Mechanism of Inhibitory Action of Cyclooxygenase-2 Inhibitors in Human Platelets

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Abstract: This study was conducted to investigate the effects of nimesulide in platelet aggregation. It shows that nimesulide (1-100 μM) inhibited platelet aggregation induced by adrenaline, (20-200 μM). It also inhibited thromboxane A2 (TXA2) formation by platelets at low concentration (IC50: 1 μM). However, much lower concentrations of nimesulide (0.01-0.1 μM) potentiated the aggregatory response of subthreshold concentrations of adrenaline (0.2-2 μM). Such an effect was blocked by Cu2+-channel blockers, verapamil and diltiazem (IC50: 7 and 46 μM, respectively), nitric oxide donor, SNAP (IC50: 2 μM) and cinecholine (10 nM) but not by genistein (up to 10 μM). These results are indicative of the concentration-dependent dual effects of nimesulide on human platelet aggregation. The synergistic effect of low doses of nimesulide and adrenaline seems to be mediated through inhibition of multiple signaling pathways.

Key words: Cyclooxygenase inhibitors, platelet aggregation, nimesulide, NS-398

INTRODUCTION

Cyclooxygenase (COX); prostaglandin-endoperoxide synthase, EC 1.14.99.1 converts arachidonic acid prostaglandins (PG) H2, which is then further metabolized by other enzymes to various PGs, prostacyclin, and thromboxanes. COX-1 is expressed constitutively whereas COX-2 is induced in cells exposed to proinflammatory agents including cytokines, mitogens and endotoxin. Human platelets predominantly express COX-1. Platelet aggregation is well known to be caused by various physiological agents, such as thrombin, adrenaline, ADP, platelet-activating factor (PAF), arachidonic acid (AA) and collagen. Most of these agonists interact with serpine receptors coupled with GTP-binding proteins (G-proteins) in platelets[10]. Activation of either Gi (e.g. with adrenaline on α2-adrenoceptors) or Gq protein (with TXA2 or PAF) has been shown to initiate the platelet aggregation process[1-3]. One common point in this cascade is elevation in cytosolic Ca2+ levels which occurs as a consequence of its release from intracellular stores or due to an increase in Ca2+-influx through platelet membrane. Thus, Ca2+ plays a crucial role in the signaling cascade mediating platelet aggregation[10].

Previous studies have shown that nimesulide exerts marked anti-inflammatory effects mainly through inhibition of COX-2 activity and thus prostaglandin biosynthesis[5-7]. Several studies have shown that nimesulide decreases synthesis of PAF and leukotriene B4 (LTB4) in neutrophils[10] and inhibits phosphodiesterase (PDE) IV[10]. Besides, nimesulide has been reported to decrease Ca2+ influx in neutrophils stimulated with calcium ionophore, A-23187 and FMLP[10]. Human platelets express COX-1. Since nimesulide is considered to be a selective inhibitor of COX-2[5], it has not been studied in detailed in relation to its effects on platelets. Here present study shows that nimesulide inhibits platelet aggregation mediated by various agonists. In contrast at low concentration it potentiates the aggregatory response of low (subthreshold) concentrations of adrenaline.

MATERIALS AND METHODS

Arachidonic acid, PAF, adrenaline, collagen, verapamil and diltiazem were purchased from Sigma Chemical Co., USA. Nimesulide and NS-398 were obtained from Cayman Chemicals Co. USA. 14C] Arachidonic acid (sp. act. = 58 m Ci/mmol) and 1H] thromboxane B2 (>120 Ci/mmol) were obtained from Amersham International Plc, UK.

Preparation of human platelets: For platelet aggregation studies, blood was taken by veinpuncture from normal human volunteers (n=18) reported to be free of medication

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Table 1: Effects of various substances on platelet aggregation induced by synergistic interaction of subthreshold concentrations of Adrenaline (1.3±0.45 μM) and Nimesulide (0.1 μM). (n = 5)

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>IC50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Verapamil</td>
<td>7±2</td>
</tr>
<tr>
<td>Diltiazem</td>
<td>46±8</td>
</tr>
<tr>
<td>SNAP</td>
<td>240±3</td>
</tr>
<tr>
<td>Wortmannin</td>
<td>0.8±0.2</td>
</tr>
<tr>
<td>Cinchozine</td>
<td>340±5</td>
</tr>
<tr>
<td>Gentienin</td>
<td>NE</td>
</tr>
</tbody>
</table>

NE indicated no effect

for 1 week. Blood samples were mixed with 3.8% (w/v) sodium citrate solution (9:1) and centrifuged at 260 g for 15 min at 20°C to obtain platelet rich plasma (PRP). Platelet count was determined by phase contrast microscopy and all aggregation studies were carried out at 37°C with PRP having platelet counts between 2.5 and 3.0x10^4 mL^-1 of plasma[11].

Measurement of platelet aggregation: Platelet aggregation was monitored using a Dual-channel Lumi-aggregometer (Model 400 Chronolog Corporation, Chicago, USA) using 0.45 mL aliquots of PRP. The volume was made up to 0.5 mL with sodium chloride (0.9%, w/v in water) or test drug and incubated for 1 min before challenge with the aggregating agent[12]. Aggregation was induced with adrenaline (200 μM), AA (0.75 mM), collagen (20 μM mL^-1) or PAF (0.8 μM). Platelets from about 30% healthy volunteers were poorly responsive to adrenaline. The resulting aggregation was recorded and expressed as percent effect compared with control after min challenge. The details of treatment of PRP with various inhibitors is given in figure legends. Statistical difference between control and drug-treated platelet preparations were determined by Student’s t-test.

Arachidonic acid metabolism by platelets: The formation of TXA2 in platelets was estimated as described[13]. The PRP was centrifuged at 1200 g for 20 min and the sedimented platelets were washed twice with an ice-cold phosphate buffer (50 mM, pH 7.4) containing sodium (0.15 M) and EDTA (0.2 mM). After centrifugation, washed platelets were resuspended in the same buffer without EDTA and homogenized at 4°C for 15 sec. The homogenate was centrifuged at 1200 g for 20 min and 300 μL of the supernatant (containing 0.4 mg of protein) was incubated with 10 μg unlabelled AA and 0.1 μCi [1-14C]-AA in the presence and absence of the test compound. After 15 min with gentle shaking in air at 37°C, the reaction was stopped by adding 0.4 mL of citric acid (0.4 M) and ethyl acetate (7.0 mL). After mixing and centrifuging at 600 g for 5 min at 4°C, the organic layer was separated and evaporated to dryness under nitrogen. Residues were dissolved in 40 μL were applied to silica gel G thin layer chromatography (TLC) plates (Analtech, Delaware, USA). The AA, TXB2 (a stable degradation product of TXA2) and 12-HETE standards were spotted separately. The plates were developed in ether/petroleum ether [boiling range 40-60°C] acetic acid (50:50:1 by volume) to a distance of 17 cm. Radioactive zones were located and quantified by use of a Berthold T.L.C. linear analyzer and chromatography data system (Model LKB511, Berthold, W. Germany).

RESULTS

Pretreatment of platelets with nimesulide at pharmacological concentrations (1-100 μM) inhibited platelet aggregation induced by the PAF and adrenaline with IC50 of 25 and 50 μM, receptively (Fig. 1). The maximum response of platelets to adrenaline varied between 20-200 μM. However, aggregation induced by ADP (4.2 μM), collagen 20 μM mL^-1) and arachidonic acid (0.75 mM) and Ca^2+ ionophore, A-23187 (6 μM) was not inhibited by nimesulide at comparable concentrations. In contrast, low concentrations of nimesulide (0.01-0.1 μM) potentiated the platelet aggregation induced by subthreshold dose of adrenaline which varied between 0.2-2 μM. Such an effect was not seen with other agonists (PAF, AA and collagen).

To examine whether the observed effect of nimesulide-mediated potentiation of adrenaline is a general mechanism or is specific to nimesulide, we used other inhibitors of COX-1 (indomethacin) and COX-2 (NS-398). None of these inhibitors in a wide range of concentrations had any synergistic effects with these agonists at any of the concentrations (data not shown). Since nimesulide is shown to inhibit prostaglandin synthesis in neutrophils[7]. The present study also investigated the effect of nimesulide on TXA2 synthesis by platelets. The results show that nimesulide inhibits TXA2 formation in platelets (IC50: 10 μM).

Earlier it was shown that adrenaline enhances Ca^2+ influx in platelets[14], it used Ca^2+ channel blockers, to examine if Ca^2+ influx is involved in adrenaline-nimesulide mediated platelet aggregation. It was evident from Fig. 2 that nimesulide-mediated potentiation of aggregation induced by subthreshold concentration of adrenaline is blocked by Ca^2+ channel blockers, verapamil and diltiazem and cinchozine and cinchozine in a concentration-dependent manner (Table 1). However, using Ca^2+-free medium or chelating
Fig 1: Concentration-dependent inhibitory effects of nimesulide on platelet aggregation induced by PAF (800 nM) and adrenaline (100-200 μM). Platelets pretreated with varying doses of nimesulide for 1 min were treated with platelet agonists (PAF or adrenaline or others) and aggregation recorded for 5 min.

Fig 2: Synergistic effects of low concentrations of nimesulide (0.1 μM) and adrenaline (2 μM) on platelet aggregation, a representative experiment (A). The effect in (A) is inhibited by verapamil (B), diltiazem (C) and cinchonine (D). Control (B-D) indicates treatment of platelets with subthreshold dose of adrenaline (0.2-2 μM) plus nimesulide (0.1 μM).
extracellular Ca" with EDTA did not show comparable potentiation of aggregation (data not shown).

DISCUSSION

The results of the present study shows that nimesulide inhibits TXA2 formation and inhibits platelet aggregation mediated by adrenaline. Previous studies have shown that nimesulide exerts a marked anti-inflammatory effects mainly through inhibition of COX-2 actively and thus prostaglandin biosynthesis. The inhibitory activity of nimesulide on platelet aggregation mediated by various agonists may be attributed not only to inhibition of COX-1 actively but also to many of its other diverse actions. Previous studies have shown multiple unrelated effects of nimesulide in various cell types including: elevation of cytosolic cAMP levels, decrease in Ca" influx and PKC activity and inhibition of TXA2 and PAF formation. In platelets, all these mechanisms are reported to decrease platelet aggregation.

Nimesulide is commonly used in inflammatory conditions like osteoarthritis and rheumatoid arthritis. However, our results show that nimesulide also exerts its effects on platelets which predominantly express COX-1. Recent studies of Riendeau et al. have concluded that COX-1 inhibitory effect could be detected for all selective COX-2 inhibitors tested by use of sensitive assay at low substrate concentrations. Depending on the assay system and cell types, nimesulide is reported to be 5-90 times more selective for microsomal COX-2 but equipotent on both COX-1 and COX-2 in whole cell assay systems.

The mechanism involved in synergism between low concentrations of nimesulide and adrenaline during platelet aggregation is not fully understood. Present results points towards the activation of Ca" signaling as this effect was abolished by pretreatment of platelets with Ca"-channel blockers suggesting an effect mediated through activation of Ca" signaling (Fig. 2 and Table 1). Elevation in cytosolic Ca" can be achieved by activation of Gq or Gi protein through stimulation of phospholipase C or by increasing the Ca" influx. It seems that nimesulide-adrenaline synergism increases the Ca" influx. This is further supported by the findings that cinchonine at extremely low concentrations inhibits this synergism. Recently study showed that cinchonine, an alkaloid isolated from cinchona bark and commonly used in combination therapy of resistant malaria caused by Plasmodium falciparum, exhibits its antiplatelet effects by inhibiting Ca" signals.

Most studies on platelets use pharmacological or full doses of agonists for observing their effects or studying the activated signal transduction pathways. It seems that the magnitude of receptor occupancy determines the final recruitment of signaling pathways. Since low receptor occupancy may occur more commonly than full occupancy, the activation of signaling mechanisms in operation at low concentrations of agonist are more physiologically relevant. Such a mechanism may be of great clinical significance in patients suffering from cardiovascular or thromboembolic disorders. Platelet response to adrenaline is reported to increase during stress and hypercholesteremia.

The potentiation of platelet aggregation induced by low concentrations of adrenaline is specific only to nimesulide as the effect was not produced by other COX-2 inhibitor, NS398. The reason for this effect is not fully understood but it seems to involve stimulation of Ca" signaling. A similar type of synergistic mechanism was observed when chelerythrine, a selective PKC inhibitor was combined with adrenaline. It is known that adrenaline interaction with α2-adrenoceptors on platelets dissociates the βγ-subunits of G-proteins in sufficient quantities which in turn can activate PLC pathway. Other possibilities of adrenaline-mediated effects in platelets include some other targets like tyrosine kinase, phosphatidylinositol 3-kinase, or Syk protein. Present results rule out these possibilities as the observed effects could be inhibited by genistein (tyrosine kinase inhibitor) or wortmannin, a selective inhibitor of PI 3-kinase (Table 1). The concentration of adrenaline used in the present studies is quite low. It seems that a minimal level of agonist stimulation is required for this phenomenon to occur as low dose of nimesulide alone had no effect on platelet aggregation.

In conclusion, it can be demonstrated that nimesulide in pharmacological concentrations inhibit platelet aggregation but at low doses potentiates the aggregation response to subthreshold concentration of adrenaline. This effect is blocked by Ca"-channel blockers indicating an effect on Ca" signaling. Since PKC and Ca" act in synergy and this study provide an indirect evidence for the involvement of Ca" signaling, it may be interesting to investigate the measurement of cytosolic Ca" and PKC activity under this condition.

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REFERENCES


