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Prevention of αIIbβ3 activation by non-steroidal antiinflammatory drugs

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Abstract We have studied the effect of non-steroidal antiinflammatory drugs (NSAIDs) on αIIbβ3 integrin activation and platelet aggregation. NSAIDs such as meloxicam, piroxicam, indomethacin and aspirin, but not aceclofenac or diclofenac interfered with the activation state of αIIbβ3. NSAIDs that inhibited αIIbβ3 activation were also able both to partially inhibit platelet primary aggregation and to accelerate platelet deaggregation. These effects of NSAIDs were not dependent on cyclooxygenase inhibition. The results obtained indicate that some NSAIDs exert a specific action on αIIbβ3 activation, and provide an additional mechanism that accounts for their beneficial effects in diseases in which platelet activation is involved.

Key words: Non-steroidal antiinflammatory drug; αIIbβ3; Platelet activation

1. Introduction

Platelet activation can be initiated by several mechanical (vessel wall injury, disruption of atherosclerotic plaques) or chemical (ADP, epinephrine, thromboxane A_2, thrombin) stimuli. In response to vessel wall injury, platelets attach to subendothelial matrix (adhesion) that is followed by fibrinogen-mediated platelet-platelet interaction (aggregation). Simultaneously, platelets release their intracellular granule contents (secretion) that lead to the recruitment of additional circulating platelets (amplification). Platelet aggregation is mediated exclusively by αIIbβ3, an integrin that binds fibrinogen, fibronectin, von Willebrand factor and vitronectin [1,2].

As other integrins, αIIbβ3 can be found in different affinity states for their ligands. Variations in the ligand-binding affinity of integrins (integrin activation) occur within seconds after cell stimulation as a consequence of intracellular signals [3,4] and are due to changes in the conformation of the extracellular domain of these molecules [5]. In this regard, several antibodies have been described that specifically recognize neoepitopes that are expressed only by activated integrins [6–8]. Upon platelet activation, αIIbβ3 undergoes a measurable conformational change in its quaternary structure [9,10], switching from low to a high-affinity state. These structural changes allow the binding of soluble fibrinogen ending in thrombi formation.

Non-steroidal antiinflammatory drugs (NSAIDs) are a heterogeneous group of chemical compounds very commonly prescribed for disorders characterized by inflammation or thrombosis. Although the inhibition of prostaglandin synthesis has been proposed to be the mechanism of action of NSAIDs, there are evidences that suggest that additional functions may be implicated. In this regard, we have recently described that some NSAIDs such as indomethacin, diclofenac, aspirin, ketoprofen and aceclofenac induce L-selectin shedding from neutrophil cell membrane, thereby preventing the adhesion of neutrophils to endothelial cells [11]. On the other hand, NSAIDs from the oxicam family are able to interfere with neutrophil L-selectin shedding and αMβ2 integrin up-regulation induced by pro-inflammatory cytokines [12]. In addition, these drugs inhibit the appearance of activation-dependent epitopes on β2 and β1 integrins in cytokine-stimulated neutrophils and T lymphocytes, respectively [12].

Although it is accepted that some NSAIDs interfere with the platelet secondary aggregation through the inhibition of platelet degranulation, recently described effects of these agents on integrin activation prompted us to examine whether NSAIDs could inhibit the platelet aggregation through the modulation of αIIbβ3 activation. In the present work, we report that some NSAIDs are able both to inhibit the primary aggregation of platelets induced by agonists through the inhibition of αIIbβ3 integrin activation as well as to increase the rate of deaggregation independently of cyclooxygenase inhibition.

2. Materials and methods

2.1. Antibodies and reagents

Prostaglandin I_2 (PGI_2), ADP, epinephrine, piroxicam, diclofenac, aspirin (acetylsalicylic acid) and indomethacin were purchased from Sigma Chemical Co (St. Louis, MO, USA). Aceclofenac was obtained from Prodesfarma (Barcelona, Spain) and meloxicam from Boëhringer Ingelheim GmbH (Ingelheim, Germany). All NSAIDs were solubilized in dimethyl sulfoxide (DMSO) (Panreac, Barcelona, Spain) and were incubated with platelets in a final concentration of 0.2% DMSO. All control data were obtained in the presence of 0.2% DMSO. Thrombin was from Boëhringer Mannheim GmbH (Germany). Acid solubilized type I collagen was from ICN Biomedicals (Costa Mesa, CA, USA). Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse Igs was purchased from Dako (Salstrup, Denmark). Thromboxane B_2 (TXB_2) Immunoassay was from R and D Systems (United Kingdom). Ro43-5054 is an αIIbβ3-specific non-peptide antagonist that has been described previously [13]. The non-inhibitory anti-αIIbβ3 mAb D57 [14] was kindly provided by Dr. M.H. Ginsberg, anti-αIIbβ3 activated mAb PAC-1 [7] was a generous gift from Dr. S. Shattil and anti-P-selectin mAb S12 [15] was kindly donated by Dr. R.P. McEver. P3X63 myeloma culture supernatant was used as negative control.

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Abbreviations: DMSO, dimethyl sulfoxide; mAb, monoclonal antibody; NSAIDs, non-steroidal antiinflammatory drugs; PGI_2, prostaglandin I_2; PRP, platelet rich plasma; TXB_2, thromboxane B_2

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2.2. Platelet isolation

Platelet isolation was carried out at room temperature from normal human venous blood mixed with buffered sodium citrate 0.13 M in the proportion 10:1. Platelet rich plasma (PRP) was obtained by centrifugation of whole blood at 1100 rpm for 10 min. To isolate washed platelets, 1 μM PGI_2 was added to PRP, and platelets were collected by centrifugation at 2200 rpm for 10 min. The pellet was gently resuspended in PBS pH 7.3, supplemented with 0.1% BSA, 1 μM PGI_2, and centrifuged at 2200 rpm for 10 min. Finally, washed platelets were gently resuspended in PBS, 0.1% BSA, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$. To isolate gel-filtered platelets, PRP was passed over a column of Sepharose 2B (Pharmacia Fine Chemicals).

2.3. Flow cytometry analysis

Binding of mAbs to resting or agonist activated platelets was analyzed by flow cytometry, using P3X63 myeloma culture supernatant as negative control. Washed platelets were incubated in some assays with NSAIDs for 20 min at 37°C. Different stimuli (ADP, epinephrine, thrombin, collagen) were added immediately. After an incubation of 5 min at 37°C, aliquots of untreated or NSAIDs pre-treated platelets were incubated with saturating concentrations of specific mAbs at room temperature. After 15 min incubation, cells were labeled with FITC-conjugated goat anti-mouse Igs (1:50 final dilution) for an additional 15 min at room temperature. Platelets were finally fixed with an equal volume of 4% formaldehyde for 2 h at 4°C, washed, and resuspended in PBS.

Flow cytometry analysis was performed with a FACScan cytofluorometer (Becton Dickinson, Mountain View, CA, USA). Linear and logarithmic fluorescence values from at least 1×10^4 platelets per sample were obtained in each experiment. In experiments where the fluorescence was recorded in linear scale, the fluorescence gain was adjusted so that 5% of cells of the sample with greatest fluorescence were positive in the highest fluorescence channel. The results are presented in some figures as the mean fluorescence intensity (MFI). In order to minimize the variations between different blood donors, in experiments with NSAIDs, data were normalized to express relative percentage of variation of MFI (r %ΔMFI) of PAC-1 binding. First, we calculate the percentage of variation of MFI (%ΔMFI) in each sample respect to basal MFI of resting platelets as follows:

$$\%\Delta MFI_{SAMPLE} = [(MFI_{SAMPLE}/MFI_{BASAL})-1]\times 100.$$

Then, r %ΔMFI of stimulated samples previously incubated with NSAIDs respect to vehicle treated samples was calculated as follows:

$$r\ \%\Delta MFI = (\%\Delta MFI_{NSAID+AGONIST}/\%\Delta MFI_{DMSO+AGONIST})\times 100,$$

where r %ΔMFI of vehicle treated (control) platelets was 100%.

2.4. Platelet dense granules secretion

Platelet secretion of dense granules ATP was monitored with a manual luminometer (Lumat LB9501, Berthold), using the ATP Bioluminescence Assay Kit HSII (Boëhringer Mannheim GmbH, Germany). Platelet ATP secretion of untreated and activated platelets was expressed as relative light units.

2.5. Platelet aggregation

Platelet aggregation studies were performed with PRP using a four-channel aggregometer (Menarini, Firenze, Italy). Autologous platelet poor plasma was prepared by centrifugating blood at 3500 rpm for 10 min at room temperature. Platelet concentration in PRP was adjusted to 3×10^5 platelets/μl by adding platelet poor plasma. To study the effect of NSAIDs on platelet primary aggregation, PRP was preincubated with these substances added in a final concentration of 0.2% DMSO for 15 min at 37°C. Then, aggregation was induced by adding 1 μM ADP to the aggregometer cuvette and stirring at 1100 rpm. Aggregation was monitored for 5 min and is expressed as percentage of change in light transmittance. The rate of deaggregation is arbitrarily expressed as percentage of change in light transmittance/min calculated during the 45 s following the maximal aggregation.

2.6. Platelet thromboxane B_2 production

Thromboxane generation was determined in gel-filtered platelets. Gel-filtered platelets were incubated with NSAIDs solubilized in 0.2% DMSO for 10 min at 37°C, and then activated with 1 mM arachidonic acid for 5 min at 37°C. Reaction was quenched by addition of ice-cold ethanol, samples were centrifuged at 14 000 rpm for 5 min at room temperature, ethanol was evaporated and the pellet resuspended in immunoassay buffer. Determination of the stable end-product of TXA_2, TXB_2, was analyzed using a TXB_2 Immunoassay according to the manufacturer's protocol (R and D Systems).

2.7. Statistical analysis

Results were expressed as arithmetic mean ± standard error of the mean (S.E.M.) or standard deviation (S.D.). Student's *t*-test for paired samples was used to determine significant differences between means.

3. Results

To determine αIIbβ3 activation state, we analyzed the expression of a specific activation-dependent epitope on this integrin by the PAC-1 mAb. This mAb recognizes the ligand-binding site of the activated form of αIIbβ3 [16], and the expression of the epitope recognized by PAC-1 indicates the ability of this integrin to bind soluble fibrinogen. Platelets were activated with low doses of different agonists (epinephrine, ADP and type I collagen), to avoid the release of endogenous agonist stored into platelet granules. The expression levels of αIIbβ3 activation-dependent epitope and P-selectin, and the ATP release from dense granules under these activation conditions are shown in Fig. 1 (A, C and D, respectively). We found that 1 μM epinephrine, 1 μM ADP or 50 μg/ml collagen activated αIIbβ3 integrin but were unable to induce P-selectin expression and dense granules secretion, while 0.02 U/ml thrombin induced a significant granules release reaction as confirmed by increase in the binding of anti-P-selectin mAb

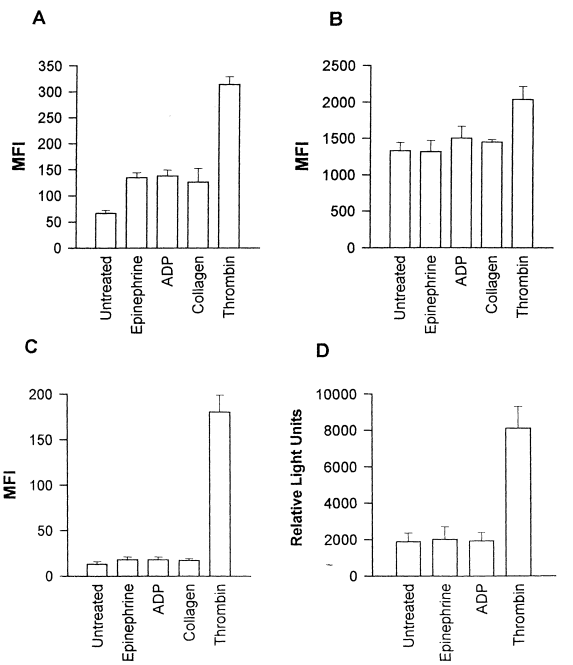

Fig. 1. Expression of activated αIIbβ3 integrin, P-selectin, and ATP release in platelets activated with low doses of agonists. Platelets were activated with either 1 μM epinephrine, 1 μM ADP, 50 μg/ml collagen or 0.02 U/ml thrombin. Platelets were then stained with PAC-1 mAb (A), D57 mAb (B) or S12 mAb (C) and bound fluorescence was analyzed by flow cytometry. Data are expressed as the arithmetic mean ± S.E.M. of the MFI from eight independent experiments. D: ATP release for untreated and agonist-treated platelets was analyzed in a luminometer. Results are expressed as the arithmetic mean ± S.E.M. of the relative light units from four independent experiments.

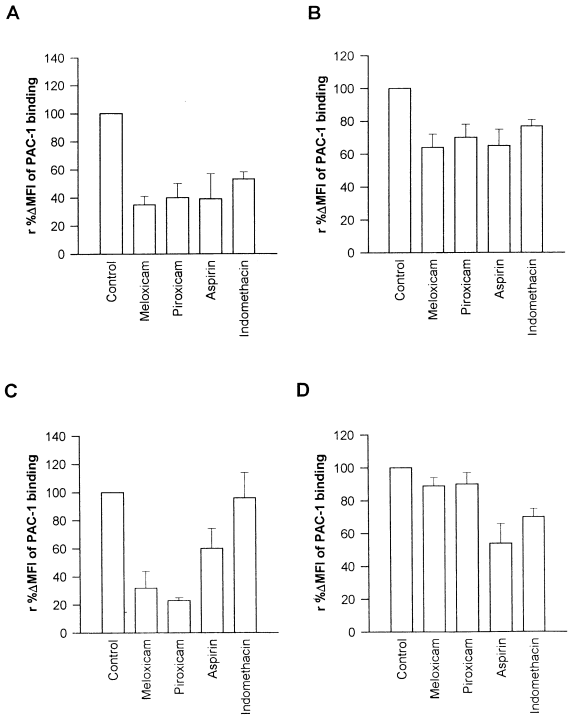

Fig. 2. Effect of NSAIDs on αIIbβ3 activation-dependent epitope expression induced by different agonists. Platelets were pretreated with meloxicam (20 μg/ml or 57 μM), piroxicam (40 μg/ml or 120 μM), aspirin (250 μg/ml or 1.4 mM), indomethacin (40 μg/ml or 112 μM) or DMSO prior to agonists addition. A: 1 μM epinephrine. B: 1 μM ADP. C: 50 μg/ml collagen. D: 0.02 U/ml thrombin. The data are expressed as the aritmethic mean ± S.E.M. of four independent experiments.

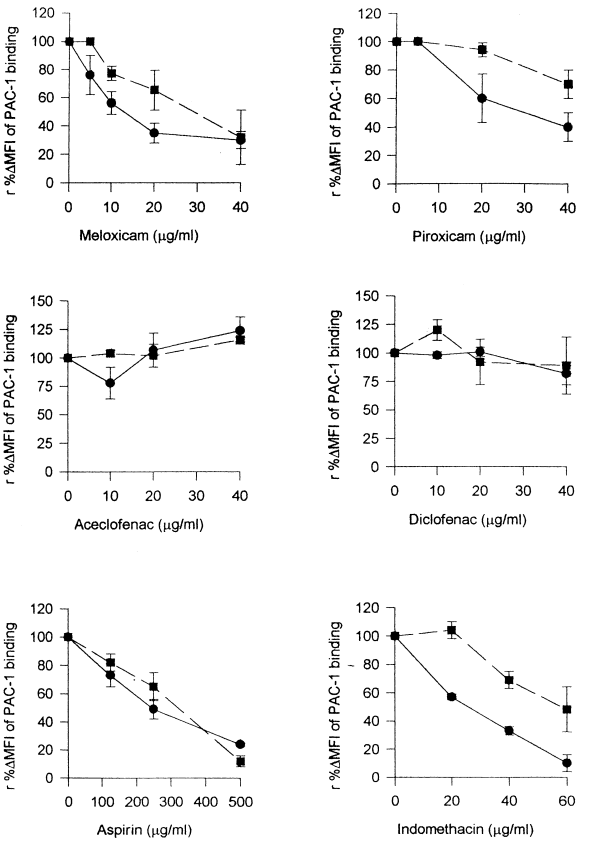

Fig. 3. Dose-response of the effect of NSAIDs on PAC-1 binding to activated platelets. Platelets were preincubated with different concentrations of NSAIDs. Then, platelets were activated with 1 μM epinephrine (●), 1 μM ADP (■) for 5 min at 37°C. PAC-1 binding was measured by flow cytometry, and data are expressed as the aritmethic mean ± S.E.M. from four experiments.

and ATP detection. The incubation of platelets with epinephrine, ADP or collagen at those doses did not modify the basal expression of αIIbβ3 as indicated by D57 mAb binding (Fig. 1B). However, the basal expression of αIIbβ3 integrin on platelet surface was slightly increased during incubation with thrombin (Fig. 1B), likely as a consequence of αIIbβ3 translocation from α and dense granules [17,18]. This point was confirmed by analysis of surface expression of P-selectin (Fig. 1C), which is also a component of both sorts of granules in platelets [15,18]. In this study we have used these mild activation conditions to analyze the effect of NSAIDs on αIIbβ3 integrin activation in the absence of platelet granule release.

To determine whether NSAIDs interfere with αIIbβ3 integrin activation in platelets, we examined the effect of different NSAIDs on the conformational changes of αIIbβ3 induced by different stimuli. Pretreatment of platelets with meloxicam, piroxicam, aspirin and indomethacin inhibited the expression of the activation-dependent epitope on αIIbβ3 induced by 1 μM epinephrine (Fig. 2A). A partial inhibitory effect of PAC-1 epitope expression was also observed when the agonist used was 1 μM ADP (Fig. 2B). The inhibitory effect of NSAIDs on αIIbβ3 integrin activation induced by either epinephrine or ADP was dose dependent, with a more noticeable inhibition on the activation induced by epinephrine (Fig. 3). The inhibitory effect of meloxicam was stronger than that of piroxicam upon β3 integrin activation. In contrast, aceclofenac and diclofenac were unable to prevent αIIbβ3 activation induced by both epinephrine and ADP (Fig. 3). Collagen-induced αIIbβ3 activation was also inhibited by meloxicam, piroxicam and aspirin but not by indomethacin (Fig. 2C). Finally, the activation of αIIbβ3 induced by the potent agonist thrombin was only partially inhibited by aspirin and indomethacin whereas no effect was found with oxicams (Fig. 2D). Moreover, indomethacin and aspirin caused a significant inhibition on P-selectin surface expression after thrombin activation (data not shown), thus indicating an inhibitory effect of these NSAIDs on granule secretion, as previously described [19,20]. In contrast, meloxicam and piroxicam did not show any significant effect on P-selectin expression (data not shown).

To study the effect of NSAIDs on platelet primary aggregation, assays were carried out in the presence of low doses of

Table 1
Effect of NSAIDs on platelet primary aggregation

	Aggregation (% change in light transmittance)
Control	37.8 ± 0.7
Meloxicam 20 μg/ml	25.0 ± 1.2*
Piroxicam 20 μg/ml	27.0 ± 1.6*
Indomethacin 20 μg/ml	28.3 ± 1.2*
Aspirin 250 μg/ml	28.2 ± 1.3*
Aceclofenac 20 μg/ml	36.5 ± 2.4
Ro 43-5054 2 μM	0

Data are expressed as the aritmethic mean ± S.E.M. obtained in six independent assays.
*$P < 0.01$ (Student's t-test for paired samples).

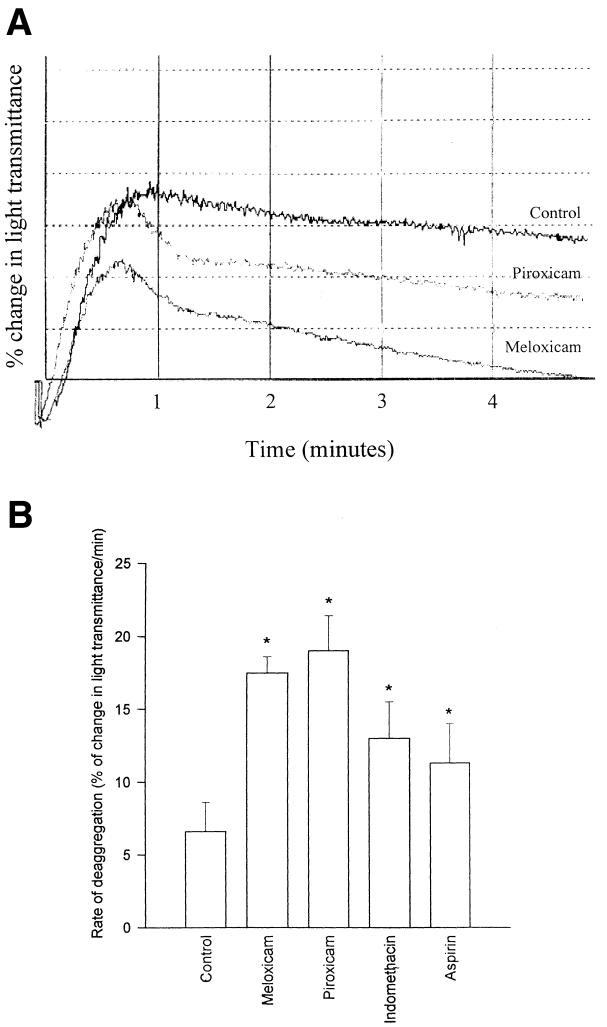

Fig. 4. Effect of NSAIDs on the rate of platelet deaggregation. PRP was incubated with meloxicam (20 μg/ml), piroxicam (20 μg/ml), indomethacin (20 μg/ml) or aspirin (250 μg/ml). A: Representative aggregometer tracing. B: Rate of deaggregation. Data are expressed as the arithmetic mean ± S.E.M. obtained in ten independent assays. $^*P < 0.01$ (Student's *t*-test for paired samples).

ADP (1 μM). The term primary aggregation refers to the direct aggregation of platelets incubated with a low concentration of agonist that does not induce granule secretion [21]. We found that the treatment of platelets with meloxicam, piroxicam, indomethacin or aspirin partially prevented primary aggregation (Table 1). In contrast, aceclofenac did not affect platelet maximal primary aggregation. Furthermore, meloxicam, piroxicam, indomethacin and aspirin also significantly increased the initial rate of platelet deaggregation ($P \leq 0.01$) (Fig. 4A and B). Platelet aggregation was solely mediated by the interaction of αIIbβ3 with fibrinogen, since the Ro43-5054 compound, that prevents the binding of fibrinogen to αIIbβ3, completely abolished the platelet aggregation in these assays (Table 1).

To ascertain whether the effect of NSAIDs on αIIbβ3 activation was dependent or not on the inhibition of cyclooxygenase, we analyzed the effect of NSAIDs on the production of TXB_2. The basal TXB_2 production was 3459 ± 802 pg/10^8 platelets and it was increased to 84 709 ± 5529 pg/10^8 platelets after activation with 1 mM arachidonic acid. Treatment of platelets with indomethacin and aspirin inhibited dramatically TXB_2 generation after arachidonic acid stimulation (Fig. 5), whereas the treatment with meloxicam did not exert any significant inhibitory effect on TXB_2 formation (Fig. 5). Together these results indicate that the inhibitory effects of different NSAIDs on platelet αIIbβ3 integrin activation and aggregation take place regardless of their effects on cyclooxygenase inhibition.

4. Discussion

Most NSAIDs are able to inhibit cyclooxygenase, interfering with the metabolism of arachidonic acid and therefore with thromboxane A_2 formation in platelets. However, these drugs have many biochemical effects that do not involve prostaglandin synthesis which indicate that the effects of NSAIDs seem to be drug- and cell-type dependent [22–24]. In this report we provide an additional mechanism that may account for NSAIDs beneficial effect in diseases in which platelet aggregation is involved. Hence, we found that some NSAIDs are able to inhibit platelet aggregation through the inhibition of αIIbβ3 integrin activation in a cyclooxygenase-independent manner.

Signals elicited by weak agonists (ADP, epinephrine, collagen) do not induce granules release reaction by themselves and cause a partial reversible platelet aggregation. Strong agonists (thrombin) activate phospholipase C, increasing cytoplasmic free calcium and activating protein kinase C, and these events synergistically stimulate granules secretion and cause a complete non-reversible platelet aggregation [25]. The low doses of agonists employed in this report allowed us to study the regulation of αIIbβ3 integrin activation in the absence of a complete platelet activation, as determined by the failure to increase P-selectin expression and ATP release, indicating that this integrin activation is a very early and rapid event during platelet activation. The signalling mechanisms involved in the conformational changes that activate αIIbβ3 integrin are not completely understood, but involve at least seven transmembrane protein receptors, G proteins, phospholipases, calcium release, arachidonic acid metabolism, protein kinase C, adenyl and guanyl cyclases, and other protein kinases and phosphatases [26]. Our data

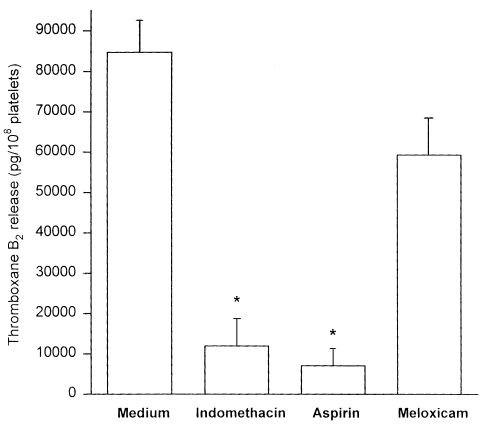

Fig. 5. Thromboxane B_2 production is inhibited by some NSAIDs. Gel-filtered platelets were preincubated with different NSAIDs (250 μg/ml aspirin; 20 μg/ml indomethacin and meloxicam). TXB_2 production is expressed as arithmetic mean ± S.D. of three independent experiments. $^*P < 0.01$ (Student's *t*-test for paired samples).

indicate that oxicams only prevent integrin activation induced by weak agonists. These drugs induced their highest inhibition of αIIbβ3 activation when platelets were stimulated with collagen. Under this condition, indomethacin did not exert any effect suggesting that αIIbβ3 activation induced by collagen might be independent of arachidonic acid metabolism. Integrin activation mediated by ADP and epinephrine was partially inhibited by all NSAIDs except aceclofenac and diclofenac, probably because several intracellular pathways are elicited by these stimuli.

Interestingly, the interference of NSAIDs with integrin αIIbβ3 activation has functional consequences on platelet primary aggregation. This platelet initial aggregation is independent of agonists endogenous release and TXA_2 production. The effect of NSAIDs on platelet primary aggregation seems to be a consequence of both a partial inhibition of maximal αIIbβ3 activation and an increase in the rate of deactivation of this integrin. Hence, platelet aggregation, mediated by αIIbβ3 interaction with fibrinogen, seems to be more stable in untreated platelets than in NSAID-treated platelets. These results suggest that these drugs could directly affect regulation of αIIbβ3 activation either by interfering with intracellular signalling events that change integrin conformation through their cytoplasmic tail or by modifying integrin-cytoskeleton interactions.

Indomethacin and aspirin inhibited events mediated by both weak (integrin activation) and strong agonists (integrin activation and granule secretion). These data are in agreement with the work of Shattil and Brass [27], that describes that indomethacin inhibits the expression of the PAC-1 neoepitope in platelets treated with low doses of thrombin. In this regard, it has been described that aspirin and indomethacin decrease the fibrinogen binding to platelets induced by epinephrine, apparently by prevention of both dense granules and TXA_2 release, which may potentiate the platelet response [19,20]. Our results confirm that aspirin and indomethacin strongly inhibit TXA_2 formation, indicating that these drugs inhibit cyclooxygenase-1, the enzyme isoform present in platelets. Nevertheless, the inhibitory effect on cyclooxygenase is not common to all NSAIDs, as we show that meloxicam did not significantly inhibit cyclooxygenase at the doses used in this study. Therefore, our data support the issue that the effects of NSAIDs on αIIbβ3 activation do not correlate to their capability to inhibit cyclooxygenase, and indicate that each NSAID may have more than one mechanism of action. Thus, we conclude that some NSAIDs can interfere with one of the first events of platelet activation: fibrinogen receptor activation, an effect that diminishes platelet aggregation triggered by low doses of agonist. The molecular mechanisms responsible of the action of NSAIDs on αIIbβ3 activation remain to be elucidated. This novel effect of NSAIDs could play an important role during pathological processes where traces of platelet agonists could lead to microthrombi formation.

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