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Involvement of Cyclooxygenase, Phospholipase C and Map Kinase Pathways in Human Platelet Aggregation Mediated by the Synergistic Interaction of Platelet Activating Factor and Arachidonic Acid

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Abstract: This study was conducted to examine the mechanism(s) of synergistic interaction of platelet-activating factor (PAF) and arachidonic acid (AA) in platelet aggregation. We found that the synergism in platelet aggregation mediated by subthershold concentration of PAF (5-40 nM) and AA (0.1-0.2mM) was inhibited by PAF receptor blocker (WEB 2086, IC_{50}= 0.6 μM) showing that the effect is receptor mediated. To examine the role of the downstream signaling pathways, we found that such an interaction was inhibited by calcium channel blockers, diltiazem (IC_{50}= 15 μM) and verapamil (IC_{50} = 20 μM), as well as by low concentrations of phospholipase C (PLC) inhibitor (U73122; IC_{50} = 6 μM) and MAP kinase inhibitor, PD 98059 (IC_{50}= 3.5 μM). Inhibitor of AA-cyclooxygenase (flurbiprofen: IC_{50} = 3 μM) also inhibited PAF and AA induced aggregation showing the involvement of COX pathway. On the other hand, herbimycin A, a specific inhibitor of tyrosine light chain kinase (TLCK) and SNAP, a nitric oxide (NO) donor also inhibited PAF and AA-induced aggregation with IC_{50} values of 15 and 1.7 μM respectively. However, the inhibitor of protein kinase C (chelerythrine; 20 μM)) had no effect on aggregation induced by PAF and AA. These data suggest that the synergism between PAF and AA in platelet aggregation involves activation of PLC, COX, MAP kinase, TLCK and NO signaling pathways.

Key words: Platelet aggregation, arachidonic acid, platelet activating factor, cyclooxygenase, phospholipase C

Introduction

Different agonists act synergistically in platelet aggregation and up to date, few studies have been done in human platelets on the cooperative effects of Platelet Activating Factor (PAF) and Arachidonic Acid (AA). Interactions of different stimuli could be physiologically important *in vivo* and in isolated platelet defects in order to prevent bleeding complications.

It is now well documented that most of the platelet agonists act largely through the stimulation of G-protein coupled receptors (GPCRs). Thromboxane A-2 (TXA_2), a potent vasoconstrictor and a mediator of platelet aggregation (Tazawa *et al.,* 1996), is a major AA metabolite in platelets and induces aggregation by binding to specific receptors on the platelet membrane (Okuma *et al.,*1996). TXA_2 receptor stimulation causes phospholipase C activation and increase in [Ca^{2+}] via G-protein of the Gq-11 family leading to aggregation by Ca^{2+} influx (Ohkubu *et al.,* 1996). It has been postulated that the main proaggregatory effects of TXA_2 are mediated by the inhibition of adenylate cyclase-cAMP complex (Somova, 1996).

Most of the actions of PAF have been reported to occur through the stimulation of pertussis toxin insensitive G-proteins (Gq-11) resulting in the stimulation of phospholipase C (PLC) and thus generation of second messenger diacylglycerol (DAG) and inositol-1, 4,5-triphosphate (IP_3), which results in the activation of protein kinase C (PKC) and the mobilization of intracellular Ca^{2+}, respectively (Obberghen-Schilling and Pouyssegur, 1993). Both Ca^{2+} and PKC stimulate platelet aggregation and also elicit synergism in platelets (Crabos *et al.,*1992). Consistent with the potential involvement of G_q-PLC pathway, the deficiency of G_q protein in transgenic mice leads to impairment of agonist-induced platelet aggregation (Offermanns *et al.,* 1997).

PAF is a strong platelet activator and human platelets show high affinity binding sites for this agonist. It induces adhesion of platelets to the endothelium in the presence of activated leukocytes (Hirafuji and Shinoda, 1991). PAF is also known to play an important role in various patho-physiological conditions that include modulation of blood pressure, cardiac dysfunction in cardiac anaphylaxis, hemorrhagic, traumatic and shock syndromes (Anderson *et al.,* 1991; Montrucchio *et al.,* 2000).

In addition, PAF also stimulates TXA_2 production in human platelets. It enhances vasoconstriction of the

coronary arterioles (DeFily *et al.,* 1996) and at the inflammatory coronary lesions *in vivo* by itself as well as in a synergistic manner with other agonists like epinephrine and 5-HT (Shah *et al.,* 2000). Because of the close interaction between many agonists and their importance in thrombosis, hypertension and atherosclerosis, this study was conducted to examine the synergism between PAF and AA to elucidate the possible signaling mechanism(s) involved during this synergism. We show that synergistic interaction of PAF and AA is mediated through multiple signaling pathways including PLC-Ca^{2+}, COX, NO and MAP kinase pathways and is modulated by nitric oxide.

Materials and Methods

PAF, Arachidonic acid, diltiazem, verapamil, herbimycin A and chelerythrine were purchased from the Sigma Chemical Co. (St. Louis, Mo. USA). U73122 and SNAP were from Alexis LC Labs (UK). All other chemicals used were of the highest purity grade available.

Preparation of Human Platelets: Blood was taken by vein-puncture from normal human volunteers reported to be free of medication for one 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 3.0 $x10^8 ml^{-1}$ of plasma (Shah and Saeed, 1995).

Measurement of Platelet Aggregation: Aggregation was monitored using a Dual-channel Lumi-aggregometer (Model 400 Chronolog Corporation, Chicago, USA) using 0.45 ml aliquots of PRP. The final volume was made up to 0.5 ml with the test drug dissolved either in normal saline or appropriate vehicle known to be devoid of any effect on aggregation. Aggregation was induced with PAF and sub-threshold concentration was determined. To obtain the synergistic effect of PAF and AA, we added low concentrations of these agonists. The anti-aggregatory effects of different compounds were studied by pretreatment of PRP with various inhibitors for one min followed by addition of the sub-threshold concentrations of PAF and AA. The resulting aggregation was recorded for 5 min after challenge by the change in light transmission as a function of time. Once the anti-platelet activity of various inhibitors against agonists was established, dose-response curves were constructed to calculate the IC_{50} values of the agonists and inhibitors.

Thromboxane formation in platelets: Arachidonic acid metabolism and TXA_2 formation were studied with the co-addition of PAF (40 nM) and AA (0.2 mM) using methods described previously (Saeed *et al.*, 1997). For these studies, human blood platelets were routinely obtained in plastic bags containing 30-40 ml of concentrated PRP from The Aga Khan University Hospital Clinical laboratory, Karachi. Statistical analysis was done using Students-*t*-test. Differences were considered significant when probability (p) was $p<0.05$.

Results

The results demonstrated that treatment of PRP with PAF (5-800 nM) or AA (0.1-1.73 mM) showed concentration-dependent increase in platelet aggregation. However, simultaneous addition of subthreshold concentrations of PAF (40 nM) and AA (0.2 mM) exhibited a synergistic effect (Fig. 1A). Such an effect was comparable to that obtained by higher concentrations of PAF (800 nM) or AA (1.73 mM) alone. The synergism between PAF and AA was inhibited by pre-treatment of PRP with a potent PAF antagonist, WEB 2086 (IC_{50} = 0.6 μM) (Fig. 1B) indicating that the synergistic effect is receptor mediated. Recent studies show that concomitant activation of Gi and Gq protein-linked signaling pathways results in aggregation of human platelets. However, the present data demonstrate that activation of Gq protein by two different agonists at subthreshold concentrations is equally potent in eliciting the aggregation response by platelets. In platelets thromboxane A2 derived from PAF and AA cause stimulation of Gq protein followed by activation of PLC. We used PLC inhibitor (U73122) to examine if the PAF and AA mediated effects involved the activation of PLC. Results show that pretreatment of PRP with U73122 completely inhibited the synergistic effect of PAF and AA with an IC_{50} of 6 μM (Fig. 1C). Since activation of PLC leads to an increase in cytosolic Ca^{2+} due to its release from internal stores by inositol triphosphate (IP_3) or through store-depleted Ca^{2+} influx (Heemskerk and Sage, 1994), we examined the effect of Ca^{2+} channel blockers (verapamil and diltiazem) on platelet aggregation and found that the synergistic effect of PAF and AA was inhibited by both verapamil and diltiazem (IC_{50}=15 and 20 μM respectively) as shown in Fig. 1D.

As stimulation of the G-protein-Ca^{2+} cascade leads to mitogen activated protein (MAP) kinase signaling (Heemskerk and Sage, 1994), we used the selective MEK inhibitor PD 98059 in PAF and AA synergism. Results show that pretreatment of PRP with PD98059 exhibited strong inhibitory effects (IC_{50} = 3.5 μM) on platelet aggregation by co-addition of subthreshold concentration of PAF and AA (Fig. 2).

Since PD98059 has been recently reported to directly inhibit purified cyclo-oxygenase (COX-1 and 2) (Borsch-aubold *et al.*, 1998), we examined the effect of PD98059 on AA metabolism and TXA_2 formation (data not shown).

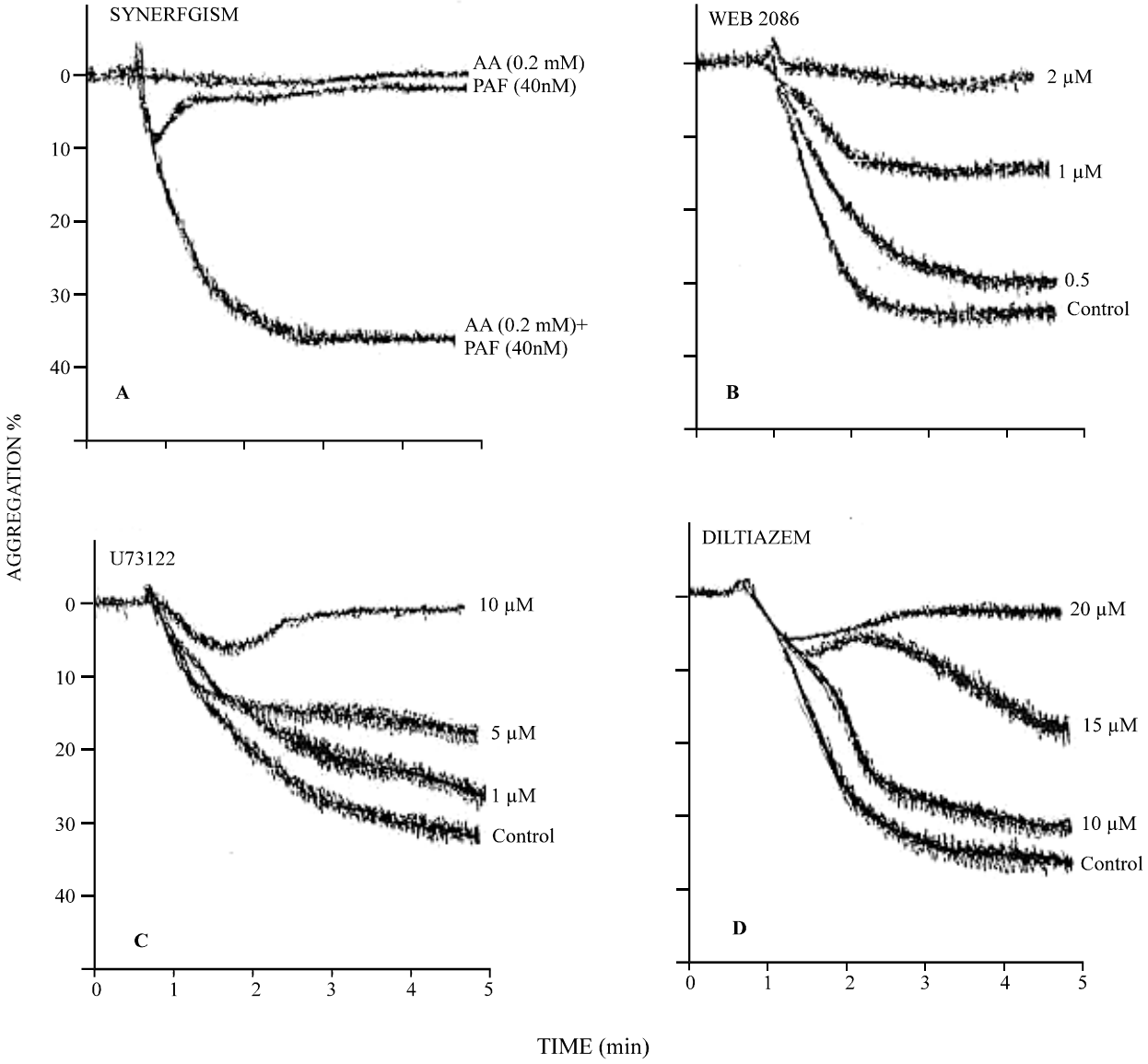

Fig. 1: (A) Tracings from representative experiments showing synergism of AA (0.2 mM) and PAF (40 nM). (B) The synergistic effect of AA and PAF on platelet aggregation is blocked by PAF receptor antagonist, WEB 2086. © phospholipase C inhibitor, U73122 and (D) calcium channel blocker, diltiazem. Inhibitors were added one minute before the agonists. Control means platelet aggregation induced by AA (0.2 mM) plus PAF (40 nM). n=5

An analysis of results show that PD98059 also inhibits agonist-induced TXA_2 production with an IC_{50} of 2.5 μM. To determine the role of COX be used flurbiprofen (AA-COX) inhibitor which inhibited PAF and AA induced aggregation with IC_{50} value of 3 μM (Fig. 3A). Recent studies indicate an important role of nitric oxide (NO) in modulating platelet aggregation (Shah *et al.*, 1999). Our results show that NO donor, SNAP, completely blocked platelet aggregation mediated by the synergistic interaction of PAF and AA (Fig. 3B). These data provide evidence in support of an important role for NO in negatively modulating the human platelet aggregation. Herbimycin A, a specific inhibitor of tyrosine kinase also inhibited PAF and AA-induced aggregation with IC_{50} of 15 μM indicating the involvement of tyrosine kinase in this cascade (Fig. 4). However, the inhibitor of protein kinase C (chelerythrine; 20 μM) had no effect.

Discussion

Platelet agonists produce their effects by interacting with

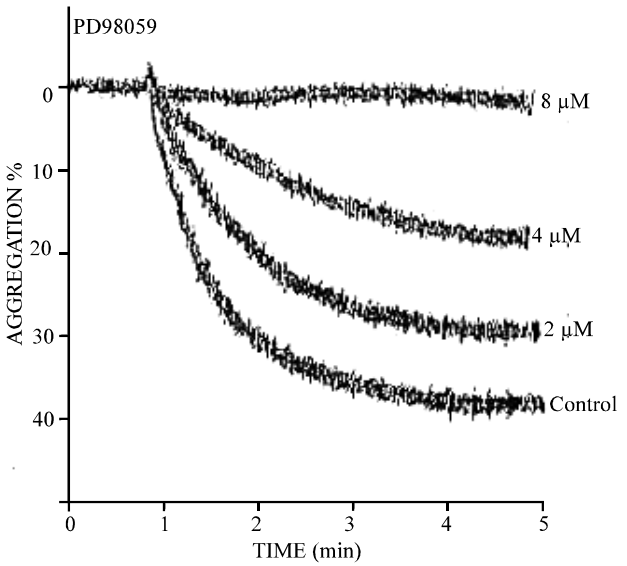

Fig. 2: The effect of MEK inhibitor, PD98059 on the synergistic interaction on AA (0.2 mM) and PAF (40 nM). n=7

G-protein coupled receptors and through activation of either Gq-PLC or Gi-adenylyl cyclase in platelets (Brass *et al.*, 1993; Siess, 1989). Both PAF and AA are the potent mediators of platelet aggregation. Platelet membrane contains specific PAF receptors, linked with Gq proteins. Arachidonic acid released by phospholipase A_2, the substrate for the sequential activities of platelet cyclooxygenase and thromboxane synthetase results in the formation of TXA_2. When AA is added to PRP it results in the formation of a prostanoid, TXA_2 formed via cyclooxygenase. TXA_2 in an autocrine fashion binds with surface TXA_2 receptors which coupled with Gq proteins stimulate PLC and further enhance platelet aggregation. Other compounds corresponding to the lipoxygenase (LOP) pathway 12-hydroxyeicosatetraenoic acid and 12-20-dihydroxyeicosatetraenoic acids can be important for platelet function, even though their aggregating capacity has not been demonstrated. Nevertheless, it has been shown that products of LOP pathway also enhance platelet aggregation (Dutilh *et al.*, 1979; Gimeno *et al.*, 1983).

PAF is a phospholipid mediator of anaphylaxis described initially as originating from basophiles (Benveniste *et al.*, 1972). Platelet aggregation induced by PAF is mediated through COX pathway (Chesney *et al.*, 1982). The results show that PAF and AA when added in PRP in subthreshold concentration act synergistically to produce platelet aggregation. This effect is dependent on transmembrane PAF receptors (Ostermann *et al.*, 1984).

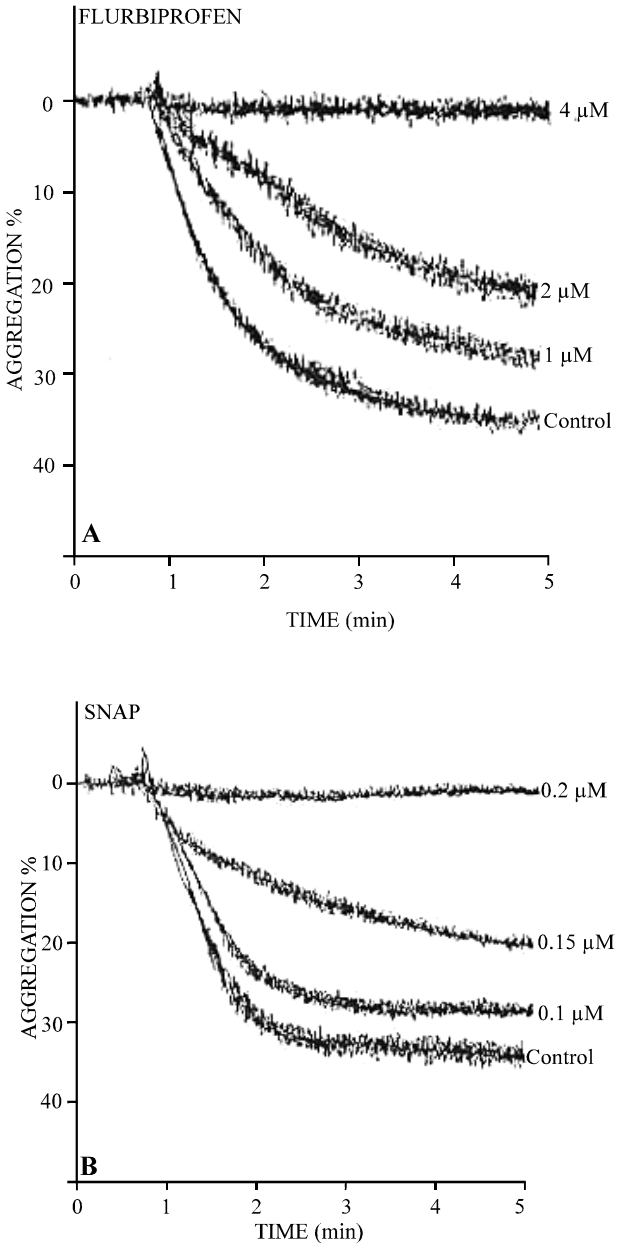

Fig. 3(A): Concentration-dependent effects of cyclooxygenase inhibitor, flurbiprofen and (B): Nitric oxide donor, SNAP on platelet aggregation induced by co-addition of AA and PAF. Control means platelet aggregation induced by AA (0.2 mM) plus PAF (40 nM). n=6

The synergism between PAF and AA was inhibited by calcium channel blockers, PAF receptor antagonist and inhibitors of PLC, MAP kinase and COX pathways. It is well documented that in platelets PAF receptors are linked

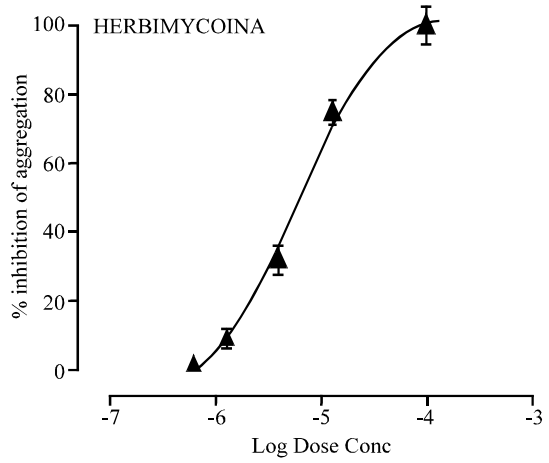

Fig. 4: Dose-response curve of specific tyrosine kinase inhibitor, herbimycin A and on co-addition of sub-threshold concentrations of AA and PAF. n =7

to Gq protein followed by the activation of PLC. This explains why U73122, a selective inhibitor of PLC, shows strong inhibitory effects on platelet aggregation induced by co-activation of these agonists. Further, PLC activation leads to generation of IP_3, release of Ca^{2+} from internal stores and eventually store-depleted Ca^{2+} influx (Heemskerk and Sage, 1994) that was inhibited by Ca^{2+} channel blockers (verapamil and diltiazem). Moreover, the increase in cytosolic Ca^{2+} causes activation of PLA_2 and COX-1 activity, thus stimulating TXA_2 formation (Heemskerk and Sage, 1994). Since the synergism of these agonists was inhibited by flurbiprofen, a selective COX-1 inhibitor, it seems that agonist-mediated synergism follows activation of COX-1 distal to PLC-Ca^{2+} activation. We tested if increasing the intracellular nitric oxide levels by NO donor and thus activating cGMP kinase has any inhibitory effect on platelet aggregation. Our results show that NO donor, SNAP, inhibited platelet aggregation (IC_{50} = 1.7 μM) suggesting that PAF and AA synergism is highly sensitive to NO generation in human platelets. However, the role of PKC in the present study was excluded, as PKC inhibition had no effect on the synergism of PAF and AA in platelets.

The cyclic nucleotides, cAMP and cGMP, through activation of cGMP-dependent protein kinases, down-regulate the Ca^{2+} responses and thus inhibit platelet aggregation (Heemskerk and Sage, 1994). Infact platelets are abundant in cAMP and cGMP dependent protein kinases and these kinases can inhibit PLC induced IP_3 production through inactivation of IP_3 and TXA_2 receptors (Wang *et al*., 2000). The inhibition of PAF and AA synergism by MEK inhibitor, PD98095, suggests the involvement of MAP kinase that is known to be distal to

Table 1: The effect of various inhibitors on subthershold concentration of and platelet activating factor (40nM) and arachidonic acid (0.2mM) induced platelet aggregation

Inhibitors	IC_{50} μM±SEM
WEB 2086	0.6±3.5
U73122	6.0±0.1
Diltiazem	15.0±2.5
Verapamil	20.0±4.2
PD98056	3.5±0.01
Flurbiprofen	3.0±0.2
SNAP	1.5±0.02
Herbimycin A	15.0±1.5
Chelerythrine	NE

Data are mean + SEM (n=5-7) and is indicated as half-maximal effect (IC_{50}) of the inhibitors. NE = No effect

Gq-PLC (Della *et al*., 1997; Della Rocca *et al*., 1999). Infact, $cPLA_2$ is also a potential target of activation by increase in cytosolic Ca^{2+}. Taken together, it appears that both Ca^{2+} signaling and MAP kinase play an important role during synergistic interaction of PAF and AA in human platelets. The selective MEK inhibitor, PD98095, is also known to inhibit COX-1 and 2 activities. Using purified COX-1 and 2 (McNicol *et al*., 1998), showed the formation of TXA_2 at quite low concentrations (IC_{50} = 0.8 μM). Under our experimental conditions, PD98059 inhibited both TXA_2 production and platelet aggregation with IC_{50} of 5 and 3.5 μM, respectively. Therefore the possibility that inhibition of agonist-induced platelet aggregation by PD98059 may be due to blockade of COX activity cannot be ignored (Table 1).

Many studies show that activation of platelets by some agonists increase the level of tyrosine phosphorylation resulting in the appearance of a new set of tyrosine-phosphorylated proteins (Ferrel and Martin, 1988; Golden and Yamamura, 1989). Increase in the phosphorylation of tyrosine residues are early events in the signal transduction pathway for stimulation of platelets by PAF (Animesh *et al*., 1990). To investigate the involvement of tyrosine kinase in present study we used herbimycin A, a known and specific inhibitor of tyrosine kinase which blocked PAF and AA-induced aggregation in a concentration-dependent manner (IC_{50} = 15 μM) showing that the synergism may also be due to the TLCK activation.

The mechanism of synergism among various platelet agonists is reported to occur due to activation of Ca^{2+} signaling cascade. A rise in Ca^{2+} induced by first agonist primes platelets for an enhanced functional response to the second agonist (Ware *et al*., 1987; Shah *et al*., 1997). Ca^{2+} plays pivotal role in platelet aggregation. The synergism among various platelet agonists in the blood is of great clinical significance as it can lead to marked potentiation of the platelet activation thus alter the cardiovascular physiology. In conclusions, our studies show that subthreshold concentrations of PAF

potentiates platelet aggregation mediated by AA. It seems to follow the PLC-Ca^{2+}, COX and MAP kinase pathways activation and is negatively modulated by nitric oxide donor, indicating a potential regulatory role of nitric oxide in platelet function during this synergism.

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