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Cooperativity between platelet-activating factor and collagen in aggregation of bovine platelets III

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In cooperative aggregation of bovine platelets induced by simultaneous addition of PAF and collagen at subthreshold concentrations the following observations were made. (i) Formation of phosphatidic acid and arachidonic acid metabolites, which characterize PAF and collagen alone-induced aggregation, respectively, was observed very obviously. (ii) A thromboxane antagonist did not completely block the cooperative aggregation. The extent of residual aggregation activity was dependent on concentration of collagen used in the simultaneous administration with PAF. These results suggest that both signal transduction pathways activated by PAF and collagen alone at high concentrations are attained by simultaneous addition of both agonists at subthreshold concentrations through unknown mechanisms.

Platelet-activating factor; Collagen; Cooperativity; Phosphatidic acid; Thromboxane; Platelet aggregation

1. INTRODUCTION

Various agonists induce aggregation of platelets through different mechanisms. Several papers reported that two different agonists demonstrate cooperativity in the aggregation [1-3]. However, mechanisms of this cooperativity have not been elucidated thoroughly. We previously found cooperativity between platelet-activating factor (PAF) and collagen using bovine platelets [4]. Cooperativity was observed at various steps of signal transduction pathway activated by PAF and collagen alone including changes of cytosolic Ca^{2+} concentration, thromboxane (TX) formation and phosphorylation of 47 kDa protein (manuscript in preparation). In bovine platelets, collagen-induced aggregation was fully dependent on endogenously formed TXA_2, whereas PAF-induced one was not at all.

We show here the results suggesting that collagen and PAF at subthreshold concentration enhanced one another's activation pathway when they were added together.

2. MATERIALS AND METHODS

2.1. *Materials*

PAF, collagen (type I), and TXB_2 were purchased from Avanti Polar Lipids (Pelham, AL), Hormon-Chemie GmbH. (Munich, FRG) and Cayman Chemical (Ann Arbor, MI), respectively. 12*S*-Hydroxyeicosatetraenoic acid (12-HETE) was chemically synthesized [5]. ONO-3708 was kindly provided from Ono Pharmaceutical Co. (Osaka, Japan). All other reagents were of analytical grade.

2.2. *Preparation of bovine washed platelets*

Washed platelet suspension in Tris-acid citrate dextrose (ACD) buffer was prepared from bovine blood anticoagulated with ACD as described previously [6].

2.3. *Measurement of platelet aggregation*

Platelet aggregation was monitored as increase of light transmission in the presence of 8 mM $CaCl_2$ and 1 mg/ml bovine serum albumin (essentially fatty acid free; Sigma) using a Nikoh Bioscience aggregometer NKK HEMA Tracer 601 (Tokyo, Japan).

2.4. *Measurement of phosphatidic acid (PA) and arachidonic acid metabolites*

Formation of PA and arachidonic acid metabolites was detected using [1-^{14}C]arachidonic acid (50 mCi/mmol, DuPont-New England Nuclear)-labeled platelets according to the method described previously [7]. Lipids were extracted from stimulated platelets and were separated by thin-layer chromatography (TCL) in the solvent system of benzene/dioxane/acetic acid (20:20:1.5). Each ^{14}C-labeled product was detected with Berthold Analytical Instruments radio TLC scanner model LB 284 (Nashua, NH) and was identified according to the R_f values of unlabeled authentic TXB_2 (R_f = 0.4) and 12-HETE (R_f = 0.62), or the R_f value of [^{14}C]dipalmitoyl PA (R_f = 0.2), which was made from cleavage of [^{14}C]dipalmitoyl phosphatidylcholine (DuPont-New England Nuclear) with phospholipase D (Boehringer Mannheim).

3. RESULTS AND DISCUSSION

As reported in a previous paper, cooperative aggregation showed aggregation patterns without lag time and disaggregation, which characterize the

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simultaneous manifestation of both collagen and PAF alone-induced aggregation [4]. This unique aggregation pattern implicated that the aggregation was induced by stimulating both activation pathways of these agonists. This implication was corroborated in this study.

Fig. 1 shows a thin-layer chromatogram of lipids derived from [^{14}C]arachidonic acid-labeled platelets stimulated with PAF, collagen or their combination at subthreshold concentrations. Although 0.5 nM PAF or 2 μg/ml collagen alone did not induce aggregation and formation of metabolites derived from [^{14}C]arachidonic acid-labeled phospholipids (panel A), when added together, they induced extensive aggregation and very obvious formation of both PA and arachidonic acid metabolites including TXB_2 and 12-HETE (panel B). With high concentration of PAF (4 nM) alone, very obvious aggregation was observed and only PA was formed and arachidonic acid metabolites were not formed at all (panel C). The situation was completely opposite when high concentration of collagen (16 μg/ml) alone was used to aggregate platelets (panel D). Arachidonic acid metabolites were detected very distinctly, but PA was not detected at all. With this radioisotopic technique, radioactivity is preferentially incorporated in phosphatidylcholine [8], therefore, arachidonic acid metabolites we detected here should be derived mainly from phosphatidylcholine probably by the activation of phospholipase A_2. Thus, we can say that PAF alone does not activate phospholipase A_2, but that PAF even at very low concentration is able to support its activation by collagen at subthreshold concentration. This action of PAF might contribute to the cooperativity with collagen in aggregation. We could not explain why PA was not

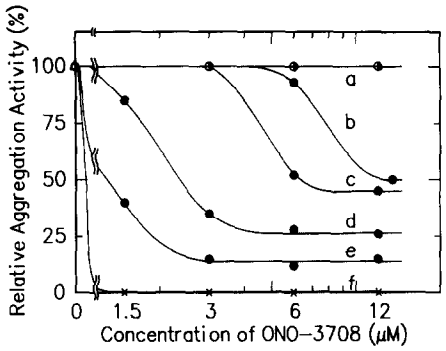

Fig. 2. Effect of ONO-3708 on platelet aggregation. Washed bovine platelets were preincubated with various concentrations of ONO-3708 (1.5–12 μM) for 5 min at 37°C, followed by the addition of 1.6 nM PAF (a), 16 μg/ml collagen (f) or with simultaneous addition of 0.4 nM PAF plus 6 μg/ml (a), 4 μg/ml (b), 2 μg/ml (c), 1 μg/ml (d) and 0.5 μg/ml (e) collagen. Maximal aggregation activity within 7 min was plotted against concentration of ONO-3708 as relative to that obtained in the absence of it in each case.

formed from ^{14}C-labeled phospholipids in collagen-induced aggregation. It was reported that PA formation was detectable along with collagen-induced aggregation of human and rat platelets using ^{32}P as the probe, in which system combined action of phospholipase C and diacylglycerol kinase should be represented [9,10]. Indeed, we also obtained the same preliminary results using ^{32}P-incubated bovine platelets (not shown). It is obvious that there are two different pathways for the formation of PA in bovine platelets, which can be detected by [^{14}C]arachidonic acid-labeling and ^{32}P-labeling techniques. One of them, formation from [^{14}C]arachidonic acid-labeled phospholipids, is operative only with PAF. It becomes of interest to note

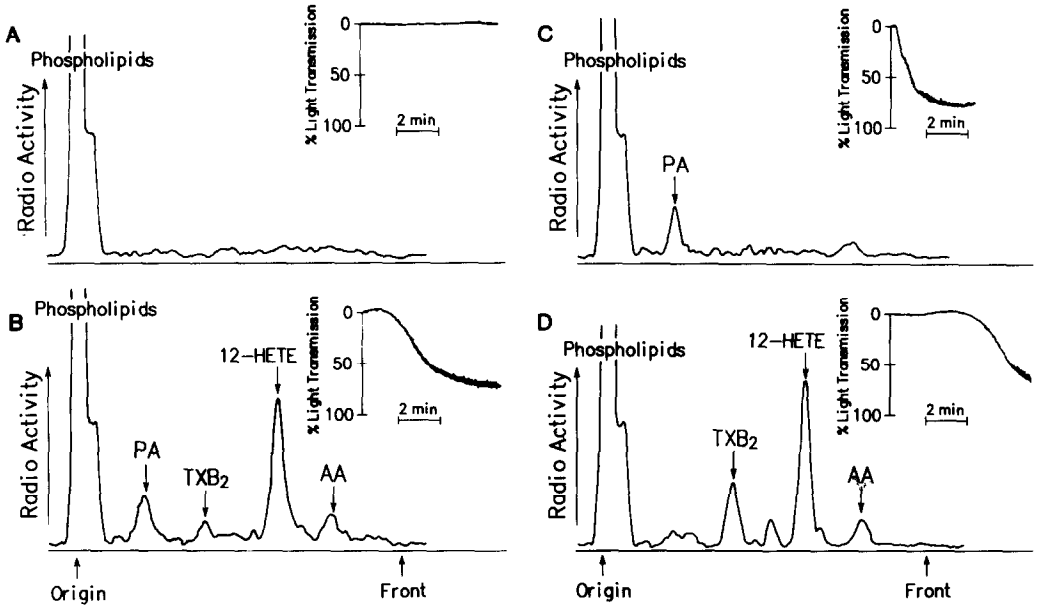

Fig. 1. Radio TLC profiles of lipids produced by platelets (2.5×10^8 platelets/500 μl) stimulated by following agonists. (A) 0.5 nM PAF or 2 μg/ml collagen alone for 7 min. (B) 0.5 nM PAF + 2 μg/ml collagen for 7 min. (C) 1.6 nM PAF alone for 4 min. (D) 16 μg/ml collagen alone for 7 min.

that both activation pathways of two agonists, namely the formation of [^{14}C]PA and arachidonic acid metabolites, function very actively when PAF and collagen are added simultaneously at subthreshold concentrations.

Fig. 2 shows the effect of a TXA_2 antagonist, ONO-3708 [11], on platelet aggregation. Aggregation induced by high concentration of collagen (16 μg/ml) alone was almost completely inhibited at 1.5 μM ONO-3708 (curve f), whereas high concentration of PAF (1.6 nM) alone-induced aggregation was not affected even at 12 μM (line a). In the case of cooperative aggregations induced by simultaneous addition of PAF at subthreshold concentration (0.4 nM) and collagen at various concentrations (0.5–6 μg/ml), the effect of antagonist was in-between these two extreme cases. The lower the concentration of collagen, the higher was the effect of the antagonist. In combination of 6 μg/ml collagen and 0.4 nM PAF, the effect of ONO-3708 was nil as was observed with PAF alone (line a). The same kind of results were obtained with aspirin (not shown). Collagen and PAF alone used in cooperative aggregation could not induce aggregation at all under our experimental conditions. This result suggests that cooperative aggregation was induced by combination of two activation pathways, one dependent on TXA_2 and the other independent. Detailed mechanisms of this interesting cooperativity between PAF and collagen remain to be elucidated.

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