Effect of Single Oral Administrations of Non Steroidal Antiinflammatory Drugs to Healthy Volunteers on Arachidonic Acid Metabolism in Peripheral Polymorphonuclear and Mononuclear Leukocytes

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Abstract — The effects of a single oral administration of acetylsalicylic acid (500 mg), indomethacin (50 mg) and piroxicam (40 mg) to healthy volunteers on functional and biochemical parameters of platelets, polymorphonuclear (PMN) and mononuclear (MNL) leukocytes were evaluated. Blood was collected before and two hours after the drug intake and blood cells separated according to conventional techniques. The considered drugs almost completely suppressed the aggregation of platelets, whereas they did not affect either PMN and MNL aggregation. Superoxide anion generation by leukocytes was significantly increased after aspirin intake, whilst a significant reduction (PMN), or no effect (MNL) was observed after piroxicam and indomethacin respectively. The formation of arachidonate metabolites via the 5-lipoxygenase pathway by PMN and MNL challenged with 10 μM A23187 was unchanged following aspirin and indomethacin. In this respect a selective increase of 5-HETE and LTC₄ synthesis by MNL only was detected after piroxicam administration. The three drugs similarly reduced TXB₂ synthesis by platelets and PMN (-80% for aspirin and indomethacin, and -40% for piroxicam). As far as MNL is concerned, aspirin inhibited this metabolite by 80%, while indomethacin reduced it by 40% only. In contrast piroxicam increased TXB₂ synthesis by stimulated MNL. It can be concluded that the considered antiinflammatory drugs 1) differently affect the cyclooxygenase enzyme in platelets and leukocytes; 2) at variance with the situation in platelets, the inhibition of thromboxane formation by leukocytes is not related to modifications of cellular function; 3) the formation of arachidonate metabolites via the 5-lipoxygenase pathway is affected by piroxicam only.
**Introduction**

Aspirin and other non steroidal antiinflammatory drugs (NSAIDs) are effective inhibitors of the cyclooxygenase enzyme (CO) in various tissues (1). As such they may inhibit the production of arachidonic acid (AA) metabolites, involved in the inflammatory process. Circulating cells, and in particular polymorphonuclear and mononuclear leukocytes, convert AA to several biologically active products not only via the CO pathway, but also via the lipoxygenase (LO) enzyme. The compounds, formed by the 5-lipoxygenase pathway (5-LO), i.e. 5-hydroxyeicosatetraenoic acid (5-HETE), leukotriene B\(_4\) (LTB\(_4\)) and leukotriene C\(_4\) (LTC\(_4\)), are known to be important mediators of the acute inflammatory response and have been implicated in vascular damage in several pathological conditions (2, 3, 4, 5).

Experimental evidence indicates a key role of 5-LO products formed by polymorphonuclear and mononuclear leukocytes in the amplification of the acute and chronic inflammatory event (6, 7, 8).

Aspirin and other NSAIDs, at concentrations completely suppressing prostaglandin and thromboxane synthesis, have been shown, in vitro, either to stimulate or not to have effects on the 5-LO product formation in activated neutrophils (9, 10). At present, no data are available about the effects of these molecules on 5-LO metabolism by leukocytes, after their administration to human subjects.

In the present investigation, the possible effects of molecules from three different chemical series, i.e. aspirin, indomethacin and piroxicam, on the formation of AA products via the CO and 5-LO enzymes by polymorphonuclear (PMN) and mononuclear (MNL) leukocytes were evaluated and considered in relation to other functional parameters of these cells.

**Methods**

*Study design:* Fifteen male volunteers, aged 20–30 ys, 60–80 Kg b.w., free of any pharmacological treatment for at least ten days prior to the study, received orally, according to a randomized protocol, one of the following drugs: acetylsalicylic acid, ASA (Aspirina, Bayer, FGR), 500 mg; indomethacin, INDO (Metacen, Chiesi Farm., Italy) 50 mg; piroxicam, PIR (Feldene, Pfizer, USA) 40 mg. Blood was drawn, while fasting, before and two hours after the drug intake, into sodium citrate 3.8% (9:1).

*Platelet and leukocyte preparation:* Citrated blood was centrifuged at 100 xg for 18 min to obtain platelet rich plasma (PRP). Platelet poor plasma (PPP) was obtained after further centrifugation at 600 xg for 18 min. After the removal of PRP and PPP, the residue was processed for leukocyte isolation by adding a 1:1 volume of citrated phosphate buffered saline (PBS, Flow Labs., U.K.). PMN and MNL were separated using the standard technique of dextran (Dextran T-500, Pharmacia Fine Chemicals, Uppsala Sweden) sedimentation, centrifugation on Ficoll Paque (Pharmacia) and hypotonic lysis of red cells (11). Isolated cells were resuspended at 10\(^6\)/ml in PBS containing 0.05% glucose and 0.25% bovine serum albumin (Sigma, St. Louis, Mo, USA). Leukocyte and platelet counts were performed by phase contrast microscopy. All PMN preparations contained more than 97% PMN, platelet contamination being less than 1%, as determined by phase contrast microscopy. MNL suspensions consisted of 10–38% monocytes, as defined by cytochemical reactivity for alpha-naphthyl-acetate esterase (12), the remainder being lymphocytes with minimal (<3%) PMN contamination. Platelet contamination in MNL preparations was less than 1:8. Cell viability was determined by Trypan blue exclusion.

*Platelet studies:* Platelet aggregation was carried out in PRP samples by the Born turbidimetric technique (13), using threshold concentrations of collagen (Mascia Brunelli, Milan, Italy) and adrenaline (Angelini S.P.A., Roma, Italy), as previously described (14). Determination of thromboxane B\(_2\) (TXB\(_2\)) level was performed in PRP samples stimulated with 10 \(\mu\)g/ml collagen, using a specific radioimmunoassay (15).

*PMN and MNL aggregation studies* were performed, using a modification (16) of the method originally described by Craddock (17), in an Elvi aggregometer (Elvi Logos, Milan, Italy), connected to a linear recorder (Omniscribe, Texas Instruments, Tx. USA). The following aggregating agents were used: calcium ionophore A23187 (Calbiochem, Behring Corp., La Jolla, Ca, USA) and n-formyl-methionyl-leucyl-phenylalanine (fMLP, Sigma, St. Louis,
Mo, USA). Stock solutions, in dimethylsulfoxide and ethanol for fMLP and calcium ionophore A23187 respectively, were divided into aliquots, kept at -80°C and dilutions freshly made in PBS. For fMLP induced aggregation PMN samples were preincubated at room temperature with 5 µg/ml cytochalasin B (Sigma) for 3 min. Leukocyte (7.5 x 10⁶ cells/ml) aggregation was carried out in the presence of CaCl₂ (1.2 mM) and MgCl₂ (0.4 mM). The aggregation induced by calcium ionophore A23187 was expressed as the amplitude (cm) calculated at 5 min after the addition of the stimulus, using a scale of 100, in which the cell suspension was 10 and the cell poor blank was 100, whereas the aggregation elicited by fMLP was measured by calculating the area under the aggregation curve, with a graphic tablet connected to a personal computer (Apple IIe, Apple Computer, Ca, USA) and the results were expressed in cm²/min.

**Arachidonic acid metabolism by PMN and MNL:**
PMN and MNL samples (1 ml, 5 x 10⁶ cells), were incubated at 37°C (1000 rpm) for 1 min, in the presence of the divalent cations, as described in the previous section, and stimulated by 10 µM calcium ionophore A23187. The incubation was carried out for 7 min. Incubation time and calcium ionophore concentration were chosen on the basis of dose and time response curves. In the selected experimental conditions, the formation of 5-LO products was at plateau level. For TXB₂ determination the incubation was stopped with 2.5 ml methanol. TXB₂ levels were determined using a specific radioimmunoassay (15). For TXB₂ determination the incubation was stopped with 2.5 ml methanol. TXB₂ levels were determined using a specific radioimmunoassay (15).

For LO product determination the incubation was stopped by acidification (pH 3) with 1 M citric acid and by adding 1.5 ml methanol. To each sample 750 pmoles PGB₂ (Upjohn Co, Kalamazoo, Mi, USA) and 600 pmoles oleic acid (Sigma) were added as internal standards for LTB₄ and LTC₄ and for 5-HETE respectively. Synthetic LTB₄, LTC₄ and 5-HETE were purchased from Upjohn Co (Kalamazoo, Mi, USA). The various standards were purified by high pressure liquid chromatography (HPLC, Gilson, mod.330, connected with a UV detector) and the purity was assessed spectrophotometrically by UV absorbance. Supernatants were filtered through a Sep-pak C18 cartridge (Waters Associates, Milford, Mass, USA) previously washed according to Powell (18).

Leukotrienes were eluted with methanol:water:acetic acid 70:30:0.01, containing tetrabutylammonium phosphate 5 mM (PIC-A, Waters Associates, Milford, Mass, USA) and pH adjusted to 5.7 with acetic acid, whereas monohydroxyacids were eluted with ethylacetate (19). Elution fractions were evaporated under N₂ and injected into the HPLC column under the following conditions: column (250 x 4.6 mm), packed with Spherisorb ODS2 5 µm (Phase Separation, U.K.); mobile phase for LTB₄ and LTC₄ as used for the elution of the same compounds; flow rate for LTB₄ and LTC₄ 1 ml/min and for 5-HETE 1.5 ml/min. The retention times of the compounds were the following: PGB₂ 8 min; LTC₄ 10 min; LTB₄ 14 min; oleic acid 29 min; 5-HETE 41 min. The detector was at 280 nm for LTB₄ and LTC₄ and at 235 nm for 5-HETE respectively. A calibration curve was prepared for each analysis by adding 750 pmoles PGB₂, 600 pmoles oleic acid and increasing amounts of LTB₄, LTC₄ and 5-HETE to the unstimulated samples. Each standard sample was then processed and analyzed. For each 5-LO metabolite a linearity curve was obtained by plotting the ratio of peak intensity of internal standard against the concentration of the added 5-LO products. The regression coefficient was 0.99 for all 5-LO metabolites. The amounts of 5-LO metabolites in the biological samples were calculated from the peak ratios on the basis of the slope of the linearity curve. The detection limit range was 6-8 pmoles for LTB₄ and 1 LTC₄ and 8-10 pmoles for 5-HETE. Superoxide anion generation:

Release of O₂⁻ was measured by reduction of ferricytochrome c (Sigma), monitored at 550 and 468 nm in a dual beam recording spectrophotometer (Shimadzu, Kyoto, Japan), according to Babior (20). Each 1 ml cell suspension contained 3 x 10⁶ PMN or MNL, CaCl₂ 0.6 mM, MgCl₂ 0.1 mM and 1 mg/ml ferricytochrome c dissolved in saline. Samples were preincubated at 37°C with 5 µg/ml cytochalasin B for 10 min and stimulated with fMLP 10⁻⁷ M. Incubations were carried out for 30 min. The amounts of reduced cytochrome c, found in the presence of 30 µg/ml superoxide dismutase (SOD, Sigma), were subtracted from samples without SOD to establish specificity for O₂⁻. A millimolar extinction coefficient of 2.1 x 10³ mol x 1⁻¹ x cm⁻¹ for the difference between the oxidized and the reduced forms was used to calculate superoxide production. The results were expressed as nmol O₂⁻/10⁶ cells/30 min.
Statistical analysis
The paired t-test was used to compare values obtained before and after the drug intake.

Results
Platelet aggregation and TXB₂ formation: 2 hours after administration, ASA, INDO and PIR inhibited the aggregation of platelets, elicited by threshold concentrations of collagen and adrenaline, by more than 70% (Table 1).

| Table 1 Effects of NSAIDs on PRP Aggregation Induced by Collagen and Adrenaline. |
|---------------------------------|-----------------|-----------------|-----------------|
|                                  | **collagen**     | **adrenaline**  |
|                                  | % (aggregation at 3') | % (aggregation at 3') |
| **Baseline**                    | **2 hrs**       | **%I**         |
| ASA (n = 5)                     | 55.2 ± 6.3      | 4.5 ± 2.2*     | 92              |
| INDO (n = 5)                    | 60.0 ± 1.8      | 1.0 ± 0.7*     | 98              |
| PIR (n = 5)                     | 64.4 ± 2.6      | 3.3 ± 1.7*     | 95              |

PRP aggregation was induced by threshold concentrations of collagen and adrenaline (see results)

n = number of the subjects, %I = inhibition percent.
*p < .001 vs baseline

The threshold aggregating concentration (TAC) was defined as the minimal concentration of aggregating agent able to elicit 40% aggregation for collagen and the second wave for adrenaline. In the subjects participating in the study, TACs for collagen were between 0.125 and 0.5 &μl and 0.06–0.5 &μg/ml for adrenaline. In addition, ASA and INDO administration markedly inhibited the formation of TXB₂ in PRP (-96%) stimulated with collagen, whereas in subjects taking PIR, TXB₂ formation was inhibited by only 40% (Table 2).

PMN and MNL aggregation: A23187 added to cell suspensions aggregated both PMN and MNL. The aggregation of PMN and MNL induced by increasing amounts (8–24 &μM) of A23187 was not affected by the administration of the drugs (data not shown).

In our experimental conditions fMLP induced the aggregation of PMN only. As shown in Table 3, the drugs differently influenced the aggregation of PMN elicited by fMLP 10 &μM: an increase in the amplitude of aggregatory tracings was detected after INDO and PIR (p < .05 and p < .005 respectively), whereas no effect could be detected after ASA.

Superoxide anion generation: fMLP (10⁻⁷ M) induced the formation of O₂⁻ in both leukocyte subpopulations. The synthesis of O₂⁻ by MNL was lower with respect to PMN, when compared in terms of absolute cell number. O₂⁻ formation by PMN and MNL was significantly increased (p < .05 and p < .001 respectively) following ASA intake, whereas it was unaffected by the

| Table 2 Effects of NSAIDs on TXB₂ Formation by PRP Stimulated by 10 &μg/ml Collagen. |
|---------------------------------|----------------|----------------|
|                                  | **TXB₂**        | **%I**         |
|                                  | (pmol/ml)       |                |
| **Baseline**                    | **2 hrs**       | **%I**         |
| ASA (n = 5)                     | 200 ± 32.8      | 1.3 ± 0.4*     | 99              |
| INDO (n = 5)                    | 248 ± 19.5      | 13.1 ± 2.5*    | 95              |
| PIR (n = 5)                     | 304 ± 37.0      | 185.0 ± 39.0** | 40              |

n = number of the subjects
*p < .001 vs baseline
**p < .05 vs baseline

PMN

| Table 3 Effects of NSAIDs on PMN Aggregation Induced by fMLP. |
|---------------------------------|-----------------|-----------------|
|                                 | **fMLP 10⁻⁷ M** (cm²/min) | **2 hrs**       |
|                                 | **Baseline**    | **ASA (n = 5)** |
| ASA (n = 5)                     | 8.8 ± 1.3       | 9.4 ± 1.7       |
| INDO (n = 5)                    | 11.4 ± 1.7      | 12.7 ± 1.9*     |
| PIR (n = 5)                     | 7.8 ± 0.6       | 10.1 ± 1.0**    |

*p < .05 **p < .001
Values represent the mean ± S.E.M. of PMN aggregation evaluated at the baseline and 2 hours after the intake of the drug.

Table 4 Effects of NSAIDs on Superoxide Anion Generation by PMN and MNL.

<table>
<thead>
<tr>
<th>PMN</th>
<th><strong>Baseline</strong></th>
<th><strong>2 hrs</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>ASA (n = 5)</td>
<td>55.2 ± 7.9</td>
<td>69.0 ± 8.9*</td>
</tr>
<tr>
<td>INDO (n = 5)</td>
<td>73.7 ± 11.4</td>
<td>78.2 ± 11.8</td>
</tr>
<tr>
<td>PIR (n = 5)</td>
<td>85.2 ± 11.1</td>
<td>66.3 ± 11.2*</td>
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* p < .05 ***p < .001
Values represent the mean ± S.E.M. of O₂⁻ generation evaluated at the baseline and 2 hours after the intake of acetylsalicylic acid (ASA, 500 mg), indomethacin (INDO, 50 mg) and piroxicam (PIR, 40 mg). Data are expressed as nmol. O₂⁻·10⁶ cells/30 min. Cells were stimulated by fMLP 10⁻⁷ M.
administration of INDO. In subjects taking PIR, a significant (p < .05) decrease of O^-2 generation by PMN was noted. The generation of O^-2 in FMLP elicited MNL was not influenced by this latter drug (Table 4).

Arachidonic acid metabolism: different amounts of TXB2 were formed by PMN and MNL in response to stimulation with A23187. TXB2 formation by MNL was greater than that of similarly challenged PMN (0.83 ± 0.07 and 0.38 ± 0.08 pmol/10^6 cells for MNL and PMN respectively, n = 15, p < .001). TXB2 synthesis by stimulated PMN was reduced by more than 70% in subjects taking ASA and INDO. The extent of the inhibition of TXB2 formation in PMN of subjects taking PIR was lower with respect to ASA and INDO, and comparable to that recorded in platelets from the same subjects (−45%). As far as MNL were concerned, inhibition of TXB2 biosynthesis in subjects taking ASA was similar to that observed for PMN, whereas INDO reduced it only by 45%. Increased formation of TXB2 was detected in MNL of subjects who ingested PIR (Fig. 1).

The incubation of PMN and MNL with A23187 resulted in the activation of the 5-LO pathway with the concomitant formation of 5-HETE, LTB4 and LTC4. Synthesis of 5-LO products by MNL was lower than that of similarly challenged PMN (5-HETE: 30.7 ± 5.3 and 78.3 ± 13.1, n = 15, p < .005; LTB4: 10.9 ± 1.6 and 31.1 ± 4.3, n = 15, p < .001; LTC4: 24.9 ± 2.8 and 67.7 ± 8.6, n = 15, p < .001, expressed as pmol/10^6 MNL and PMN respectively).

Figure 2 shows the mean levels of 5-LO products in PMN stimulated by ionophore A23187 before and after the intake of the three considered NSAIDs. 5-HETE, LTB4 and LTC4
synthesis by PMN, two hours after the intake of ASA, INDO and PIR were comparable to baseline. Similarly, the synthesis of 5-LO products by MNL stimulated with A23187 was not influenced by ASA and INDO. In subjects who were administered PIR, a significant increase (p < .05) of 5-HETE and LTC₄ synthesis by MNL was detected, whereas LTB₄ formation was not affected by this drug (Fig. 3).

Discussion
The results reported in this study show that ASA, INDO, and PIR, differently affect AA metabolism by PMN, MNL and platelets. These drugs inhibited TXB₂ synthesis by these cells to different extents. TXB₂ formation by platelets and PMN was equally inhibited by ASA and INDO, whereas PIR inhibited it by 40%. When the inhibitory effect of the three NSAIDs on TXB₂ synthesis by PMN and MNL was compared, it appeared that the degree of inhibition of the CO enzyme achieved 2 hours after ASA administration, was comparable for the two leukocyte subpopulations, whereas the inhibition exerted by INDO was greater for PMN than for MNL. In contrast PIR did not inhibit, but even stimulated, the formation of TXB₂ by MNL. Although the interaction of INDO and PIR with the leukocyte CO requires further investigation, the reported data suggest that the tested NSAIDs display differential activities on the enzyme(s) responsible for TXB₂ formation.

Data in the literature on the effects of NSAIDs on leukocyte 5-LO product formation are mainly related to the effects of these compounds on the synthesis of lipooxygenase metabolites by PMN. Since mononuclear cells are known to be involved in the pathogenesis of chronic inflammatory diseases, and in particular monocytes have been shown to be able to metabolize AA via the CO and the LO enzymes, upon stimulation with A23187 (8), the effects of ASA, INDO and PIR, were evaluated not only in PMN, but also in mononuclear cells.

Results on the effects of the NSAIDs on 5-LO product synthesis by PMN and MNL stimulated with A23187 indicated that the inhibitory activities of ASA and INDO were selective for the CO enzyme, since no difference was recorded in the formation of 5-LO metabolites two hours after the administration of the drugs. Although PIR intake did not modify the formation of 5-LO product by PMN, the drug selectively increased 5-HETE and LTC₄ synthesis by MNL.

In vitro studies have suggested that in conditions of CO inhibition, an increased formation of 5-LO products by PMN, due to the shunting of the substrate from the prostaglandin into the leukotriene pathway, could be detected (21, 22). In addition, stable prostaglandins have been shown, in vitro, to inhibit LTB₄ release from activated neutrophils (23). Thus, in our study, following the administration of NSAIDs (ASA
ARACHIDONIC ACID METABOLISM IN PERIPHERAL POLYMORPHONUCLEAR AND MONONUCLEAR LEUKOCYTES

and INDO), which almost completely suppressed cyclic AA metabolites by activated PMN, an increased formation of 5-LO metabolites would have been expected. This was not true for A23187 stimulated PMN and MNL after ASA and INDO intake, suggesting that no control of leukotriene formation by prostaglandins was exerted in these experimental conditions. In contrast, PIR, which increased TXB₂ formation by MNL, selectively raised 5-HETE and LTC₄ synthesis in these cells, enhancing the overall amount of AA converted. The inhibition of CO enzyme exerted by NSAIDs in PMN and MNL, however, was not related to modifications of leukocyte function. In fact, two of the tested NSAIDs inhibited, although to a different extent, TXB₂ formation in the considered cells, without affecting the aggregation of PMN and MNL induced by calcium ionophore.

Because calcium ionophore A23187 is known to stimulate protein-kinase C directly, through calcium mobilization (24), the aggregation of PMN was also studied using the chemoattractant fMLP which is known to act by a receptor-mediated mechanism (25). Using this stimulus, PMN aggregation was either unaffected (ASA) or even significantly increased (PIR, INDO). Also, the effects of NSAIDs on O⁻² generation by PMN and MNL in response to fMLP were not consistent. PIR significantly reduced O⁻² generation by PMN but did not influence this parameter in MNL. No effect or even stimulation of oxygen burst was observed after ASA and INDO respectively.

Kaplan et al. (26), previously reported that INDO and PIR in vitro and ex vivo inhibited neutrophil aggregation induced by fMLP, whereas O⁻² was affected by PIR only. Even if these authors used a treatment schedule different from ours, the findings reported in the present study, as far as O⁻² is concerned, are in agreement with their results. At variance with them, we did not find an inhibitory effect of these drugs on fMLP induced aggregation. It is thus conceivable to hypothesize that a different dosage and/or a repeated administration of the drugs, as in the schedule adopted by Kaplan et al., might be responsible for the different cellular responses to the various NSAIDs.

Moreover, a link between changes of functional parameters of elicited PMN and MNL after the administration of the drugs and the formation of 5-LO metabolites is difficult to assess, since the amounts of LTB₄ formed by these cells upon stimulation by fMLP have been reported to be relatively low and often below the detection limit of the techniques employed (27).

It is not possible, on the basis of our data to explain the mechanism by which PIR increases 5-HETE and LTC₄ synthesis by MNL. However, since LTC₄ possesses vasoconstrictor activity, thus playing a role in the control of oedema (28), the selective increase of this metabolite, following PIR intake, might be relevant for the antiinflammatory properties of this compound.

It can be concluded that single oral administrations of ASA, INDO and PIR, which inhibit, although to a different extent, both platelet and leukocyte CO, do not affect the metabolism of AA via the 5-LO pathway in PMN. Similar findings are observed in MNL from the same subjects, with the exception of 5-HETE and LTC₄ after PIR intake. These changes cannot be related to modifications of cellular function following NSAIDs intake, indicating that other mechanisms, such as movement of intracellular calcium and/or activation of the phosphatidylinositol cycle which precedes the metabolism of AA are responsible for the effects displayed by these compounds.

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References


