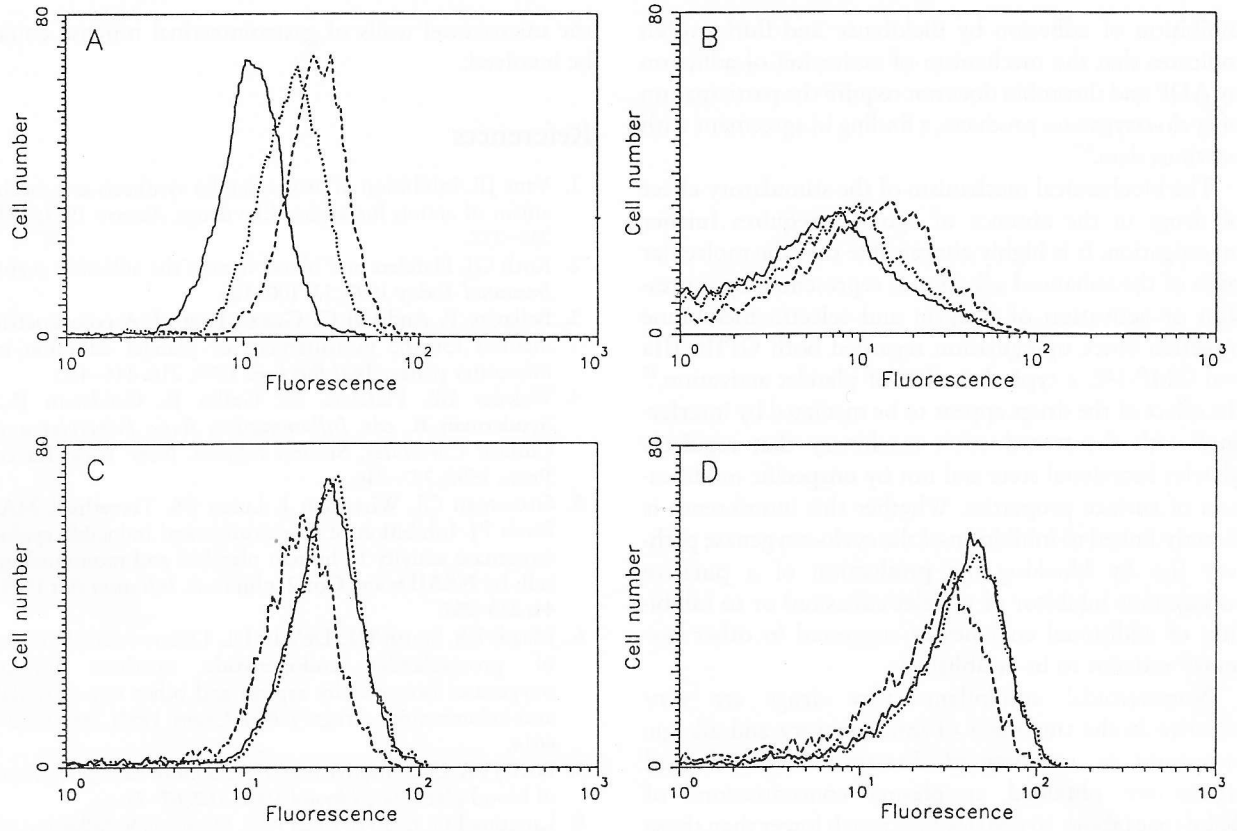

Figure 2. Typical GPIIb/IIIa (A: resting platelets; C: 0.05 U/ml thrombin-activated platelets) and GMP-140 (B: resting platelets; D: 0.05 U/ml thrombin-activated platelets) distribution histograms by immunocytofluorimetry. Solid line: no drug; dotted line: + 100 μM diclofenac; dashed line: + 500 μM diclofenac. In all experimental conditions, the fluorescence peaks of platelets treated with control IgG1 mAbs (not shown) was lower than 3.5 fluorescence units and was not modified by any dose of diclofenac nor by thrombin.

(> 500 μM) of diclofenac. At doses ranging from 50 to 300 μM, diclofenac was particularly active in stimulating adhesion of resting platelets. Figure 1B describes the results of a series of experiments, showing highly significant stimulatory effects of diclofenac on resting platelets. In addition, Figure 1B shows that similar stimulatory effects were found using flurbiprofen (200–500 μM). Higher doses (1 mM) of the latter drug markedly inhibited both resting and ADP- or thrombin-stimulated adhesion.

Immunocytofluorimetry demonstrated that diclofenac dose-dependently (100–500 μM) induced the expression of GPIIb/IIIa and GMP-140 on the membranes of unstimulated platelets (Figure 2A and B). High doses (> 500 μM) of diclofenac inhibited thrombin-induced expression of the two glycoproteins (Figure 2C and D).

Discussion

Human blood platelets circulate as resting packets of granules containing vasoactive materials. However, the plasma membrane of platelets is easily activated by many types of injurious or inflammatory stimuli. Since platelet activation is involved in a number of pathophysiological processes, including inflammation,[4] considerable efforts have been addressed to its pharmacological regulation.

Our results show that agonist-stimulated platelet adhesion is not inhibited by diclofenac and flurbiprofen at doses that are known to inhibit cyclo-oxygenase enzymes.[5,6] Control experiments done in our laboratory using conventional aggregometry[7] on the same platelet preparations used in adhesion assays showed that arachidonic-acid induced aggregation is markedly inhibited by 50–100 μM diclofenac. On the other hand, in the absence of agonists platelet adhesion was stimulated by diclofenac and flurbiprofen, a paradoxical effect that, to our knowledge, has never been described. Very high doses (up to 500–1000 μM) of the same drugs inhibited both spontaneous and agonist-stimulated adhesion.

Non-steroidal anti-inflammatory drugs have several pharmacologic activities. First of all, they are inhibitors of cyclo-oxygenase and thus inhibit the formation of prostaglandins and thromboxanes. However, lack of

inhibition of adhesion by diclofenac and flurbiprofen indicates that the mechanism of activation of adhesion by ADP and thrombin does not require the participation of cyclo-oxygenase products, a finding in agreement with previous data.[8,9]

The biochemical mechanism of the stimulatory effect of drugs in the absence of agonists requires further investigation. It is highly conceivable that the molecular basis of the enhanced adhesion is represented by expression or activation of integrin and selectin membrane moieties. Since upregulation regarded both GPIIb/IIIa and GMP-140, a typical marker of platelet activation,[10] the effect of the drugs appear to be mediated by interference with the transduction machinery that regulates platelet functional state and not by unspecific modification of surface properties. Whether this interference is directly linked to inhibition of the cyclo-oxygenase pathway (i.e. by blocking the production of a putative endogenous inhibitor of platelet adhesion) or to inhibition of additional enzymes as suggested in other systems[11] remains to be established.

Non-steroidal anti-inflammatory drugs are very effective in the treatment of inflammatory and allergic symptoms in a variety of diseases. Pharmacological effects are obtained at plasma concentrations of diclofenac (about 10 μM) that are much lower than those affecting platelet adhesion *in vitro*, indicating that the effects described in this report could not occur *in vivo* during standard anti-inflammatory therapy. However, patient compliance is often compromised by gastrointestinal side-effects associated with NSAIDs ingestion. Our results may provide a possible explanation for such toxicity, particularly when high concentrations are reached (i.e. in tissues where absorption occurs). Among the various mechanisms, increased platelet adherence to the microvessel walls of gastrointestinal mucosa could be involved.

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