Inflammation, Vol. 21, No. 5, 1997

STUDY ON PARADOXICAL EFFECTS OF NSAIDs ON PLATELET ACTIVATION

GIUSEPPE ANDRIOLI,[1] SABRINA LUSSIGNOLI,[1] STEFANIA GAINO,[1] GIUSEPPINA BENONI,[2] and PAOLO BELLAVITE[1]

[1]*Institute of Clinical Chemistry*
[2]*Institute of Pharmacology*
University of Verona, Italy

Abstract—We recently described a stimulatory effect of high doses (>100 μmol/L) diclofenac on platelet adhesion. In this study we extend our research to the possible biochemical mechanisms of the observed effects, to other non steroidal anti-inflammatory drugs (NSAIDs) (flurbiprofen, indomethacin, acetylsalicylic acid, ibuprofen, nitrofenac and nitroflurbiprofen) and to the effect of high doses diclofenac and flurbiprofen on platelet aggreation. We observed that high doses of diclofenac and of flurbiprofen, but not of the other tested NSAIDs, increased platelet adhesion at doses ranging from 100 to 500 μmol/L, an effect completely removed by the 12-lipoxygenase-inhibitor nordihydroguaiaretic acid. Moreover, they had no pro-aggregating effect, inhibiting platelet aggregation induced by 10 μmol/L arachidonic acid and dose-dependently increasing the $[Ca^{2+}]_i$. Finally, whereas no basal nitric oxide release by washed platelets was detected, when platelets were incubated by 500 μmol/L diclofenac or flurbiprofen, the production of nitric oxide, as measured by amounts of nitrite released, was 4.4 ± 0.5 and 3.8 ± 0.4 pmol/5 × 10^8 platelets/min, respectively. Our data indicate that high doses diclofenac and flurbiprofen are promoters of the early phases of platelet activation, probably through the 12-lipoxygenase pathway.

INTRODUCTION

The majority of patients with significant inflammatory processes, for instance rheumatic diseases, require either prolonged or recurrent treatment with non steroidal anti-inflammatory drugs (NSAIDs). However, the therapeutical use of these compounds is limited primarily by their capacity to produce unpleasant or frankly dangerous toxic side effects, the most frequent can be referred to the upper gastrointestinal lesions (1, 2). The pathogenesis of these side effects is not well understood. Much evidence supports the hypothesis that the major

0360-3997/97/1000-0519$12.50/0

therapeutic and toxic effects of NSAIDs are due to the inhibition of the enzyme cyclo-oxygenase: consequently, suppression of endogenous prostacyclin formation and subsequent reduction in mucosal blood flow has been proposed to be one of the most likely mechanisms underlying the tissue damage induced by NSAIDs (3). In recent years, it has become increasingly clear that neutrophils and neutrophil-derived factors must play an important role in the gastrointestinal damage induced by aspirin like-drugs (4, 5). Moreover, some observations suggest that inhibition of the lipoxygenase pathway prevent the high doses indomethacin-induced leukocyte adherence to vascular endothelium (6).

On the other hand, little is known about the effects of higher doses of NSAIDs on platelet physiology, above all as regards the early phase of platelet activation, and about the possible role played by these cells in the damages induced by these drugs. To our knowledge, it is reported an enhanced platelet deposition on the rabbit veins from animals treated with high doses acetylsalicylic acid as compared to animals treated with low doses acetylsalicylic acid. The authors referred this phenomenon to the reduced vessel wall PGI_2 production induced by high doses aspirin rather than a direct effect on platelet function (7).

In a preliminary report, we showed a stimulatory effect of high doses (>100 μmol/L) diclofenac on platelet adhesion in vitro associated with upregulation in the expression of two major adhesion glycoproteins, i.e. GPIIb/IIIa and GMP-140 (8). Since platelets have potential pro-inflammatory properties (9), this observation may have important implications for the unwanted side-effects of these widely used drugs.

With the present report we extended the study to the effects on platelet adhesion of other classical NSAIDs (flurbiprofen, indomethacin, acetylsalicylic acid, ibuprofen) and of new nitric oxide-releasing derivatives such as nitrofenac and nitroflurbiprofen. The latter drugs have been reported to have markedly reduced ulcerogenic properties in animal models (10, 11). Moreover, we tried to define the biochemical mechanism of the observed platelet activation. We investigated the effects of diclofenac and flurbiprofen on platelet intracellular free calcium ($[Ca^{2+}]_i$) as well as nitric oxide production and aggregation. Finally, these data are discussed with regard to the possible pathophysiological role of platelets in the toxicity by high doses of NSAIDs, i.e., gastrointestinal side effects.

MATERIALS AND METHODS

Platelet Preparation. Platelets were harvested from blood of healthy human volunteers, who had not been taking any drugs for at least 3 weeks, by differential centrifugation. A final volume of 30 mL of blood were drawn by venipuncture in 4.98 mL of anticoagulant 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 at the indicated concentrations in a buffer composed by 145 mmol/L NaCl, 5 mmol/L KCl, 10 mmol/L Hepes, 0.5 mmol/L Na_2HPO_4 and 6 mmol/L glucose, pH 7.4 (buffer A). When assay of adhesion was performed, 0.2% serum albumin was added to buffer A. The platelet suspensions were kept at room temperature and utilized within one hour. Ten min before use, platelets were warmed up to 37°C.

Assay of Adhesion. Platelet adhesion was evaluated as previously described (12). Briefly, sterile 96-well microtiter plates with flat-bottom wells (Linbro type) (Sigma Chemical Company) were coated overnight with 0.2 mg/mL human fibrinogen (type I) (Sigma Chemical Company) in Dulbecco's phosphate-buffered saline (PBS) (Gibco Ltd) and washed twice with physiological saline. Immediately after coating and washing, the wells were supplemented with 25 μL of the tested drugs (3 × the final desired concentration, in buffer A supplemented with 2 mmol/L $CaCl_2$ and 2 mmol/L $MgSO_4$). Diclofenac, flurbiprofen, indomethacin, ibuprofen and acetyl salicylic acid were purchased from Sigma Chemical Company; nitrofenac (diclofenac 4-nitroxybutylester) and nitroflurbiprofen (flurbiprofen 4-nitroxybutylester) were synthesized and kindly supplied by NICOX Ltd (London, UK). Plates were then brought to 37°C, and 50 μL of the platelet suspension (2.5×10^6 platelets), pre-warmed at 37°C, were added to each well using a multichannel pipette. Plates were incubated 40 min under static conditions in humidified thermostat (37°C, 5% CO_2) and then they 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 mol/L citrate buffer, pH 5.4, containing 5 mmol/L p-nitrophenyl-phosphate and 0.1% Triton X-100. After incubation at room temperature for 60 min, the reaction was stopped and the colour 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 platelets free blank. The percentage of adherent cells was calculated on the basis of a standard curve obtained with a defined number of platelets of the same donor.

Platelet Intracellular Free Calcium. The cytosolic calcium concentration was measured in platelets with a fluorescent indicator, Fura 2 (Calbiochem), as previously described by Pollock et al. (13). After loading with 2 μmol/L Fura 2 AM in platelet-rich plasma (PRP), the platelets were collected by centrifugation and suspended (2×10^7 platelets/mL) in buffer A. The external calcium and magnesium concentration was restored to 1 mmol/L. Platelet $[Ca^{2+}]_i$ variations induced by drugs were measured recording the fluorescence variations at 37°C by F-2000 fluorescence spectrophotometer (Hitachi), using 340 nm excitation wavelength and 500 nm emission wavelength.

Measurement of Platelet Nitrite Production. The fluorimetric method of Misko et al. (14), with some modifications, was used to measure nitrite generated by platelet as index of nitric oxide production. Washed platelets (5×10^8 platelets/mL) were incubated for 1 h at 37°C with or without the indicated drugs in the presence of exogenous 300 μmol/L L-arginine. After incubation, the samples were immediately centrifuged at 5600 g for 3 min and 900 μL of the reaction supernatant were collected; then, 900 μL of freshly prepared 2,3-diaminonaphthalene (0.05 mg/mL in 0.62 mol/L HCl) were added and mixed immediately. After 10 min incubation at +4°C, the reaction was stopped with 45 μL of 2.8 N NaOH. the fluorescence intensity was measured by the F-2000 fluorescence spectrophotometer, using 365 nm excitation wavelength and 450 nm emission wavelength.

Platelet Aggregation. Platelet aggregation was monitored by a standard nephelometric technique in which 0.5 mL of platelet suspension (2×10^8 platelets/mL) were incubated at 37°C and stirred at 1,000 rpm in a four-channel aggregometer (Aggrecorder II, Daiichi). Drugs- and agonist-induced aggregation was quantified by measuring the increase in light transmission (% T). Before the addition of a drug or an agonist, 1 mmol/L $CaCl_2$ and 1 mmol/L $MgSO_4$ were added to the platelet suspension. To test the effect of NSAIDs on platelet aggregation induced by agonist, platelets were pre-incubated 15 min at 37°C with the drugs.

Statistical Analysis. Data are expressed as Mean ± Standard Error of the Means (*SEM*).

Means were compared using Student's *t*-test for paired data. *P* values <0.05 were taken as significant.

RESULTS

Effects of NSAIDs on Platelet Adhesion. Figure 1 shows platelet adhesion to fibrinogen coated wells in dependence of increasing doses of diclofenac, flurbiprofen and their nitroderivatives, nitrofenac and nitroflurbiprofen. In the absence of drugs, only 3 to 4% of platelets adhered to the fibrinogen-coated surface of the incubation wells, as expected because the assay was carried out in the absence of agonists. At doses ranging from 100 to 500 μmol/L, diclofenac and flurbiprofen were particularly active in stimulating adhesion of platelets thus achieving peak adhesion of 13% to 18%, values that are comparable with those induced by 10 μmol/L ADP or 5 μmol/L arachidonic acid (12% and 14% respectively, data not shown). These stimulatory effects were not present using the nitric oxide-releasing nitrofenac and nitroflurbiprofen. On the contrary, these compounds reduced the basal value of adhesion, a phenomenon particularly evi-

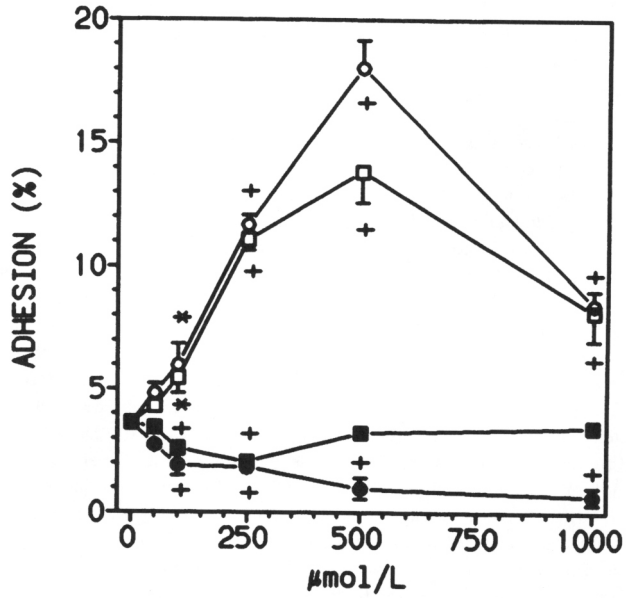

Fig. 1. Effects of diclofenac (○), flurbiprofen (□), nitrofenac (●) and nitroflurbiprofen (■) on platelet adhesion (n = 12; $^*P < 0.02$, and $+P < 0.001$ vs basal).

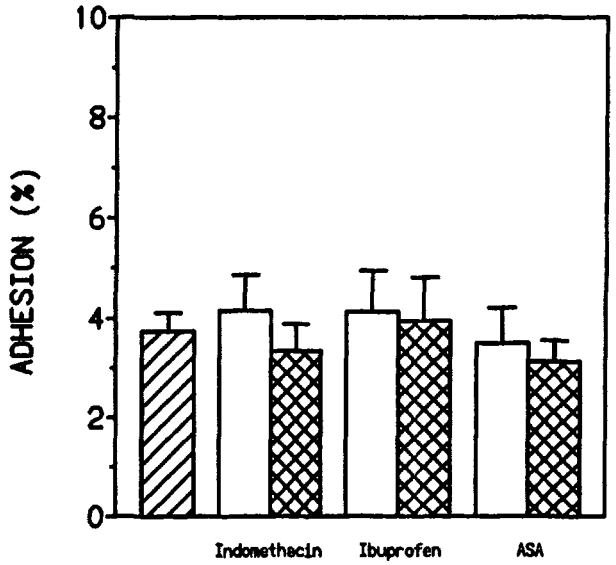

Fig. 2. Effects on platelet adhesion of diluent solution (rising to right bas), 100 μmol/L (open bars) indomethacin, ibuprofen and acetylsalicylic acid and 500 μmol/L (diagonal crosshatch bars) indomethacin, ibuprofen and acetylsalicylic acid (n = 12).

dent for nitrofenac. Incubation of platelets with drugs diluent or high doses of the others tested NSAIDs-acetylsalicylic acid, indomethacin and ibuprofen (0 to 1 mmol/L)-did not increase the platelet adhesion (Figure 2).

Effects of Diclofenac and Flurbiprofen on Platelet Aggregation, Platelet $[Ca^{2+}]_i$ *and Nitric Oxide Production.* In a second set of experiments, diclofenac and flurbiprofen, the two NSAIDs with increasing effect on platelet adhesion, were tested on platelet aggregation. These compounds had no direct platelet pro-aggregating effect, evaluated by conventional aggregometry (Figure 3A). On the contrary, the same drugs showed a marked inhibition on platelet aggregation induced by 10 μmol/L arachidonic-acid, as expected (Figure 3B).

The platelet intracellular free calcium, measured by fluorescent probe Fura 2 AM, was dose-dependently increased by diclofenac and flurbiprofen in a concentration range from 100 to 500 μmol/L (basal; 67.2 ± 2.4; 500 μmol/L diclofenac: 122.6 ± 3.7*; 500 μmol/L flurbiprofen: 111.2 ± 3.0* nmol/L; *$P < 0.01$ vs basal, n = 5) (Figure 4).

In the present study, a basal nitric oxide release by washed platelets was not detected. When the platelets were activated by 10 μg/mL collagen, the production of nitric oxide as measured by amounts of nitrite released was 15.3 ± 1.6 pmol/5 × 10^8 platelets/min (n = 8). When the platelets were incubated with

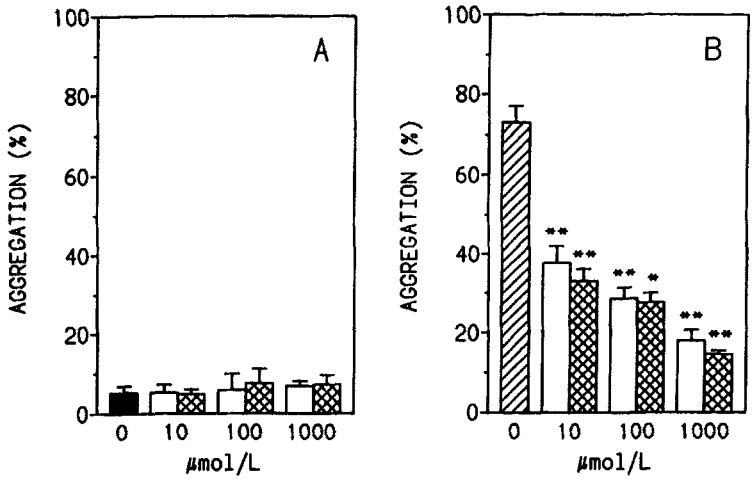

Fig. 3. A: Effects of diclofenac (open bars) and flurbiprofen (diagonal crosshatch bars) on aggregation of resting platelets. Solid bar indicates platelet aggregation in the absence of drugs. B: Effects of increasing doses of diclofenac (open bars) and flurbiprofen (diagonal crosshatch bars) on platelet aggregation induced by 10 μmol/L arachidonic acid. Rising to right bar indicates platelet aggregation by 10 μmol/L arachidonic acid without drugs (n = 8; $^*P < 0.02$ and $^{**}P < 0.01$ vs 0 μM drugs).

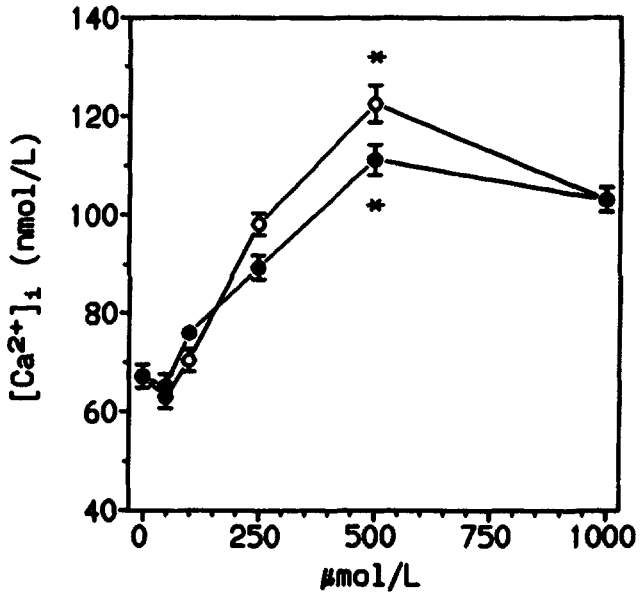

Fig. 4. Effects of diclofenac (○) and flurbiprofen (●) on platelet $[Ca^{2+}]_i$ (n = 8; $^*P < 0.01$ vs basal).

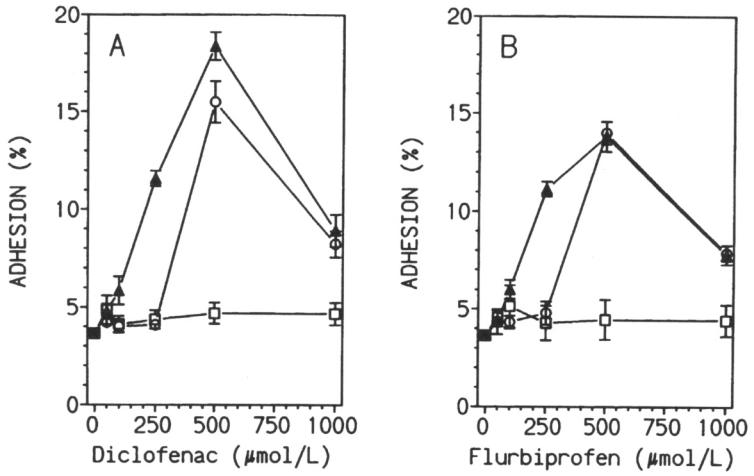

Fig. 5. Effects of aristolochic acid (○) and of nordihydroguaiaretic acid (□) on platelet adhesion in dependence of increasing doses of diclofenac (A) or flurbiprofen (B). ▲ indicates the control without inhibitors.

500 μmol/L diclofenac or flurbiprofen in the absence of collagen, the amounts of nitrite generated were 4.4 ± 0.5 and 3.8 ± 0.4 pmol/5 × 10^8 platelets/min, respectively (n = 8).

Effects of Phospholipase A_2- and Lipoxygenases-Inhibitors on Stimulated Platelet Adhesion. Figure 5 reports the effects of diclofenac and flurbiprofen on the adhesion of platelets preincubated for 15 min at 37°C with either 50 μmol/L aristolochic acid, a phospholipase A_2 inhibitor or 50 μmol/L nordihydroguaiaretic acid, a selective inhibitor of lipoxygenases (IC_{50} = 0.2, 30 and 30 μmol/L for 5-, 12- and 15-lipoxygenase respectively) over cyclo-oxyenase (IC_{50} = 100 μmol/L). The nordihydroguaiaretic acid completely inhibited the platelet adhesion activated by diclofenac and flurbiprofen, whereas the inhibitory effect of aristolochic acid were not detectable when diclofenac and flurbiprofen were tested at concentration exceeding 250 μmol/L.

DISCUSSION

In this study we described the influence of several NSAIDs on various platelet functional responses. In particular, our results showed that diclofenac and flurbiprofen but not other "classical" NSAIDs-indomethacin, acetylsalicylic acid and ibuprofen—were able to trigger the early step of platelet activation, i.e. adhesion, but also that they had no stimulating effects on later step of platelet

activation, i.e., aggregation. On the contrary, platelet aggregation by arachidonic acid was inhibited by diclofenac and flurbiprofen, as expected on the basis of their cyclo-oxygenase-inhibiting activity. Besides, our results suggest that the diclofenac- and flurbiprofen-induced platelet adhesion is possibly mediated through a 12-lipoxygenase-dependent mechanism. This contention is based on the observation that the phenomenon was largely prevented by pre-treatment of platelets with the nordihydroguaiaretic acid, an inhibitor of platelet lipoxygenase (15).

Platelet adherance to the fibrinogen depends on the expression of surface activated GPIIb/IIIa and this is the result of the activating process in response to specific agonists or physical/chemical stimuli. Unlike platelet aggregation, the adhesion does not require the participation of cyclo-oxygenase products, i.e. thromboxane A_2 (16, 17). Perhaps diclofenac and flurbiprofen might alter the platelet plasma membrane and consequently induce a greater tendency of adhere, as suggested by the morphological studies of Djaldetti et al. (18), referring an increased and a marked elongation of platelet pseudopodia after incubation with diclofenac, and by our previous observation of increased expression of GPIIb/IIIa after treatment with this drug (8). However, considering also the ability, even if weak, of diclofenac and flurbiprofen to increase the $[Ca^{2+}]_i$, our results are indicating a real platelet activation rather than a simple plasma membrane "perturbation." During the platelet activation, the release of arachidonic acid from platelet-membrane phospholipids initiates the synthesis of eicosanoids by cyclo-oxygenase and 12-hydroxyeicosatetraenoic acid by 12-lipoxygenase. In the platelet physiology, various mediators can alternatively promote or inhibit platelets adherence. Before all, it is possible that platelet adherence by diclofenac and flurbiprofen is a consequence of the removal of such an inhibitory factor and so it leads to an imbalance between the production of proadhesive and antiadhesive products of the arachidonic acid cascade. As concern the cyclo-oxygenase-derived inhibitory compound, i.e., PGI_2, a reduction of its synthesis is unlikely to be involved in the mechanism of increased platelet adhesion because the concentrations of diclofenac and flurbiprofen required to effect enhancement are far greater than those necessary to inhibit cyclo-oxygenase activity and, in addition, indomethacin, acetylsalicylic acid and ibuprofen, others cyclo-oxygenase inhibitors, did not enhance platelet adhesion.

At high concentrations in vitro, NSAIDs do not inhibit phospholipase A_2, which controls arachidonic acid formation from phospholipids, and have negligible effects on the 5-, 12- and 15-lipoxygenase (19–21). So, it is conceivable that, in our conditions, the platelet lipoxygenase product 12-hydroxyeicosatetraenoic acid could be synthetized in abundance during diclofenac and flurbiprofen administration. In this connection, although the direct product of platelet arachidonate lipoxygenase, 12-hydroperoxyeicosatetraenoic acid, is known to inhibit platelet functions (22, 23), there are other studies indicating that platelet

lipoxygenase may play a significant role in platelet activation (24–26). To our knowledge, there is no direct evidence that inhibition of cyclo-oxygenase activity leads to a "shunting" of arachidonic acid through the 12-lipoxygenase pathway. However, whereas activation of membrane phospholipases is usually accompanied by synthesis of eicosanoids from arachidonic acid, the two processes are not always related. Platelets rendered incapable of producing thromboxane or other eicosanoids (as after aspirin treatment) still undergo the release reaction on contact with appropriate strong stimuli such as collagen and thrombin, as a result of direct phospholipase C stimulation (9). However, the activation of 12-lipoxygenase pathway by arachidonic acid shunting can not be the only explanation of the adhesion induced by diclofenac and flurbiprofen, because it remains to be explained why not all the NSAIDs tested in this work have the capacity to activate. A reason could be that acetylsalicylic acid and indomethacin interfere with the peroxidase activity in platelets and thereby inhibit the conversion of 12-hydroperoxy-eicosatetraenoic acid to 12-hydroxy-eicosatetraenoic acid whereas diclofenac and flurbiprofen do not seem to have this effect (27).

We cannot exclude that diclofenac and flurbiprofen exert at least part of their effects on platelet adhesion via a mechanism that is independent of both cyclo-oxygenase and lipoxygenase and probably through the inhibition of some endogenous inhibitory metabolite. For this reason, we investigated the possible connection between the described NSAIDs activating effect on platelet adhesion and the production of nitric oxide that others have shown to be produced by platelets (28) and to have regulating effects on these cells. The possibility was explored on one hand that higher doses of diclofenac or flurbiprofen increased platelet adhesion affecting platelet nitric oxide production, on the other that the simultaneous administration in large quantities of the two NSAIDs and nitric oxide could result in a normal platelet adhesion. As regards the first hypothesis, no basal nitric oxide production by washed platelets was detected, and this is in agreement with previous observations (29–31). However, from our data it is difficult to support a reduced platelet production of this metabolite by diclofenac and flurbiprofen since it appears that high doses of the two drugs increase platelet nitric oxide production rather than reduce it.

Investigating the second hypothesis, we used two nitric oxide-releasing NSAIDs, i.e., nitrofenac and nitroflurbiprofen. These compounds are new anti-inflammatory drugs obtained by the incorporation of a nitroxybutyl moiety through an ester linkage to the carboxylic group of diclofenac and flurbiprofen, respectively. Nitric oxide-releasing NSAIDs are part of the strategies that, over the past few years, have been employed in order to reduce the gastrointestinal side effects by NSAIDs (32–34). These compounds may provide important alternatives to existing anti-inflammatory agents since they had showed markedly less gastrointestinal ulcerogenic activity but comparable anti-inflammatory properties to the parent compounds, both in acute and in chronic models of inflammation in

the rat (10, 11). An interesting result of the present study is the absence of platelet activation by the diclofenac and flurbiprofen nitric oxide-releasing analogs, nitrofenac and nitroflurbiprofen. Moreover, they rather showed an inhibitory effect on platelet adhesion that for nitrofenac was particularly evident. The anti-adhesive and antiaggregatory effects on platelet physiology of nitric oxide have been described several years ago and repeatedly confirmed. More recent evidences indicate that, in intact human platelets, nitric oxide selectively inhibits 12-lipoxygenase over that cyclo-oxygenase and suggest that such inhibition may be an important regulatory mechanism (35).

It is conceivable that the adherence of platelets to the vascular endothelium could be a critical step in the pathogenesis of NSAIDs-induced gastropathy. In the present study, diclofenac and flurbiprofen were used at concentrations which could readily be achieved in the gastric mucosal interstitium after ingestion of high therapeutic doses of the two drugs, particularly in patients whose compliance is compromised. Although achieved in vitro, the results of this study reinforce the hypothesis that various pathogenetic mechanisms are to the basis of the side effects produced by high doses of NSAIDs and particularly with the regard to the different aspirin-like drugs effects to the various kind of cells (platelet, leukocyte and endothelial cell).

In conclusion, our data are indicating that high doses diclofenac and flurbiprofen are promoter of platelet adhesion and this proadhesive action appears to be mediated by the 12-lipoxygenase pathway. This platelet activation by diclofenac and flurbiprofen may be completely opposed by derivatization of the molecular with nitric oxide. Clearly, the real pathophysiological role of platelets in the gastrointestinal side effects by high doses of diclofenac and flurbiprofen should be investigated in vivo models, where the complex cell-cell interactions and the pharmacodynamic properties of these drugs and their derivatives can be taken into account.

Acknowledgments—We gratefully acknowledge Dr Piero Del Soldato of Nicox, UK, for providing nitrofenac and nitroflurbiprofen. This work was supported by the Ministero Universitá Ricerca Scientifica (fondi 60%).

REFERENCES

1. IVEY, K. J. 1988. Mechanisms of NSAID-induced gastric damage: actions of therapeutic agents. *Am. J. Med.* **84**(Suppl 2A): 41–48.
2. ALLISON, M. C., A. G. HOWATSON, M. B. CAROLINE, J. TORRANCE, F. D. LEE, and R. I. RUSSELL. 1992. Gastrointestinal damage associated with the use of non-steroidal anti-inflammatory drugs. *New Engl. J. Med.* **327**:749–754.
3. ROBERT, A. 1979. Cytoprotection by prostaglandins. *Gastroenterology* **77**:761–767.
4. WALLACE, J. L., C. M. KEENAN, and D. N. GRANGER. 1990. Gastric ulceration induced

by nonsteroidal anti-inflammatory drugs is a neutrophil-dependent process. *Am. J. Physiol.* **259:**G462–467.

5. ASAKO, H., P. KUBES, J. WALLACE, R. E. WOLF, and D. N. GRANGER. 1992. Modulation of leukocyte adhesion in rat mesenteric venules by Aspirin and salicylate. *Gastroenterology* **103:**146–152.
6. ASAKO, H., P. KUBES, J. WALLACE, T. GAGINELLA, R. E. WOLF, and D. N. GRANGER. 1992. Indomethacin-induced leukocyte adhesion in mesenteric venules: role of lipoxygenase products. *Am. J. Physiol.* **262:**G903–908.
7. GERSHLICK, A. H., Y. D. SYNDERCOMBE COURT, A. J. MURDAY, C. T. LEWIS, and P. G. MILLS. 1985. Adverse effects of high dose aspirin on platelet adhesion to experimental autogenous vein grafts. *Cardiovasc. Res.* **19:**770–776.
8. ANDRIOLI, G., S. LUSSIGNOLI, R. ORTOLANI, P. MINUZ, F. VELLA, and P. BELLAVITE. 1996. Dual effects of diclofenac on human platelet adhesion in vitro. *Blood Coag. Fibrin.* **7:**153–156.
9. WESKLER, B. B. 1992. Platelets. *In* Inflammation: Basic Principles and Clinical Correlates. 2nd Edition. J. I. Gallin, I. M. Goldstein, and R. Snyderman, editors. Raven Press, New York, 727–746.
10. WALLACE, J. L., B. REUTER, C. CICALA, W. MCKNIGHT, M. B. GRISHAM, G. CIRINO. 1994. Novel NSAID derivatives with markedly reduced ulcerogenic properties in the rat. *Gastroenterology* **107:**173–179.
11. MARIOTTO, S., M. MENEGAZZI, A. CARCERERI DE PRATI, L. CUZZOLIN, A. ADAMI, H. SUZUKI, and G. BENONI. 1995. Protective effect of NO on gastric lesions and inhibition of expression of gastric inducible NOS by flurbiprofen and its nitro-derivative, nitroflurbiprofen. *Br. J. Pharmac.* **116:**1713–1714.
12. BELLAVITE, P., G. ANDRIOLI, P. GUZZO, P. ARIGLIANO, S. CHIRUMBOLO, F. MANZATO, and C. SANTONASTASO. 1994. A colorimetric method for the measurement of platelet adhesion in microtiter plates. *Anal. Biochem.* **216:**444–450.
13. POLLOCK, W. K., T. J. RINK, and R. F. IRVINE. 1986. Liberation of ^{3}H arachidonic acid and changes in cytosolic free calcium in fura-2-loaded human platelets stimulated by ionomycin and collagen. *Biochemical J.* **235:**869–877.
14. MISKO, T. P., R. J. SCHILLING, D. SALVEMINI, W. M. MOORE, and M. G. CURRIE. 1993. A fluorimetric assay for the measurement of nitrite in biologicals samples. *Anal. Biochem.* **214:**11–16.
15. CERLETTI, C. 1985. Interaction of non-steroidal anti-inflammatory drugs on platelet COX and lipoxygenase activity. *Int. J. Tissue React.* **7:**309–312.
16. LAPETINA, E. G., B. REEP, N. G. READ, and S. MONCADA. 1986. Adhesion of human platelets to collagen in the presence of prostacyclin, indomethacin and compound BW755C. *Thromb. Res.* **41:**325–335.
17. DE CLERCK, F. 1986. Blood platelets in human hypertension. *Agents & Actions* **18:**563–580.
18. DJALDETTI, M., P. FISHMAN, D. CRETER, and I. NOTTI. 1982. Ultrastructural and functional studies on human platelets incubated with diclofenac sodium (Voltaren). *Acta. Haematologica* **68:**285–294.
19. SIROIS, P., C. SAURA, H. SALARI, and P. BORGEAT. 1984. Comparative effects of etodolac, indomethacin and benoxaprofen on eicosanoid biosynthesis. *Inflammation* **8:**353–364.
20. MARCUS, A. J. 1994. Multicellular eicosanoid and other metabolic interactions of platelets and other cells. *In* Hemostasis and Thrombosis: Basic Principles and Clinical Practice. Third Edition. R. W. Colman, J. Hirsh, V. J. Marder, and E. W. Salzmann, editors. JB Lippincott, Philadelphia. 590–602.
21. KU, E. C., W. LEE, H. V. KOTHARI, and D. W. SCHOLER. 1986. Effect of diclofenac sodium on the arachidonic acid cascade. *Am. J. Med.* **80**(Suppl. 4B):18–23.
22. AHARONY, D., J. B. SMITH, and M. J. SILVER. 1982. Regulation of arachidonate-induced platelet

aggregation by the lipoxygenase product, 12-hydroxyperoxyeicosatetraenoic acid. *Biochim. Biophys. Acta.* **718:**193–200.

23. CROSET, M., and M. LAGARDE. 1983. Stereospecific inhibition of PGH_2-induced platelet aggregation by lipoxygenase products of eicosaenoic acids. *Biochem. Biophys. Res. Commun.* **112:**878–883.
24. DUTILH, C. E., E. HADDEMAN, J. A. DON, and F. TEN HOOR. 1981. The role of arachidonate lipoxygenase and fatty acids during irreversible blood platelet aggregation in vitro. *Prostaglandin Med.* **6:**111–126.
25. NISHIGAWA, E. E., D. J. WILLIAMS, and C. L. CONNELL. 1983. Arachidonate induced aggregation of rat platelets may not require prostaglandin endoperoxides or thromboxane A_2. *Thromb. Res.* **30:**289–296.
26. CARLINI, C. R., J. A. GHIMARAES, and J. M. C. RIBEIRO. 1983. Platelet release reaction and aggregation induced by canatoxin, a convulsant protein: Evidence for the involvement of the platelet lipoxygenase pathway. *Br. J. Pharmac.* **84:**551–560.
27. SIEGEL, M. I., R. T. MCCONNELL, N. A. PORTER, and P. CUATRECASAS. 1990. Arachidonate metabolism via lipoxygenase and 12L-hydroperoxy-5,8,10,14-eicosatetraenoic acid peroxidase sensitive to anti-inflammatory drugs. *Proc. Natl. Acad. Sci. U.S.A.* **77:**308–312.
28. ZHOU, Q., G. R. HELLERMANN, and L. P. SOLOMONSON. 1995. Nitric oxide release from resting human platelets. *Thromb. Res.* **77:**87–96.
29. RADOMSKI, M. W., R. M. PALMER, and S. MONCADA. 1990. An L-arginine/nitric oxide pathway present in human platelets regulates aggregation. *Proc. Natl. Acad. Sci. U.S.A.* **87:**5193–5197.
30. MALINSKI, T., M. W. RADOMSKI, Z. TAHA, and S. MONCADA. 1993. Direct electrochemical measurement of nitric oxide released from human platelets. *Biochem. Biophys. Res. Comm.* **194:** 960–965.
31. LANTOINE, F., A. BRUNET, F. BEDIOUI, J. DEVYNCK, and M. A. DEVYNCK. 1995. Direct measurement of nitric oxide production in platelets: relationship with cytosolic Ca^{2+} concentration. *Biochem. Biophys. Res. Comm.* **215:**842–848.
32. WALLACE, J. L., Q. J. PITTMAN, and G. CIRINO. 1995. Nitric oxide-releasing NSAIDs: A novel class of GI-sparing anti-inflammatory drugs. *A.A.S.* **46:**121–129.
33. MINUZ, P., C. LECHI, S. BONAPACE, S. GAINO, A. ADAMI, L. CUZZOLIN, DEL P. SOLDATO, and G. BENONI, 1996. Effects of a new class of NO-releasing NSAIDs on platelets and isolated arteries. *Inflammopharmacology* **4:**83–90.
34. LECHI, C., G. ANDRIOLI, S. GAINO, R. TOMMASOLI, V. ZULIANI, R. ORTOLANI, M. DEGAN, G. BENONI, P. BELLAVITE, A. LECHI, and P. MINUZ. 1996. The antiplatelet effects of a new nitroderivative of acetylsalicylic acid—An in vitro study of inhibition on the early phase of platelet activation and on TXA_2 production. *Thromb. Haemost.* **76:**791–798.
35. NAKATSUKA, M., and Y. OSAWA. 1994. Selective inhibition of the 12-lipoxygenase pathway of arachidonic acid metabolism by L-arginine or sodium nitroprusside in intact human platelets. *Biochem. Biophys. Res. Commun.* **200:**1630–1634.