Contact Activation Triggers Stimulation of the Monocyte 5-Lipoxygenase Pathway Via Plasmin

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The purpose of this study was to characterize the stimulus that activates the 5-lipoxygenase pathway in human peripheral monocytes (PM) during the process of contact activation. Incubation of PM, but not of polymorphonuclear leukocytes (PMN), in contact-activated, recalcified plasma induced a time-dependent release of leukotrienes (LT). The presence of platelets was required for the generation of cysteinyl-LT, but LTB4 formation also proceeded in their absence, although to a lesser extent. Plasmin, presumably generated via the intrinsic fibrinolytic pathway, was liable for the 5-lipoxygenase stimulation during contact activation inasmuch as (1) the 5-lipoxygenase pathway in PM was stimulated by contact-activated, recalcified, autologous or homologous plasma, but not by factor XII-deficient or prekallikrein-deficient plasma; (2) lysine analogs such as N'-acetyl-L-lysine, 6-aminohexanoic acid (6-AHA), or trans-4-(aminomethyl)cyclohexane-1-carboxylic acid (t-AMCA), which inhibit plasminogen binding to PM plasminogen binding sites, concentration-dependently reduced the cysteinyl-LT release; (3) plasminogen activators such as urokinase or streptokinase concentration-dependently enhanced the cysteinyl-LT release up to 10 and 1,000 IU/mL, respectively, while higher concentrations were less effective leading to bell-shaped concentration-response curves; (4) plasmin inhibitors such as aprotinin or α2-antiplasmin concentration-dependently inhibited the cysteinyl-LT release; and (5) preincubation of plasma with monoclonal antibodies directed against plasminogen and capable of preventing plasminogen activation blocked the contact-mediated 5-lipoxygenase stimulation. Moreover, incubation of PM with plasmin, but not with plasma kallikrein, in Hanks' balanced salt solution (HBSS)-bovine serum albumin (BSA) 0.4% triggered a concentration-dependent release of LTB4 up to 0.1 caseinolytic units (CU)/mL, with higher concentrations being less effective. By contrast, release of cyclooxygenase metabolites such as thromboxane (TX) B2 and prostaglandin (PG) E2 was not stimulated by plasmin, indicating specificity for the 5-lipoxygenase pathway. With plasmin as a hitherto unknown stimulus of the 5-lipoxygenase pathway in PM, a novel link between contact activation and inflammation has been established.

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MATERIALS AND METHODS

Materials. Urokinase (87% high-molecular weight form), streptokinase, plasminogen, α2-antiplasmin, and factor XII-deficient plasma were from Behring-Werke (Marburg, Germany), while prekallikrein-deficient plasma was purchased from George King BioMedical (Overland Park, KS). Monoclonal antibodies (TC 4PG) directed against plasminogen were obtained from Technoclone (Vienna, Austria). Human plasma (lot no. 370177) was purchased from Fluka (Buchs, Switzerland), and human plasma kallikrein (lot no. HPKa 390 L, 25 U/mg) from Enzyme Research Laboratories (South Bend, IN). Aprotinin was a gift from Dr Faßbender, Bayer AG (Wuppertal, Germany). Eicosanoid standards were obtained from Cascade (Reading, UK). Solvents were all of HPLC grade and other chemicals were of analytical grade.

Preparation of human blood cells, PRP, and PPP. Blood was
collected by free-flow technique from the antecubital vein of medication-free, apparently healthy, male volunteers (age, 21 to 33 years) with normal differential blood cell counts.

Monocytes were isolated on Percoll gradients basically as previously described. In brief, mononuclear cells were prepared from EDTA-anticoagulated blood by centrifugation (300g, 20 minutes, 20°C) against 61% Percoll-NaCl (vol/vol). After washing once with NaCl 150 mM, mononuclear cells were resuspended in PPP anticoagulated with 5 mM EDTA. This cell suspension was centrifuged (800g, 40 minutes, 4°C) through discontinuous EDTA (5 mM)-PPP-Percoll gradients of 25% and 39% Percoll (vol/vol), respectively. PM collected in a band of 94.0% purity at the 25%/39% interphase. The cells were washed twice in HBSS-bovine serum albumin (BSA) 0.4% (wt/vol). Two percent to 6% lymphocytes were found. Platelet satellism, ie, platelets associated with the PM membrane, was less than seven platelets per 100 monocytes. Cell purity was generally checked by phase-contrast microscopy and after staining with Trypan blue exclusion. PM were further characterized by staining for α-naphthyl-acetate esterase (>95% positive staining). Furthermore, transmission and scanning electron microscopy have been used for the identification of PM and for the determination of platelet satellism.

PMN were prepared from heparin-anticoagulated blood (20 μL) by centrifugation (300g, 20 minutes, 20°C) through two-step Percoll-NaCl (150 mM)-gradients of 61% and 75% Percoll (vol/vol). Mononuclear cells and PM collected at the 61% and 75% Percoll phase, respectively. After washing twice in NaCl 150 mM, remaining erythrocytes were removed by hypotonic lysis (NaCl 0.2% [wt/vol] for 1 minute). PMN were washed once more and purity was more than 98% with less than 2% lymphocytes.

Washed platelets were prepared as basically described by Marcus. However, the platelets were washed in CaCl2- and Mg2+-free HBSS, and were finally resuspended in HBSS-BSA 0.4%. PRP was prepared from blood anticoagulated with trisodium citrate 3.8% (wt/vol), 1 vol trisodium citrate + 9 vol blood) (310g, 20 minutes, 20°C). Except for the experiments with various platelet concentrations, PRP was generally adjusted with PPP to 3 × 10⁸ platelets/mL. PPP for cell incubations and dilution of PRP was prepared from PRP by centrifuging plasma twice (2,500g, 20 minutes, 20°C) against 61% Percoll-NaCl (vol/vol). After washing twice in NaCl 150 mM, remaining erythrocytes were removed by hypotonic lysis (NaCl 0.2% [wt/vol] for 1 minute). PMN were washed once more and purity was more than 98% with less than 2% lymphocytes.

Experimental protocol. When PM or PMN were incubated in recalcified plasma, 100 μL of HBSS-BSA 0.4% containing the desired cell number were pipetted into polystyrene glass tubes, and 900 μL of autologous PRP or PPP were added. Plasma was recalcified with 0.3 mol/L CaCl2 solution, resulting in a final concentration of 1.1 mol/L of free Ca2+ ions as determined by a Ca2+-sensitive electrode (Orion Research, Cambridge, MA). These samples were allowed to clot up to 120 minutes at 37°C, as indicated.

In experiments with factor XII-deficient or prekallikrein-deficient plasma, homologous plasma had been used. In experiments with recalcified plasma, 100 μL of HBSS-BSA 0.4% containing the desired cell number were pipetted into borosilicate glass tubes, and 20 and 100 μL, respectively.

In experiments with HBSS-BSA 0.4%, PM were incubated in polystyrene tubes for 60 minutes at 37°C in 500 μL. Plasmin dissolved in HBSS and kallikrein dissolved in NaCl 150 mM were added in a volume of 20 μL. In control experiments, PM in HBSS-BSA 0.4% were stimulated for 20 minutes with 1 μmol/L ionophore A 23187 dissolved in dimethylsulfoxide. Experimental controls always received the appropriate solvents.

At the time points indicated, incubations were stopped by rapid centrifugation at 4°C and the supernatants were used for further analysis.

Analytical procedures. Serum or HBSS-BSA levels of cysteinyl-LT, LTB4, TXB2, or prostaglandin (PG) E2 were determined radioimmunologically as previously described. The validity of the assays for LT for LT had been established by RP-HPLC. Before analysis of cysteinyl-LT, proteins were precipitated by addition of 3 vol of precooled acetone. After storage at −80°C, centrifugation and evaporation of the supernatants under reduced pressure, the residues were resuspended in Tris-HCl 50 mM, pH 7.4, and finally used for the radioimmunological determination of cysteinyl-LT contents. The anticysteinyl-LT antiplasma recognizes mainly LTC4, but exhibits 78% and 49% relative cross-reaction with LTD4 and LTE4, respectively, whereas other eicosanoids do not interfere with the assay. Cysteinyl-LT were calculated in terms of LTC4-like material, since upon RP-HPLC, immunoreactivity coeluted mainly with the reten-

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cysteinyl-LT into the serum samples was observed upon incubation of $15 \times 10^5$ PM/mL. In these samples, the cysteinyl-LT formation was significantly higher at 30 and 60 minutes as compared with incubations with the lower PM number ($n = 6$ each, $P < .05$, Fig 1A), although it was not triplicated, as one might have expected.

In contrast, incubation of PM in Ca²⁺-PPP at cell densities of 5 or $15 \times 10^5$/mL did not induce any detectable cysteinyl-LT release during the 120-minute incubation period (Fig 1B).

Similar to cysteinyl-LT formation, a time-dependent release of immunoreactive LTB₄ was found when PM (5 or $15 \times 10^5$/mL) were incubated in Ca²⁺-PRP (Fig 2A). At $5 \times 10^5$ PM/mL, LTB₄ serum levels significantly increased from 125 ± 19 pg/mL at 15 minutes to 465 ± 47 pg/mL at 60 minutes and further to $694 \pm 73$ pg/mL at 120 minutes ($n = 5$ each, $P < .005$). The higher cell number of PM ($15 \times 10^5$/mL) resulted in a much larger LTB₄ release relative to the lower cell number of PM ($5 \times 10^5$/mL) when compared with the cysteinyl-LT production shown in Fig 1A. The increased release of LTB₄ with the higher cell number of PM ($15 \times 10^5$/mL) was significant at any time point of the incubation period ($P < .01$). The difference in LTB₄ formation between the two PM cell densities was highest at 15 minutes with a factor of 3.25, and steadily decreased up to 120 minutes, reaching a factor of 2.12 (Fig 2A).

In contrast to Fig 1B, where we could not detect any cysteinyl-LT release when PM (5 or $15 \times 10^5$/mL) were incubated in Ca²⁺-PPP, these samples did contain immunoreactive LTB₄, although at a clearly reduced level (Fig 2B).
With $5 \times 10^5$ PM/mL, immunoreactive LTB$_4$ serum levels were 128 ± 43 pg/mL and 477 ± 73 pg/mL at 30 and 120 minutes, respectively ($n = 5$ each). At 60 and 120 minutes, the LTB$_4$ release into serum was significantly higher ($P < .05$) with $15 \times 10^5$ PM/mL as compared with $5 \times 10^5$ PM/mL (Fig 2B).

Incubation of PM 5 or $15 \times 10^5$/mL in HBSS-BSA 0.4% for the same length of time as in the experiments with Ca$_{2+}$-plasmas did not result in any detectable cysteinyl-LT or LTB$_4$ formation (data not shown).

When PMN 5 or $15 \times 10^5$/mL were incubated up to 120 minutes at 37°C in either Ca$_{2+}$-PRP, Ca$_{2+}$-PPP, or HBSS-BSA 0.4%, neither any cysteinyl-LT nor LTB$_4$ formation could be detected (data not shown).

The lack of a detectable cysteinyl-LT release in the absence of platelets, ie, in contact-activated Ca$_{2+}$-PPP samples containing PM, while considerable amounts of LTB$_4$ were formed at the same time, hinted to a potential transcellular biosynthesis of cysteinyl-LT under these circumstances. Indeed, when a fixed number of PM ($15 \times 10^5$/mL) were incubated for 60 minutes in autologous, contact-activated Ca$_{2+}$-PRP containing various concentrations of platelets, the cysteinyl-LT release was clearly dependent on the platelet number (Fig 3A). At a platelet concentration of $10^5$/mL, equivalent to a 30-fold dilution of the usual platelet number of $3 \times 10^9$/mL, release of immunoreactive cysteinyl-LT was no longer detectable, ie, there was a more than 87% reduction (Fig 3A). Similarly, incubation of various concentrations of PM in autologous, contact-activated Ca$_{2+}$-PRP containing a fixed number of platelets ($3 \times 10^9$/mL) demonstrated the dependency on the PM number (Fig 3B). However, the slope of this curve is not as steep as that for the platelets. A 30-fold dilution of the PM number from $15 \times 10^5$ to $0.5 \times 10^5$/mL led only to a 56.4% ± 9.5% ($n = 4$) reduction in cysteinyl-LT release (Fig 3B). In the absence of PM, no cysteinyl-LT could be detected (data not shown).

Characterization of immunoreactive LT by RP-HPLC. Serum from Ca$_{2+}$-PRP containing $15 \times 10^5$ PM/mL allowed to clot for 60 minutes contained immunoreactive material coeluting with the retention times of synthetic LTCA, LTDb, and LTEa. LTCa represented the largest portion, but also considerable amounts of LTDb and LTEa could be detected (Fig 4A). Such serum samples also contained immunoreactive material that coeluted in a single peak with the retention time of synthetic LTB$_4$ (Fig 4C).

Dependence on contact activation. Clear evidence for the necessity of contact activation was obtained in experiments with prekallikrein-deficient or factor XII-deficient plasma incubated under contact activation conditions. Incubation of PM ($15 \times 10^5$/mL) in contact-activated, autologous or homologous Ca$_{2+}$-PPP for 60 minutes induced release of practically identical amounts of LTB$_4$ into the serum samples (Fig 5). In contrast, incubation of PM in either 100% (vol/vol) prekallikrein-deficient or factor XII-deficient Ca$_{2+}$-plasma under otherwise identical conditions was not accompanied by any detectable release of LTB$_4$ (<98 pg/mL, $n = 4$ each, $P < .005$). When 50% (vol/vol) of the autologous Ca$_{2+}$-PPP was replaced by either calcified prekallikrein-deficient or factor XII-deficient plasma, a moderate but significant ($P < .005$) inhibition of the LTB$_4$ release was observed (Fig 5). Both deficiency plasmas had no direct effect on the monocyte 5-lipoxygenase activity, because stimulation of PM with ionophore A23187 (1 μmol/L) for 20 minutes in either Ca$_{2+}$-plasma triggered generation of identical amounts of LTB$_4$ as compared with autologous plasma (data not shown).

Effects of lysine analogs. To evaluate the possible contribution of plasmin to the contact activation-related stimulation of the 5-lipoxygenase pathway in PM, the effects of various lysine analogs on the contact-mediated cysteinyl-LT production were investigated. All of the compounds tested...
Fig 4. RP-HPLC profile of immunoreactive cysteinyl-LT from serum samples obtained after a 60-minute incubation of autologous PM (15 x 10^6/mL) in recalcified PRP at 37°C in vitro (A). RP-HPLC profiles of immunoreactive LTB₄ from serum samples of recalcified PRP (B) or PPP (C) containing PM as aforementioned. Arrows indicate the retention times of appropriate standards. The results of representative experiments are shown. Each experiment was performed at least three times, with qualitatively identical results.

Fig 5. Inhibitory effects of prekallikrein-deficient or factor XII-deficient plasma on contact-related stimulation of the monocyte 5-lipoxygenase pathway as compared with autologous or homologous plasma. PM (15 x 10^6/mL) were incubated for 60 minutes in recalcified plasmas at 37°C in vitro. Incubation in the deficiency plasmas proceeded either in the presence of deficiency plasma alone (100%) or in a mixture of autologous plasma containing 50% (vol/vol) deficiency plasma. **P < .005 as compared with autologous plasma. Results are the mean ± SEM of four experiments each; nd, not detectable, <28 pg/mL.

were effective inhibitors of cysteinyl-LT formation when 5 x 10^7 PM/mL were incubated for 60 minutes in Ca²⁺-PRP (Fig 6). The weakest inhibitor was N-acetyl-L-lysine (IC₅₀, 2.23 mmol/L; range, 1.99 to 2.50 mmol/L; n = 4) while t-AMCA was the most potent compound (IC₅₀, 55 μmol/L; range, 43 to 69 μmol/L; n = 5). As expected, 6-AHA had an intermediate potency (IC₅₀, 604 pmol/L; range, 395 to 922 μmol/L; n = 4) (Fig 6). The highest concentrations of the lysine analogs used did not inhibit cysteinyl-LT release when PM (5 x 10^7/mL) incubated in HBSS-BSA 0.4% were stimulated with 1 μmol/L ionophore A23187 for 20 minutes (data not shown).

The most potent lysine analog, t-AMCA, was used at a concentration of 100 μmol/L to test whether the LTB₄ formation by PM (5 x 10^7/mL) incubated in Ca²⁺-PPP for 60 minutes would be inhibited as well. Under these conditions, LTB₄ release into the serum samples was significantly reduced by 69.1% ± 3.8% (n = 3, P < .001; 100% value, 295 ± 56 pg/mL).

Effects of plasminogen activators. Further evidence for a potential role of plasmin in the contact-mediated 5-lipoxygenase activation came from experiments with plasminogen activators such as urokinase and streptokinase. Indeed, urokinase led to a concentration-dependent stimulation of cysteinyl-LT release into the serum samples, with a maximum effect at approximately 10 IU/mL (Fig 7A). Beyond that concentration, cysteinyl-LT serum levels decreased again, resulting in a bell-shaped concentration response curve in the range between approximately 3 and 100 IU/mL, while at a concentration of 300 IU/mL no stimulatory activity could be detected (Fig 7A). By contrast, when streptokinase
was used as a stimulus, no activation of the cysteiny-LT production was observed between 0.1 and 30 IU/mL (data not shown). However, at streptokinase concentrations between 300 and 3,000 IU/mL, similar to the urokinase experiments, a bell-shaped concentration-response curve was observed, with a maximum at approximately 1,000 IU/mL (Fig 7B). Neither urokinase 10 IU/mL nor streptokinase 1,000 IU/mL triggered any release of LTB4 or cysteiny-LT from 5 \times 10^5 PM/mL incubated for 20 minutes (data not shown).

Effects of plasmin inhibitors. As shown in Fig 8A, apro-arin concentration-dependently reduced the serum levels of cysteiny-LT (IC_{50} = 316 KIU/mL; range, 208 to 481 KIU/mL; n = 5). Similarly, exogenous \( \alpha_2 \)-antiplasmin as highly specific plasmin inhibitor concentration-dependently inhibited the contact activation-mediated 5-lipoxygenase stimulation (IC_{50} = 566 nmol/L; range, 473 to 678 nmol/L; n = 5) (Fig 8B). On the other hand, both compounds had no effect on the ionophore A23187 (1 \mu mol/L)-stimulated cysteiny-LT release from 5 \times 10^5 PM/mL incubated in HBSS-BSA 0.4% for 20 minutes (data not shown).

The specific plasmin inhibitor \( \alpha_2 \)-antiplasmin was also used with PM 5 \times 10^5/mL incubated for 60 minutes in Ca^{2+}-PPP. Under these conditions, 1 \mu mol/L of \( \alpha_2 \)-antiplasmin significantly reduced the LTB4 release by 78.4% \pm 5.5% (n = 3, P < .001; 100% value, 295 \pm 56 pg/mL).

Effects of monoclonal antiplasminogen antibodies. In our final approach regarding the role of plasmin under these experimental conditions, we tested the effects of monoclonal antibodies (TC 4PG) on the release of cysteiny-LT into serum samples from contact-activated Ca^{2+}-PRP containing 5 \times 10^5 PM/mL incubated in HBSS-BSA 0.4% for 60 minutes (data not shown).

As shown in Fig 9A, after 60 minutes, control samples contained considerable amounts of cysteiny-LT (496 \pm 32 pg/mL, n = 4), while in the presence of TC 4PG (8 pmol/L), which is known to protect plasminogen from activation to plasmin, no detectable release of cysteiny-LT could be found (<75 pg/mL, n = 4, P < .001) (Fig 9A). Similar results were obtained with respect to LTB4 release from 15 \times 10^5 PM/mL incubated in contact-activated, autologous Ca^{2+}-PPP (501 \pm 27 pg/mL < 98 pg/mL in TC 4PG pre-treated plasma, n = 4, P < .001) (Fig 9B).

Effects of plasmin and kallikrein on eicosanoid formation by PM. Incubation of 15 \times 10^5 PM/mL in HBSS-BSA 0.4% alone for 60 minutes did not induce any detectable
release of immunoreactive LTB4. However, when PM were incubated in the presence of plasmin 0.001 to 1.0 caseinolytic units (CU)/mL, a significant (P < .01) and concentration-dependent stimulation of LTB4 release was observed, reaching a maximum of 554 ± 78 pg/mL at a plasmin activity of 0.1 caseinolytic units (CU)/mL (Fig 10A). Beyond 0.1 CU/mL of plasmin, LTB4 formation decreased again. At a plasmin activity of 1.0 CU/mL, LTB4 levels were significantly decreased to 101 ± 22 pg/mL (n = 6, P < .01) as compared with 0.1 CU/mL (Fig 10A).

Immunoreactive LTB4 produced by PM in HBSS-BSA 0.4% upon stimulation with 0.1 CU/mL of plasmin for 60 minutes, after RP-HPLC coeluted in a single peak with the retention time of synthetic LTB4 (data not shown). The concentrations used, both compounds had no effect on the ionophore A 23187 (1 μmol/L)-stimulated LTB4 formation (data not shown).

In the absence of platelets, release of cysteinyl-LT from PM (5 × 10^7/mL) incubated in HBSS-BSA 0.4% for 60 minutes in the presence of purified plasmin was close to or below the detection limit of the assay (data not shown). However, when PM coincubated with washed platelets (3 × 10^7/mL) were stimulated with plasmin 0.01, 0.1, or 1.0 CU/mL, a concentration-dependent release of cysteinyl-LT was observed (221 ± 31 pg/mL, 524 ± 42 pg/mL, and 198 ± 41 pg/mL, respectively, n = 4), which roughly paralleled the concentration response curve of plasmin for LTB4 release (compare Fig 10A).

In contrast to plasmin, human plasma kallikrein in concentrations between 0.06 and 0.5 μmol/L did not trigger any detectable release of immunoreactive LTB4 from PM 15 × 10^7/mL incubated in HBSS-BSA 0.4% for 60 minutes (n = 3, data not shown).

To investigate a potential stimulatory effect of plasmin on the cyclooxygenase pathway of arachidonic acid metabolism, we determined the release of the two major prostanoids generated by PM. As shown in Fig 10B, plasmin did not significantly affect the release of immunoreactive TXB2 or PGE2, indicating selectivity for the 5-lipoxygenase pathway.

**DISCUSSION**

We have previously demonstrated that clotting of human whole blood in vitro is accompanied by LT production due to activation of the monocyte 5-lipoxygenase pathway of arachidonic acid metabolism. In extension of these studies, our present results show that the complex system of contact-activated human whole blood can be successfully reduced to the essential components, namely isolated PM as the cellular source of 5-lipoxygenase activity and recalcified, contact-activated plasma providing the trigger mechanism for 5-lipoxygenase stimulation. It was this experimental setup that permitted us to perform crucial experiments, such as those with prekallikrein-deficient or factor XII-deficient plasma. These experiments clearly indicate that contact activation,
PM, the platelet number is a critical determinant in our system. Apart from transcellular biosynthesis, PM themselves do possess the capacity for generation of cysteinyl-LT in the absence of platelets, which was actually observed in our control experiments with 1 μmol/L ionophore A 23187–stimulated PM. Because LTC₄ release may be as low as 20% of the LTB₄ formation when physiologic stimuli are being used, the moderate stimulation of the 5-lipoxygenase pathway in PM by contact-activated Ca²⁺-PPP may have led to cysteinyl-LT serum levels well below the detection limit of the assay.

The presence of platelets was required for the generation of detectable amounts of cysteinyl-LT, but it also significantly enhanced the release of LTB₄. Since platelets do not seem to convert LTA₄ into LTB₄, the enhanced release of LTB₄ observed is due to further activation of the PM 5-lipoxygenase pathway. Similar to cysteinyl-LT formation, this stimulatory effect was dependent on the platelet number. However, it was not due to any potential substrate feeding, since labeling of platelets with [³H]-arachidonic acid before contact activation did not lead to any [³H]-labeled LT formation upon RP-HPLC profiling. 12(S)-hydroperoxy-10-trans-5,8,14-cis-eicosatetraenoic acid (12-HPETE), which as a platelet-derived arachidonic acid metabolite has been reported to stimulate LT biosynthesis in human leukocytes, had no stimulatory effect in concentrations up to 10 μmol/L (unpublished results). Thus, in line with other investigators, we could not detect any metabolism of platelet intermediates by PM, nor did we find any 5-lipoxygenase activation in PM by 12-HPETE.

Several other studies have shown an effect of platelets on a specific activity of PM. Thus, tissue factor activity was found to be higher in platelet-enriched as compared with platelet-poor mononuclear cell preparations. Although clear evidence has been presented for a role of 12-HETE under these conditions, its generation in our experiments is unlikely to contribute to the stimulatory effect of platelets, since, similar to 12-HPETE, up to 10 μmol/L of 12-HETE did not enhance LTB₄ release from PM incubated in contact-activated Ca²⁺-PPP (unpublished results). The presence of platelets has also been reported to enhance the rate of cholesterol esterification, as well as the cholesteryl ester accumulation in PM and U937 cells. In addition, PM-platelet interactions may serve to increase specifically PM synthesis of the adhesive protein thrombospondin, which mediates platelet-PM cell adhesion via glycoprotein IV. Therefore, in future studies concerning the role of platelets in the contact-mediated activation of the PM 5-lipoxygenase pathway, a potential role of thrombospondin will have to be considered. On the other hand, the stimulatory effect of platelets could also be brought about by enhancing and/or accelerating effects on contact activation, although such effects have been debated.

As a test system for further studies on the contact-mediated trigger mechanism, we chose to incubate PM in contact-activated Ca²⁺-PRP. Under these conditions, the presence of platelets warranted more physiological activation of the intrinsic coagulation than in Ca²⁺-PPP. Since, in our hands, the determination of cysteinyl-LT serum levels was much
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less critical than that of LTB₄, we routinely used the cysteinyl-LT release as a parameter of 5-lipoxygenase activation. However, to show that the results obtained could be extended to PPP and LTB₄ in some instances experiments were also performed with Ca²⁺-PPP and LTB₄ was determined.

Initially, we considered components of the intrinsic coagulation cascade assembled on PM membranes¹²² as a potential trigger mechanism. However, we could not gain any evidence for a stimulatory effect of factor Xa or factor Va. Contact activation is known to stimulate the intrinsic fibrinolytic pathway in plasma.¹²²,²⁸ Consequentially, when we became aware of the work of Miles et al showing that membranes of PM and related monocytoid U937 cells possess binding sites for plasminogen,²⁹⁻³¹ we tested the effects of lysine analogs in our system. These compounds are known to inhibit plasminogen binding to specific receptors on PM membranes, presumably by interaction with the high-affinity lysine binding site of the plasminogen molecules.²⁹⁻³¹ Indeed, the rank order of the inhibitory potency of the lysine analogs on cysteinyl-LT release was comparable to that as inhibitors of plasminogen binding to U937 cells.²⁹

Further experiments demonstrating that plasminogen activators such as urokinase and streptokinase concentration-dependently enhanced the cysteinyl-LT release strengthened the hypothesis that plasmin might be involved in the activation mechanism. Urokinase, as well as streptokinase, led to bell-shaped concentration-response curves, which might be due to proteolytic digestion of the plasmin binding sites. In fact, plasmin is a rather unspecific protease that might, for example, degrade platelet adhesive receptors such as glycoprotein Ib.¹³ Although at present the molecular mechanisms leading to the bell-shaped expression of the concentration response curves remain unknown, other investigators have also observed this phenomenon. Both urokinase³³ and streptokinase³⁴ have been reported to trigger plasmin-mediated effects in citrated plasma, thereby exhibiting bell-shaped concentration-response curves. The profile of the urokinase concentration-response curve with a maximum at 10 IU/mL and a loss of stimulatory activity at 300 IU/mL would suggest that plasma levels of urokinase attained during fibrinolysis (ie, 500 to 2,000 IU/mL)³⁵ should be beyond the concentrations resulting in PM stimulation. By contrast, with a maximum effect at 1,000 IU/mL, streptokinase required much higher concentrations for stimulation of the cysteinyl-LT release. This may be, at least partially, to complex formation of the activator with plasmin.³⁶ In line with this assumption, we found that the stimulatory effect of plasmin 0.175 Committee on Thrombolytic Agents (CTA) units/mL in HBSS-BSA 0.4% on PM-derived LTB₄ formation was inhibited by streptokinase, with an IC₅₀ of 164 IU/mL. Therapeutic dosage of streptokinase 1.5 x 10⁷ IU/h may well lead to plasma levels of 400 IU/mL,³⁶ which under our in vitro conditions slightly enhanced cysteinyl-LT release already. However, whether this effect may be relevant to clinical situations remains to be established.

The significance of plasmin for the contact-mediated stimulation of the PM 5-lipoxygenase pathway was further demonstrated by experiments with plasmin inhibitors. Both the rather unspecific aprotinin, which in contrast to α₂-antiplasmin is even able to inhibit cell-bound plasmin,³⁷ as well as the highly specific and physiological plasmin inhibitor, α₂-antiplasmin,³⁸ concentration-dependently inhibited the contact-mediated 5-lipoxygenase activation to nearly complete blockade.

In our last approach regarding the role of plasmin in the contact-activated 5-lipoxygenase stimulation in PM, we were able to show that monoclonal antibodies directed against miniplasminogen and able to inhibit plasminogen activation⁰ completely blocked the contact-mediated 5-lipoxygenase stimulation in PM incubated in both PRP or in the absence of platelets in PPP. Thus, by four independent pharmacological approaches using several compounds in three of them, we present convincing evidence that plasmin generated during the process of contact activation plays a critical role for the stimulation of the PM 5-lipoxygenase under these conditions. It is reasonable to assume that the plasmin formation occurs via the contact-activated intrinsic fibrinolytic pathway.¹²²,²⁸ First, LT production could not be detected when PM were incubated in HBSS-BSA 0.4% under resting conditions. Second, the dextrane sulfate—dependent and therefore contact-mediated fibrinolysis in whole human plasma has been shown to depend on the presence of both factor XII and prekallikrein,³⁹ as does the contact-mediated LT formation. Similarly, a recently described although not fully characterized contact-dependent plasminogen activator, allegedly liable for a substantial part of the contact-dependent intrinsic fibrinolysis, appears to be strictly dependent on activation via factor XII and prekallikrein.⁴⁰ Moreover, contact-mediated activation of plasma pro-urokinase, which also appears to be involved in the intrinsic fibrinolytic pathway,¹²,⁴¹ has also been shown to be clearly dependent on the presence of both factor XII and prekallikrein.³² Third, addition of aprotinin (6000 KIU/mL) to PM-containing Ca²⁺-PRP as early as 3 minutes after contact activation did not reduce LT release over a further 57-minute incubation, while addition at time 0 nearly completely blocked LT release, indicating that the activation occurs during the very early phase of contact activation (unpublished results).

From the experiments discussed so far, it is evident that plasmin plays a pivotal role in the contact-mediated stimulation of the 5-lipoxygenase pathway in PM. In further experiments in which the cells were incubated in HBSS-BSA 0.4%, purified plasmin proved to trigger LT release from PM, yielding a concentration-response curve reminiscent of that obtained with plasminogen activators. Similar results were obtained with respect to cysteinyl-LT production when the plasmin stimulation was performed in the presence of washed platelets. The effectiveness of lysine analogs under these conditions indicates that the stimulatory activity of plasmin requires association of the molecule with the PM receptors via its lysine binding sites. It is of considerable interest that plasmin acts relatively selectively on the 5-lipoxygenase pathway, while the release of the two major cyclooxygenase products, TXB₂ and PGE₂,¹⁸ was not significantly affected. It has previously been suggested that release of cyclooxygenase or lipoxygenase metabolites may not necessarily reflect the extent of phospholipase activation.
in PM. Evidence exists, indeed, that within certain limits PM and macrophages might be capable of differential release of cyclooxygenase and lipoxygenase products of arachidonic acid metabolism. Under certain conditions, enhanced LT production might actually be accompanied by reduced PGE2 formation. These effects might be attributable to compartmentalization of both, the endogenous arachidonic acid liberation, and the 5-lipoxygenase enzyme. Such compartmentalization of phospholipases and/or arachidonic acid metabolizing enzymes could easily explain the relative selectivity of plasmin for the 5-lipoxygenase pathway as found in our study.

Plasmin has previously been shown to affect various cell functions. Thus, low concentrations of plasmin (0.1 to 0.5 CU/mL) appear to inhibit platelet functions at least in part by blocking the mobilization of arachidonic acid from membrane phospholipid pools. Higher concentrations of plasmin (≥1.0 CU/mL), on the other hand, trigger platelet activation independent from TXB2 formation, but accompanied by intracellular Ca2+ elevation, phospholipase C activation, and protein phosphorylation. In this context, peculiar effects of plasmin have been observed on arachidonic acid metabolism. In platelets, as well as in endothelial cells, plasmin seems to cause mobilization of endogenous arachidonic acid, but without significant metabolism to the major cyclooxygenase products formed by these cells. In endothelial cells, plasmin has also been shown to inhibit tissue-type plasminogen activator release. Although in our hands plasmin did not stimulate any LT or prostanoid formation by PMN, these cells apparently do respond to plasmin challenge with cell aggregation and enhanced adherence to endothelial cells. Because little is known so far about potential signal transduction mechanisms in plasmin-mediated effects, further studies are underway to characterize the mechanism leading to PM 5-lipoxygenase activation.

In conclusion, by several independent approaches, our study clearly demonstrates that contact activation of re-calculated human plasma triggers activation of the 5-lipoxygenase pathway in PM via plasmin. Since LT formation is a proinflammatory signal, plasmin generated during contact activation may represent a new link between contact activation and the PM component of inflammatory reactions. Contact activation is indeed a common feature in numerous inflammatory diseases, including rheumatoid arthritis, suggesting that the contact-mediated proinflammatory activation of PM might be of considerable pathophysiological importance. Furthermore, these findings indicate a novel pathophysiological role for the contact-activated intrinsic fibrinolytic pathway, which is too weak for efficient fibrinolysis but apparently strong enough for proinflammatory activation of PM.

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Contact activation triggers stimulation of the monocyte 5-lipoxygenase pathway via plasmin

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