Decreased arachidonic acid metabolism in human platelets by autologous neutrophils: possible role of cell adhesion

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The amount of the 12-lipoxygenase and cyclo-oxygenase products, 12(*S*)-hydroxy-(*Z,Z,E,Z*)-5,8,10,14-eicosatetraenoic acid (12-HETE) and 12(*S*)-hydroxy-(*E,E,Z*)-5,8,10-heptadecatrienoic acid (HHT), in human platelets stimulated by thrombin (0.1 and 2.5 units/ml), was studied in the presence of autologous neutrophils. A decreased formation of both products was induced by unstimulated neutrophils or neutrophils challenged with *N*-formylmethionyl-leucyl-phenylalanine (0.1 μ M) or Ca^{2+} ionophore A23187 (0.15 μ M). The effect of neutrophils was observed only in the presence of Ca^{2+} . 12-HETE and HHT were also produced in platelets stimulated with thrombin in the absence of Ca^{2+} and/or Mg^{2+} , but their level was not altered by neutrophils. 12(*S*),20-Dihydroxy-(*Z,Z,E,Z*)-5,8,10,14-eicosatetraenoic acid (12,20-DHETE), the cytochrome *P*-450 product from 12-HETE in neutrophils, was hardly detected, and its level did not compensate for the decrease in 12-HETE observed after platelet and neutrophil co-incubation. 5(*S*),12(*S*)-Dihydroxy-(*E,Z,E,Z*)-6,8,10,14-eicosatetraenoic acid (5(*S*),12(*S*)-DHETE), the 5-

lipoxygenase product of 12-HETE in neutrophils, was never detectable. In addition, the inhibition of 12-HETE and HHT formations appeared not to be due to degradation or thrombin uptake by neutrophils, nor was the decrease observed when the two cell populations were physically separated. A monoclonal antibody against the human platelet glycoprotein GMP140 (CD62), mediating Ca^{2+} -dependent platelet–neutrophil adhesion, mimicked the inhibitory effect of neutrophils in a dose-dependent fashion. Furthermore, the 12-HETE and HHT productions were not affected when platelets were stimulated in the presence of neutrophils previously incubated with sialidase, which removes the sialic acid from a sialyl Lewis^x structure assumed to be the neutrophil receptor for platelet GMP140. We conclude that the decrease in thrombin-stimulated 12-HETE and HHT formation observed when platelets were co-incubated with autologous neutrophils might be the consequence of platelet–neutrophil adherence, presumably through platelet GMP140.

INTRODUCTION

Platelets and neutrophils are implicated in several pathological processes, such as inflammation and atherosclerosis, in which these cells are activated. Moreover, the pathological role of platelets and neutrophils in these diseases may be amplified during their interaction. The relevance and the possible mechanisms of platelet–neutrophil interactions in the pathogenesis of thrombosis and inflammation have been reviewed (Robertson and Page, 1987; Marcus, 1990; Bazzoni et al., 1991; Faint, 1992).

The inflammatory potency of platelets and neutrophils may be mediated by the arachidonic acid metabolites produced after cell stimulation by numerous agonists. Leukotriene B_4 , the main 5-lipoxygenase product in neutrophils, is an inflammatory active molecule (Borgeat et al., 1985). Arachidonic acid is also metabolized in platelets to prostaglandins and thromboxane through the cyclo-oxygenase pathway, and to 12(*S*)-hydroxy-(*Z,Z,E,Z*)-5,8,10,14-eicosatetraenoic acid (12-HETE) via the 12-lipoxygenase pathway (Marcus, 1987). The same stimuli that activate platelet adhesion and aggregation also trigger the release of arachidonic acid from platelet membrane phospholipids, initiating eicosanoid synthesis. Thromboxane A_2 is a potent vasoconstrictive agent associated with platelet hyperactivity in thrombosis (Rittenhouse-Simmons and Deykin, 1981; Marcus, 1987). 12-HETE exhibits some biological and physiological properties that are not yet fully elucidated (Spector et al., 1988).

It has been shown that 12-HETE acts as a chemotactic agent for neutrophils (Goetzl et al., 1977; Turner et al., 1977). Other studies have revealed that 12-HETE interferes with thromboxane A_2 -induced platelet aggregation after cell stimulation. Thus 12-HETE may decrease thromboxane- and collagen-induced platelet aggregation (Croset and Lagarde, 1983; Sekiya et al., 1990) or, in contrast, may enhance platelet aggregation induced by thrombin (Sekiya et al., 1991).

Platelet–neutrophil interaction can lead to the formation of products not generated by either cell type alone (Lagarde et al., 1989). Thus 12-HETE released from platelets is converted into 5(*S*),12(*S*)-dihydroxy-(*E,Z,E,Z*)-6,8,10,14-eicosatetraenoic acid [5(*S*),12(*S*)-DHETE] by the 5-lipoxygenase in Ca^{2+} -ionophore-stimulated neutrophils (Marcus et al., 1982), and into 12(*S*),20-dihydroxy-(*Z,Z,E,Z*)-5,8,10,14-eicosatetraenoic acid [12(*S*),20-DHETE] by cytochrome-*P*-450-dependent hydroxylation in unstimulated cells (Marcus et al., 1984). On the other hand, platelets are capable of converting leukotriene A_4 , the intermediary product of arachidonic acid metabolism through the 5-lipoxygenase pathway in stimulated neutrophils, into leukotriene C_4 (Pace-Asciak et al., 1986; Maclouf and Murphy, 1988). The transcellular utilization of arachidonic acid has also been reported. Platelets challenged by physiological agonists can significantly increase the formation of leukotriene B_4 in neutrophils by providing arachidonic acid (Palmantier and Borgeat, 1991; Antoine et al., 1992). In contrast, 12-HETE production in platelets can be enhanced by arachidonic acid translocation from

Abbreviations used: 12-HETE, 12(*S*)-hydroxy-(*Z,Z,E,Z*)-5,8,10,14-eicosatetraenoic acid; HHT, 12(*S*)-hydroxy-(*E,E,Z*)-5,8,10-heptadecatrienoic acid; 12(*S*),20-DHETE, 12(*S*),20-dihydroxy-(*Z,Z,E,Z*)-5,8,10,14-eicosatetraenoic acid; 5(*S*),12(*S*)-DHETE, 5(*S*),12(*S*)-dihydroxy-(*E,Z,E,Z*)-6,8,10,14-eicosatetraenoic acid; 15-HETE, 15(*S*)-hydroxy-(*E,Z,Z*)-8,11,13-eicosatrienoic acid; fMLP, *N*-formylmethionyl-leucyl-phenylalanine; GMP140 (CD62), 140 kDa granule membrane protein; RP-h.p.l.c., reversed-phase h.p.l.c.

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neutrophils to platelets after co-incubation with Ca^{2+} -ionophore-stimulated neutrophils (McCulloch et al., 1992). Neutrophil-platelet interactions may also lead to the control of arachidonic acid metabolism in both cell populations. We have previously shown that leukotriene B_4 biosynthesis from endogenous arachidonic acid may be decreased in the presence of autologous platelets in Ca^{2+} -ionophore-stimulated neutrophils from healthy subjects (Hosni et al., 1991). In the present study, the effect of autologous neutrophils on the biosynthesis of 12-HETE and HHT [12(*S*)-hydroxy-(*E,E,Z*)-5,8,10-heptadecatrienoic acid] was investigated in stimulated washed platelets from normal human subjects.

MATERIALS AND METHODS

Materials

Thrombin (freeze-dried powder from human placenta), the chemotactic peptide *N*-formylmethionyl-leucyl-phenylalanine (fMLP), Ca^{2+} ionophore A23187 and sialidase (neuraminidase, EC 3.2.1.18; from *Clostridium perfringens*) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 12-HETE, HHT and 5(*S*),12(*S*)-DHETE were from Cayman Chemical Co. (Ann Arbor, MI, U.S.A.) and 12(*S*),20-DHETE was from Cascade Biochem (Reading, Berks., U.K.). The monoclonal antibody to human glycoprotein GMP140 (140 kDa granule membrane protein; CD62) (clone CLB-throm/6) was purchased from Monosan (Uden, The Netherlands). All solvents used were analytical grade. Methanol was purchased from Carlo Erba Farmitalia (Italy), and acetonitrile, 'far UV' for h.p.l.c., was from Fisons (U.K.). All other products used were analytical grade or better. The RGDF peptide was generously given by Dr. J. L. McGregor (INSERM U 331, Lyon, France). Thrombin was dissolved in 1 ml of water under sterile conditions to a stock concentration of 100 units/ml and stored at 4 °C. For all the experiments, the Tyrode solution without Ca^{2+} or Mg^{2+} , containing 1 mM Hepes, was prepared as described by Mustard et al. (1989) and buffered to pH 7.35. The stock solutions of fMLP (10 mM) and Ca^{2+} ionophore (3 mM) were prepared in dimethyl sulphoxide, and diluted before use in the incubation buffer at the appropriate concentration; they were used within 5 min after preparation. The dimethyl sulphoxide concentration in the incubations never exceeded 0.05 %. 15(*S*)-Hydroxy-(*E,Z,Z*)-8,11,13-eicosatrienoic acid (15-HETrE) was prepared by the action of soybean 15-lipoxygenase (Sigma) on 8,11,14-eicosatrienoic acid by the method of Funk et al. (1976) and purified by t.l.c. on Kieselgel-G plates (Merck), with the mixture hexane/diethyl ether/acetic acid (60:40:1, by vol.) as the mobile phase, followed by reversed-phase h.p.l.c. (RP-h.p.l.c.) (see 'Assay of arachidonate metabolites' below).

Cell preparation

Neutrophils and platelets were obtained from venous blood of healthy subjects, collected on to citric acid-dextrose anticoagulant in a blood bank (Centre de Transfusion Sanguine, Lyon, France). Subjects did not take any medication for 10 days, and fasted for at least 12 h before venipuncture. All operations were carried out in polypropylene tubes at room temperature. Blood was centrifuged for 20 min at 120 *g*, and platelets were isolated from the upper phase (platelet-rich plasma) as previously described (Lagarde et al., 1980) and resuspended in Tyrode-Hepes buffer (pH 7.35), without Ca^{2+} and Mg^{2+} , at a concentration of 500×10^6 cells/ml. The platelet count was determined with Unopett bottles (Becton and Dickinson, Rutherford, NJ,

U.S.A.). Neutrophils were isolated from the lower phase by Dextran sedimentation, followed by Ficoll-Paque centrifugation as previously described (Hosni et al., 1991). Contaminating erythrocytes were eliminated by hypotonic lysis, and neutrophils were washed twice in Tyrode-Hepes buffer (pH 7.4), without Ca^{2+} and Mg^{2+} , and were finally resuspended in this buffer at a concentration of 50×10^6 cells/ml. Among polymorphonuclear leucocytes, the eosinophil proportion was always below 2 %. Cell viability was greater than 95 %, as judged by the Trypan Blue exclusion method. Neutrophil contamination by platelets, measured by phase-contrast microscopy after fixation in Unopett, was about 0.5–1 platelet per neutrophil, depending on the cell preparation. The neutrophil suspension was used within 15 min after preparation.

Effect of neutrophils on 12-HETE and HHT production in platelets

In 3 ml polypropylene tubes, 600 μl of platelet suspension (300×10^6 cells) was preincubated for 5 min at 37 °C with 100 μl of Tyrode-Hepes buffer or 100 μl of neutrophil suspension (10×10^6 cells), in the presence of CaCl_2 and MgCl_2 . Then cells were stimulated for another 5 min with thrombin alone or in combination with fMLP (final volume 1 ml). Thrombin (0.1 or 2.5 units/ml) was used as a stimulating agent for platelets, and fMLP (0.1 μM) for neutrophils. Since the final volume of the incubation changed during the course of experiments, the final concentrations of CaCl_2 and MgCl_2 were continuously adjusted to 1.6 and 0.4 mM respectively. To evaluate the effect of bivalent cations, cells were also stimulated in the absence and in the presence of CaCl_2 , MgCl_2 or both. In some experiments cells were incubated without agonists or were challenged by the Ca^{2+} ionophore A23187 (0.15 μM). In addition, the cells were stimulated with thrombin in the presence or absence of a monoclonal antibody against platelet glycoprotein GMP140 (final concentrations of the antibody were 0.02, 0.2, 2 and 5 $\mu\text{g}/\text{ml}$). In other experiments, the effect of neutrophils was studied after platelet stimulation by thrombin. For this, platelets were preincubated in the presence of CaCl_2 and MgCl_2 and stimulated with thrombin (0.1 unit/ml) for 5 min at 37 °C. Then neutrophils or Tyrode-Hepes buffer were added with or without (unstimulated neutrophils) fMLP (0.1 μM), and the incubation was prolonged for another 5 min. In control assays, the reaction was terminated after platelet stimulation for 5 min with thrombin.

Effect of the separation of cell populations

Polypropylene tubes (90 mm length and 14 mm internal diameter) were cut at 2 cm from the bottom, washed and sterilized. A Transwell cell (Costar Corp., Cambridge, MA, U.S.A.) was introduced at the top of each shortened tube, and the cell stimulation was carried out with this system. In control experiments, 600 μl of platelet suspension (300×10^6 cells) and 200 μl of Tyrode-Hepes buffer were introduced into the tube, and 200 μl of buffer was deposited into the Transwell cell. When platelets and neutrophils were separately stimulated, 200 μl of neutrophil suspension (10×10^6 cells) was deposited into the Transwell cell. When platelets and neutrophils were stimulated together, 600 μl of platelet and 200 μl of neutrophil suspensions were introduced into the tubes and 200 μl of buffer into the Transwell cell. Then 50 μl of CaCl_2 and MgCl_2 solution (final concns. 1.6 and 0.4 mM respectively) was added to each tube, and tubes were placed in a shaker water-bath at 37 °C. After 5 min preincubation, 50 μl of Tyrode-Hepes buffer or 50 μl of fMLP solution (final concn. 0.1 μM) were introduced into the Transwell cell, and 3 min later, 50 μl of thrombin solution (final concn. 0.1 unit/ml) was added

Table 1 Effect of neutrophils on arachidonate metabolism in platelets

Platelets ($300 \times 10^6/\text{ml}$) were preincubated alone or with neutrophils ($10 \times 10^6/\text{ml}$) in the presence of CaCl_2 (1.6 mM) and MgCl_2 (0.4 mM) for 5 min at 37°C . Cells were stimulated for a further 5 min by thrombin (0.1 or 2.5 units/ml). 12-HETE, HHT, 5(S),12(S)-DHETE and 12(S),20-DHETE were assayed by RP-h.p.l.c. as described in the Materials and methods section. Results are expressed in nmol of metabolites produced within 5 min by 300×10^6 platelets, and each value represents the mean $\pm$ S.E.M. of n determinations: * refers to the comparison with platelets alone. Abbreviations: ND, not detectable; NS, not significant.

	Thrombin (units/ml)	Arachidonic acid metabolites produced (nmol/5 min per 300×10^6 platelets)			
		12-HETE	HHT	5(S),12(S)-DHETE	12(S),20-DHETE
Platelets alone	0.1	0.68 ± 0.09 ($n = 10$)	0.31 ± 0.06 ($n = 10$)		
Platelets + neutrophils	0.1	0.13 ± 0.05 ($n = 10$) (-81% ; $P < 0.001$)*	0.07 ± 0.03 ($n = 10$) (-77% ; $P < 0.002$)*	ND	ND
Platelets alone	2.5	5.91 ± 0.42 ($n = 5$)	4.53 ± 0.75 ($n = 5$)		
Platelets + neutrophils	2.5	3.19 ± 0.48 ($n = 5$) (-46% ; $P < 0.01$)*	3.36 ± 0.53 ($n = 5$) (-26% ; NS)*	ND	0.33 ± 0.06 ($n = 6$)

to the tubes and the incubation prolonged for 5 min at the same temperature.

Effect of neutrophil treatment with sialidase

This study was carried out by the method described by Polley et al. (1991). Neutrophils (55×10^6 per ml) were incubated for 60 min at 37°C alone or with sialidase (2 units/ml), and a third sample of neutrophils was kept at room temperature. After this period of treatment, neutrophils (10×10^6 per ml) were preincubated for 5 min at 37°C with platelets (300×10^6 per ml) in the presence of CaCl_2 and MgCl_2 (1.6 and 0.4 mM respectively), and cells were stimulated for another 5 min by thrombin with or without fMLP. In control assays, platelets were stimulated alone or in the presence of sialidase.

Assay of arachidonate metabolites

In all these experiments, the reaction was terminated by adding 600 μl of ice-cold methanol containing 15-HETRe (50 pmol) as the internal standard, and tubes were vortex-mixed and cooled at -20°C until the biochemical analysis. 12-HETE and HHT were assayed by RP-h.p.l.c. Eicostetraenoic acids, mono- (12-HETE) or di- [12,20-DHETE and 5(S),12(S)-DHETE] hydroxylated derivatives, HHT and 15-HETRe were analysed on a column of LiChrospher 100 RP-18 (length 25 cm, internal diameter 4.6 mm, particle size 5 μm) (Merck). Before the analysis, the alcoholic mixture was centrifuged at 0°C for 10 min at 1500 g , and 100 μl of supernatant was injected on to the column. For the separation of 12-HETE and HHT, the column was isocratically eluted with the mixture methanol/acetonitrile/water/acetic acid (150:225:100:2, by vol), pH 5.6 (0.5 ml/min). For separating 12(S),20-DHETE and 5(S),12(S)-DHETE, the mixture methanol/acetonitrile/water/acetic acid (125:150:200:2, by vol), pH 5.6 (0.8 ml/min) was used as the mobile phase. The lipoxigenase and cyclo-oxygenase products were detected with fixed-wave-length u.v. at 235 nm [12-HETE, HHT and 12(S),20-DHETE] or 270 nm [5(S),12(S)-DHETE]. The amounts of 12-HETE, HHT, 12(S),20-DHETE and 5(S),12(S)-DHETE were determined by integration of the corresponding peaks and comparison of peak areas obtained from calibrated standards chromatographed under the same conditions.

Data and statistical analysis

Amounts of 12-HETE and HHT released by platelets stimulated

by thrombin alone or in the presence of neutrophils were expressed in nmol (means $\pm$ S.E.M.) released after 5 min stimulation per 165×10^6 cells. When the amount of available cells was sufficient, the experiments were carried out in duplicate. In these conditions, the difference between two values never exceeded 10%. Statistically significant differences between experiments were evaluated by the non-parametric Mann-Whitney 'U' test, unless otherwise indicated.

RESULTS

Effect of autologous neutrophils on arachidonate metabolism in platelets

As depicted in Table 1, when platelets were stimulated by 0.1 unit/ml thrombin in the presence of unstimulated neutrophils, amounts of 12-HETE and HHT produced were significantly lowered by 81% ($P < 0.001$; $n = 10$) and 77% ($P < 0.002$; $n = 10$), respectively. These decreases were still observed with platelets incubated in the presence of 0.1 μM fMLP-stimulated neutrophils, or when platelets and neutrophils were co-incubated without agonists or with Ca^{2+} ionophore A23187 (0.15 μM) used as a stimulating agent for both cell populations (results not shown). The 12-HETE production was also significantly decreased, but to a lesser extent (46%, $P < 0.01$; $n = 5$), when platelets were stimulated by 2.5 units/ml thrombin in the presence of unstimulated or fMLP-stimulated neutrophils. In contrast, the HHT production was not altered in platelets stimulated by 2.5 units/ml thrombin in the presence of unstimulated or stimulated neutrophils.

It is known that 12-HETE may be metabolized in neutrophils into 5(S),12(S)-DHETE via the activated 5-lipoxygenase pathway (Marcus et al., 1982) and/or into 12(S),20-DHETE by cytochrome- P_{450} -dependent hydroxylation (Marcus et al., 1984). The decreased 12-HETE release by platelets stimulated in the presence of autologous neutrophils might be then attributed to the metabolism of 12-HETE in neutrophils. We attempted to identify the presence of these metabolites. 5(S),12(S)-DHETE was never detectable when platelets were stimulated by thrombin (0.1 or 2.5 units/ml) in the presence of neutrophils either unstimulated or stimulated by 0.1 μM fMLP (Table 1) as well as 0.15 μM ionophore A23187 (results not shown). On the other hand, 12(S),20-DHETE was never detected when platelets were stimulated by 0.1 unit/ml thrombin (Table 1). After platelet stimulation by 2.5 units/ml thrombin, 12(S),20-DHETE was formed and measurable (Table 1), but the higher amount

Figure 1 Effect of Ca^{2+} and Mg^{2+} on the decrease in 12-HETE and HHT production in platelets co-incubated with neutrophils

Platelets ($300 \times 10^6/\text{ml}$) alone or with neutrophils ($10 \times 10^6/\text{ml}$) were preincubated for 5 min at 37°C in the absence of bivalent cations or in the presence of CaCl_2 (1.6 mM), MgCl_2 (0.4 mM) or both CaCl_2 and MgCl_2 . After addition of thrombin (0.1 or 2.5 units/ml) to each incubation mixture, the incubation was prolonged for 5 min at 37°C . Results are expressed in nmol of 12-HETE (a and b) and HHT (c and d) produced in 5 min by 300×10^6 platelets, and each point represents the mean $\pm$ S.E.M. (vertical bars) of 6 (0.1 units/ml thrombin; a and c) or 4 experiments (2.5 units/ml thrombin; b and d). ■, Platelets incubated alone; ▨, platelets co-incubated with neutrophils. ^a $P < 0.02$ and ^b $P < 0.025$ for comparisons between platelets incubated alone in the absence of bivalent cations. ^c $P < 0.025$ and ^d $P < 0.05$ for comparisons between platelets incubated in the presence and absence of neutrophils.

obtained represented only 13% of the difference observed between the amount of 12-HETE produced by platelets stimulated alone and that produced in co-incubations with neutrophils.

When unstimulated or fMLP-stimulated neutrophils were added 5 min after platelets were stimulated with 0.1 unit/ml thrombin and the incubation was prolonged for another 5 min, there was no inhibition. The amounts of 12-HETE and HHT produced were increased and were not significantly different from that obtained after prolongation of platelet stimulation in the absence of neutrophils (results not shown). Moreover, during the prolongation in the presence of unstimulated or fMLP-stimulated neutrophils, the production of 12(S),20-DHETE was never detectable. Thus the decreased 12-HETE production observed when platelets were co-incubated with neutrophils could not be explained by its utilization by neutrophils (i.e. metabolism and/or re-incorporation into membrane phospholipids).

McCulloch et al. (1992) have previously observed a decrease of 12-HETE and HHT biosynthesis in platelets stimulated by thrombin (0.15 unit/ml) in the presence of autologous unstimulated neutrophils, and have attributed this decrease to a lowering of the effective concentration of thrombin caused by passive adsorption on to or metabolism by neutrophils. However, in our experiments where platelets were activated with the supernatant obtained from thrombin-incubated neutrophils, the 12-HETE and HHT productions were not modified or were slightly decreased (results not shown). Moreover, we found that these decreases were also observed with cells stimulated by the Ca^{2+} ionophore A23187 as well as with unstimulated cells.

Effect of Ca^{2+} and Mg^{2+} on neutrophil-induced decrease of 12-HETE and HHT productions

In the absence of bivalent cations, 12-HETE and HHT were

produced in platelets stimulated alone with 0.1 unit/ml thrombin, but production was not significantly altered in the presence of fMLP-stimulated neutrophils (Figures 1a and 1c). In the presence of Mg^{2+} , the production of 12-HETE was slightly, but not significantly, enhanced (by 15%). In contrast, the 12-HETE production was increased by 181% ($n = 6$; $P < 0.02$) and 222% ($n = 6$; $P < 0.02$) in the presence of Ca^{2+} and Ca^{2+} plus Mg^{2+} , respectively (Figure 1a). In the presence of fMLP-stimulated neutrophils, the Ca^{2+} -stimulated 12-HETE was significantly ($P < 0.02$) lowered to its value in the absence of cations (Figure 1a). The presence of Mg^{2+} , Ca^{2+} or Ca^{2+} plus Mg^{2+} appeared to have no effect on HHT production in platelets stimulated alone by 0.1 unit/ml thrombin, but neutrophils decreased this production by 60% ($n = 6$; $P < 0.05$) and 58% ($n = 6$; $P < 0.05$) in the presence of Ca^{2+} and Ca^{2+} plus Mg^{2+} , respectively (Figure 1c). In the presence of unstimulated neutrophils, essentially the same results were obtained (not shown). In platelets stimulated alone by 2.5 units/ml thrombin, 12-HETE production was higher than in platelets stimulated by 0.1 unit/ml thrombin, was not modified in the presence of Mg^{2+} and increased in the presence of Ca^{2+} or Ca^{2+} plus Mg^{2+} (Figure 1b). Under these conditions, in the presence of neutrophils, the 12-HETE production was also lowered only in the presence of Ca^{2+} or Ca^{2+} plus Mg^{2+} , but to a lesser extent than after platelet stimulation by 0.1 unit/ml thrombin (Figure 1b). HHT production was also increased in platelets stimulated by 2.5 units/ml thrombin, but was not enhanced in the presence of Mg^{2+} , Ca^{2+} , or Mg^{2+} and Ca^{2+} , and was unaffected in the presence of unstimulated or fMLP-stimulated neutrophils (Figure 1d).

Effect of the separation of cell populations on the arachidonic acid metabolism in platelets

The results presented in Table 2 show that the decreased 12-HETE and HHT productions in thrombin-stimulated platelets

Table 2 Effect of the separation of cell populations on the arachidonic acid metabolism in platelets

These experiments were carried out as described in the Materials and methods section. Results are expressed in nmol of 12-HETE and HHT produced within 5 min by 300×10^6 platelets, and each value represents the mean $\pm$ S.E.M. of three experiments: * $P < 0.05$ for the comparison with platelets incubated alone.

	Arachidonic acid metabolites (nmol/5 min per 300×10^6 platelets)	
	12-HETE	HHT
Platelets alone	1.58 ± 0.48	0.46 ± 0.34
Platelets + neutrophils	$0.20 \pm 0.08^*$	$0.08 \pm 0.06^*$
Separated platelets and neutrophils	1.61 ± 0.52	0.54 ± 0.38

Figure 2 Effect of a monoclonal antibody against platelet glycoprotein GMP140 on the arachidonic acid metabolism in platelets stimulated by thrombin

Platelets (300×10^6 /ml) were preincubated for 5 min at 37°C in the presence of CaCl_2 (1.6 mM) and MgCl_2 (0.4 mM). Then, cells were stimulated by thrombin (final concns. 0.1 or 2.5 units/ml) for 5 min at 37°C , in the absence or presence of antibody (final concns. 0.02, 0.2, 2 and 5 $\mu\text{g}/\text{ml}$). In a control experiment, 0 (+N), platelets were stimulated by thrombin in the presence of neutrophils (10×10^6 /ml) and absence of antibody. Results are expressed in nmol of 12-HETE (a and b) and HHT (c and d) produced within 5 min per 300×10^6 platelets, and each point represents the mean $\pm$ S.E.M. (vertical bars) of 6 (platelets stimulated with 0.1 units/ml thrombin; a and b) or 3 (platelets stimulated with 2.5 units/ml thrombin) experiments. For comparison with platelets incubated alone (0): $^aP < 0.001$; $^bP < 0.01$; $^cP < 0.03$; $^dP < 0.05$.

co-incubated with autologous unstimulated neutrophils was no longer observed when neutrophils were separated from platelets by a porous membrane. The same results were obtained with neutrophils challenged with fMLP (not shown). Thus the effect of neutrophils on arachidonic acid metabolism in platelets appears to require a physical contact between both cell populations. On the other hand, an RGDF peptide, which inhibits the fibrinogen-mediated platelet-neutrophil adherence like RGD or RGDS peptides (Plow et al., 1985), did not affect the decrease in thrombin-stimulated platelet arachidonic acid metabolism observed in the presence of autologous neutrophils (results not

Table 3 Effect of treatment of neutrophils with sialidase on the arachidonic acid metabolism in platelets after cell co-incubation

Neutrophils (55×10^6 /ml) were incubated for 5 min at 37°C in the absence or presence of sialidase (final concn. 2 units/ml). Platelets (300×10^6 /ml) were preincubated for 5 min at 37°C , in the absence or in the presence of neutrophils (10×10^6 /ml) incubated with or without sialidase, and in the presence of CaCl_2 (1.6 mM) and MgCl_2 (0.4 mM). Then, cells were stimulated for 5 min at 37°C by thrombin (0.1 units/ml). In control experiments, platelets were stimulated in the presence of sialidase or untreated neutrophils kept at room temperature during the treatment of other neutrophil samples. Results are expressed in nmol of 12-HETE and HHT produced within 5 min by 300×10^6 platelets, and each value represents the mean $\pm$ S.E.M. of six experiments: * $P < 0.01$, † $P < 0.05$ for the comparison with platelets incubated alone.

	Arachidonic acid metabolites (nmol/5 min per 300×10^6 platelets)	
	12-HETE	HHT
Platelets alone	2.22 ± 0.24	1.38 ± 0.25
Platelets + untreated neutrophils	$0.91 \pm 0.25^*$	$0.65 \pm 0.18^\dagger$
Platelets + sham-treated neutrophils	$1.04 \pm 0.29^*$	$0.67 \pm 0.22^\dagger$
Platelets + sialidase-treated neutrophils	2.31 ± 0.35	1.69 ± 0.42
Platelets + sialidase	2.69 ± 0.51	1.95 ± 0.45

shown). Thus the system platelet-GPIIb/IIIa, fibrinogen and neutrophil-CD11b/CD18 does not seem to be involved in this adherence, as postulated by other authors (Ruf et al., 1992).

Effect of a monoclonal antibody to platelet glycoprotein GMP140 on the arachidonic acid metabolism in platelets

It has been recently suggested that glycoprotein GMP140 mediates the Ca^{2+} -dependent binding of polymorphonuclear leucocytes to thrombin-activated platelets (Jungi et al., 1986; Polley et al., 1991). We have studied the effect of a monoclonal antibody directed against the GMP140 protein on the arachidonic acid metabolism in thrombin-stimulated platelets. As seen in Figure 2(a), the anti-GMP140 monoclonal antibody significantly decreased (in a dose-dependent manner) the 12-HETE production in platelets stimulated by 0.1 unit/ml thrombin, and partially mimicked the effect observed in the presence of neutrophils. With 5 $\mu\text{g}/\text{ml}$ antibody, 12-HETE production was decreased by 56% ($P < 0.03$; $n = 6$). With 2 $\mu\text{g}/\text{ml}$ antibody, platelet 12-HETE production was still decreased by 30–57%, but this difference was found significant ($P < 0.01$) only with Student's two-tailed paired t test. The anti-GMP140 monoclonal antibody (5 $\mu\text{g}/\text{ml}$) also lowered the HHT production by 51% ($P < 0.05$; $n = 6$) in platelets stimulated by 0.1 unit/ml thrombin (Figure 2c). The antibody-induced decrease of 12-HETE and HHT productions was still apparent with platelets stimulated by 2.5 units/ml thrombin in the presence of 5 $\mu\text{g}/\text{ml}$ antibody (Figures 2b and 2d), but the differences appeared significant only with Student's paired t test. Thus the interaction of the monoclonal antibody against the GMP140 expressed on platelets could have the same effect as the interaction of neutrophils with this glycoprotein, i.e. decreasing the arachidonic metabolism in platelets.

Effect of treatment of neutrophils with sialidase on the arachidonic acid metabolism in platelets

The preceding results allow us to hypothesize that the decreased arachidonic acid metabolism in thrombin-stimulated platelets co-incubated with autologous neutrophils was dependent on cell adhesion, and that the glycoprotein GMP140 expressed on

platelet membranes after stimulation by thrombin could be responsible for this adherence and the subsequent inhibition. It has been suggested that the neutrophil receptor of the glycoprotein GPIIb/IIIa is an oligosaccharide with sialyl Lewis^x structure (Tiemeyer et al., 1991). Indeed Polley et al. (1991) have found that platelet adherence to neutrophils was suppressed after treatment of neutrophils with sialidase (neuraminidase). We have investigated the effect of treatment of neutrophils with this enzyme on the decreased arachidonic acid metabolism observed after thrombin stimulation of platelets. The results, presented in Table 3, show that the production of 12-HETE in platelets stimulated by thrombin (0.1 unit/ml) was lowered by 59% in the presence of untreated neutrophils ($P < 0.01$) and by 53% in the presence of neutrophils preincubated for 60 min at 37 °C without sialidase ($P < 0.01$). In contrast, this decrease was no longer observed after treatment for 60 min at 37 °C with sialidase. On the other hand, Table 3 also shows that the HHT production was significantly lowered ($P < 0.05$) in thrombin-stimulated platelets in the presence of untreated neutrophils or neutrophils preincubated without sialidase, but was not altered in platelets activated by thrombin (0.1 unit/ml) in the presence of sialidase-treated neutrophils.

DISCUSSION

The results obtained from this study show that the production of 12-HETE and HHT may be lowered in platelets stimulated by thrombin (0.1 or 2.5 units/ml) in the presence of autologous neutrophils, either unstimulated or challenged with the neutrophil chemotactic agent fMLP (0.1 µM) or the Ca²⁺ ionophore A23187 (0.15 µM). McCulloch et al. (1992) have reported similar results and attributed the observed decrease in 12-HETE production in thrombin-stimulated platelets to its metabolism by neutrophils. Indeed, 12-HETE released by thrombin-stimulated platelets is metabolized to 12(S),20-DHETE in unstimulated neutrophils (Marcus et al., 1984) or to 5(S),12(S)-DHETE in neutrophils challenged with Ca²⁺ ionophore A23187 (Marcus et al., 1982), the conversion of 12-HETE into 5(S),12(S)-DHETE occurring only when the neutrophil 5-lipoxygenase is activated. In our experiments, when thrombin-stimulated platelets were co-incubated with neutrophils, either unstimulated or stimulated by 0.1 µM fMLP or 0.15 µM ionophore A23187, the amount of 12-HETE released from platelets was decreased, but the formation of 5(S),12(S)-DHETE was never detectable, presumably because fMLP is a weak stimulating agent of neutrophil 5-lipoxygenase pathway and because this pathway was not activated in unstimulated neutrophils or neutrophils from healthy subjects challenged with 0.15 µM ionophore A23187 (Chabannes et al., 1991). The formation of 12(S),20-DHETE was detectable only in platelets co-stimulated with neutrophils in the presence of 2.5 units/ml thrombin, but the amount produced was variable, dependent on the blood sample and never higher than 13% of the difference between the amount of 12-HETE produced by platelets alone and co-incubated with neutrophils. The metabolism of HHT by neutrophils has never been described, but its level was also altered by co-incubating platelets with neutrophils. Moreover, the 12-HETE and HHT productions were increased to the same extent during the prolongation of platelet stimulation in the absence or presence of unstimulated or fMLP-stimulated neutrophils. Our observations then suggest that neutrophils alter the production of 12-HETE and HHT, rather than degrading them. McCulloch et al. (1992) have also hypothesized that the decrease in 12-HETE and HHT production observed when platelets and neutrophils were co-incubated with thrombin may be attributed

to a lowering of the effective concentration of thrombin caused by either passive adsorption on to or metabolism by neutrophils. However, in contrast with the results reported by these authors, but in agreement with those of Salvemini et al. (1989), we found that the 12-HETE and HHT productions were not modified or were slightly decreased when platelets were activated with the supernatant obtained from thrombin-incubated neutrophils. Moreover, we found that these decreases were also observed with cells stimulated by the Ca²⁺ ionophore A23187 as well as with unstimulated cells.

The decreased 12-HETE and HHT productions in thrombin-stimulated platelets co-incubated with neutrophils was not observed when cell populations were separated. Thus the physical contact between the two different cells appears necessary. Ruf et al. (1992) have shown that O₂^{•-} generation in neutrophils was increased in a contact-dependent manner after co-incubation with autologous stimulated platelets, as mediated by the system platelet GPIIb-IIIa glycoprotein, fibrinogen and neutrophil CD11/CD18 integrin. Peptides containing the RGD sequence inhibit the fibrinogen binding to the GPIIb-IIIa complex at the platelet surface (Plow et al., 1985), inhibiting the fibrinogen-mediated platelet-neutrophil adherence. However, an RGDF peptide, which inhibits platelet-neutrophil adhesion through this mechanism, did not affect the decrease in thrombin-stimulated platelet arachidonic acid metabolism observed in the presence of autologous neutrophils (results not shown). Platelets and neutrophils might interact through other specific membrane proteins. Indeed, the glycoprotein GPIIb/IIIa (CD62 or P-selectin), contained in the α-granules of platelets, is quickly expressed on their surface after thrombin stimulation (Hamburger and McEver, 1990), and the adherence mediated through this glycoprotein is Ca²⁺-dependent. The neutrophil-mediated decrease in 12-HETE and HHT from stimulated platelets was also Ca²⁺-dependent. Neutrophil treatment with sialidase, which, among sialyl-containing glycoconjugates, cleaves the structure sialyl Lewis^x (SL^x) assumed to be the natural ligand for platelet GPIIb/IIIa (Tiemeyer et al., 1991), and reported to suppress the GPIIb/IIIa-mediated neutrophil-platelet adherence (Moore et al., 1991; Polley et al., 1991), abolished the inhibitory effect of neutrophils on 12-HETE and HHT productions in thrombin-stimulated platelets. Moreover, a monoclonal antibody directed against GPIIb/IIIa, inhibiting platelet-neutrophil adhesion, mimicked the effect of neutrophils in a dose-dependent fashion. Another monoclonal antibody directed against this glycoprotein has been reported to inhibit thrombin-induced platelet aggregation (Parmentier et al., 1991). It has been reported that monoclonal antibodies against the common β-chain (CD18) or CD11a and CD11c α-chains of the CD11/CD18 integrin are able to stimulate the respiratory burst of human neutrophils (Berton et al., 1992). These results indicate that the binding of ligands to integrins can generate signals involved in activation of the neutrophil respiratory burst. Our findings also suggest that signals, leading to the inhibition of arachidonic acid metabolism in platelets, could be generated after binding of a specific monoclonal antibody to GPIIb/IIIa. Other results have pointed out that the effect of a cell population on another one was observed only after cell-cell adherence, e.g. neutrophils may be stimulated and primed by direct contact with activated T-lymphocytes (Zhang et al., 1992).

The extent of the decreased 12-HETE and HHT productions observed in the presence of neutrophils appears weaker in platelets stimulated by 2.5 than by 0.1 unit/ml thrombin. The amount of arachidonic acid released from phospholipids is higher in platelets stimulated by 2.5 than by 0.1 unit/ml thrombin (Purdon et al., 1987), and stimulating platelets by 2.5 units/ml thrombin could well correspond to an over-stimulation less

inhibitable by neutrophils. Moreover, the neutrophil-mediated inhibition of platelet arachidonic acid metabolism was no longer observed in the presence of exogenous arachidonic acid (10 μ M) (results not shown). Thus the inhibition of thrombin-stimulated platelet arachidonic acid metabolism in the presence of neutrophils could be explained by an inhibition of arachidonic acid release from phospholipids.

In summary, when platelets were stimulated by thrombin in the presence of autologous neutrophils, unstimulated or challenged with fMLP, the 12-HETE and HHT production was decreased. This decrease could not be explained by the metabolism of 12-HETE and HHT in neutrophils, and was observed only after normal contact between the two cell populations. The neutrophil adhesion to platelet GPIIb/IIIa could be responsible for the decrease in platelet arachidonic acid oxygenation.

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