Thrombin-induced Release of von Willebrand Factor from Endothelial Cells Is Mediated by Phospholipid Methylation

PROSTACYCLIN SYNTHESIS IS INDEPENDENT OF PHOSPHOLIPID METHYLATION

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The biochemical events that lead to thrombin-stimulated release of von Willebrand factor and prostacyclin synthesis in vascular endothelial cells are examined. Treatment of human umbilical vein endothelial cells with thrombin results in an instantaneous increase in phospholipid methylation which can be blocked by 3-deazaadenosine, a methyltransferase inhibitor. 3-Deazaadenosine also blocks the thrombin-induced Ca\(^{2+}\) influx into endothelial cells and the release of von Willebrand factor, indicating that these processes are coupled. The phorbol ester 4β-phorbol 12-myristate 13-acetate (PMA) and the Ca\(^{2+}\) ionophore A23187 both bypass the phospholipid methylation and directly stimulate Ca\(^{2+}\) influx and von Willebrand factor release.

In contrast to the stimulus-induced von Willebrand factor release, the thrombin-induced prostacyclin synthesis cannot be blocked by 3-deazaadenosine. Similarly, incubation of endothelial cells with EDTA has no influence on the thrombin-induced prostacyclin synthesis, and PMA has no stimulatory effect on prostacyclin synthesis. These observations indicate that thrombin induces different metabolic responses in endothelial cells: 1) phospholipid methylation followed by a Ca\(^{2+}\) influx, which subsequently leads to release of von Willebrand factor, and 2) liberation of arachidonic acid from phospholipids for prostacyclin formation, which is independent of phospholipid methylation and Ca\(^{2+}\) influx.

During the last few years it has become increasingly apparent that vascular endothelial cells not only provide a passive antithrombotic barrier between the circulating blood and the underlying tissue but, in response to certain stimuli, may instantaneously increase their antithrombotic potential by initiating a sequence of events that involves phospholipid metabolism. We found that indeed thrombin induces phospholipid methylation and enhances Ca\(^{2+}\) influx. However, these processes are only associated with the release of von Willebrand factor, not with prostaglandin synthesis. In this respect endothelial cells distinguish themselves from other cell types (11, 13) in that the induced prostacyclin synthesis is not dependent on phospholipid methylation. Similarly, PMA, another well-known stimulus of cells, both enhances the Ca\(^{2+}\) influx and the release of von Willebrand factor but does not stimulate prostacyclin synthesis in endothelial cells.

EXPERIMENTAL PROCEDURES

Cell Culture—Human vascular endothelial cells were isolated from umbilical veins and cultured according to the method originally described by Jaffe et al. (16) with some minor modifications (17) including culturing in 20% pooled human serum. The cells were identified by their typical characteristics (16). For the experiments described in this paper, subcultures of the second passage were used as confluent monolayers (approximately 8 × 10⁶ cells/cm²). For experiments designed to study the uptake of Ca\(^{2+}\); cells were cultured on a microcarrier support system of negatively charged spherical plastic beads (Biosion, A/S Nunc, Denmark) essentially as described by Devis (18). In our experiments, human endothelial cells readily grew to a density of about 40 cells/bead under static conditions. Cell numbers were determined with a hemocytometer.

Determination of Endothelial Cell Secretion Products—Confluent monolayers of endothelial cells were washed twice with RPMI-1640 and subsequently incubated in the presence of RPMI-1640. After stimulation as indicated, samples were taken from the supernatants for the determination of von Willebrand factor, β-thromboglobulin, and 6-keto-PGF\(_{1α}\). von Willebrand factor was determined by an

1 The abbreviations used are: PMA, 4β-phorbol 12-myristate 13-acetate; 6-keto-PGF\(_{1α}\), 6-keto-prostaglandin F\(_{1α}\); Heps, 4-(2-hydroxyethyl)1-piperazineethanesulfonic acid.
immunoradiometric assay essentially following the method of Ruggeri et al. (19); instead of purified polyclonal IgG, a mixture of monoclonal antibodies coded CLB-RAg 21, CLB-RAg 35, and CLB-RAg 201 (20) were used as indicator antibodies. β-Hexosaminidase was assayed as described by De Groot et al. (21). 6-keto-PGF1α was used as indicator antibodies. 8-Hexosaminidase was assayed as described by Bareis et al. (13) with some minor modifications. In short: confluent cultures of endothelial cells were maintained for 2 days with 10 ml of methionine-free Ham's F-10 medium (Flow Laboratories, Irvine, Scotland) containing 1% human serum and 10 μCi of 3H-Methionine (70-85 Ci/mmol, Amersham, UK). The medium was then added, and after the indicated time, the medium was quickly aspirated and the cells were washed with ice-cold 150 mM NaCl solution containing 77 mM EDTA, 2 mM formic acid, and 1 mM unlabeled methionine. After adding 5 ml of the latter solution the cells were carefully scraped from the flasks with a rubber policeman and transferred into a glass tube. The cells were extracted twice with 10 ml of ethyl acetate, and the combined organic phases were evaporated to dryness under a constant stream of nitrogen.

Phospholipid Methylation—Phospholipid methylation was studied as described by Bares et al. (13) with some minor modifications. In short: confluent cultures of endothelial cells were maintained for 2 days with 10 ml of methionine-free Ham's F-10 medium (Flow Laboratories, Irvine, Scotland) containing 1% human serum and 10 μCi of 3H-Methionine (70-85 Ci/mmol, Amersham, UK). The medium was then added, and after the indicated time, the medium was quickly aspirated and the cells were washed with ice-cold 150 mM NaCl solution containing 77 mM EDTA, 2 mM formic acid, and 1 mM unlabeled methionine. After adding 5 ml of the latter solution the cells were carefully scraped from the flasks with a rubber policeman and transferred into a glass tube. The cells were extracted twice with 10 ml of ethyl acetate, and the combined organic phases were evaporated to dryness under a constant stream of nitrogen. The residues were reconstituted with 0.2 ml of chloroform/methanol (2:1), and radioactivity was measured. In a separate experiment, the residues were applied to Silica Gel G60 plates (Merck, Darmstadt, Federal Republic of Germany), and phospholipids were separated by developing the plates with chloroform/methanol/water (2:1:1, v/v). Standards, run simultaneously on separate lanes, were visualized by staining with phosphomolybdcic acid (applied as aerosol, Merck), followed by heating for 5 min at 120°C. Autoradiographs were made by exposing the plates to a Kodak x-ray film for 1 week.

Calcium Flux—Approximately 6 x 10⁶ cells, grown on microcarriers, were incubated in 500 μl of Heps buffer (150 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM glucose, 4 mM glutamine, 10 mM Heps, pH 7.4) containing 2 μCi of ⁴⁰Ca (specific activity, 0.6 Ci/mmol, Amersham Corp.). The Ca²⁺ uptake was measured essentially as described by Bareis et al. (13). After 16 min the stimulus (as indicated) was added, and the uptake of ⁴⁰Ca was stopped by adding 50 μl of 50 mM CaCl₂. The medium was immediately aspirated, and the cells were washed twice with ice-cold 150 mM NaCl solution containing 5 mM CaCl₂ and 10 mM Heps, pH 7.4. After addition of 0.5 ml of 5 mM CaCl₂/0.5% Triton X-100 the microcarriers were spun down, and the supernatants were transferred into vials for the measurement of radioactivity.

Materials—All cell cultures and culture media were obtained from Gibco Biocult (Paisley, Scotland). Thrombin (human, 3000 units/mg of protein) was purchased from Sigma. Ionophore A23187 was obtained from Boehringer (Mannheim, Federal Republic of Germany). PMA was obtained from Consolidated Midland Corp. (Brewster, NY) or Sigma. 3-Deazadenosine was obtained from Southern Research Institute, Birmingham, Ala. Homocysteinyl acid was obtained from Calbiochem. All other reagents were from the purest grade available.

RESULTS

Stimulus-induced von Willebrand Factor Release and Prostacyclin Synthesis—As was shown previously (8), the addition of thrombin (2 units/ml) or the Ca²⁺ ionophore A23187 (10 μM) to confluent monolayers of cultured endothelial cells resulted within 10 min in a significant increase in the rate of accumulation of von Willebrand factor and 6-keto-PGF₁α, the stable hydrolysis product of prostacyclin in the culture medium (Table I). Only upon prolonged incubation with PMA (more than 8 h) a slight increase of prostacyclin production was noted. On the other hand, PMA (100 ng/ml) stimulated the release of von Willebrand factor but had no effect on the synthesis of 6-keto-PGF₁α (Table I). The release of von Willebrand factor was dose dependent and occurred at concentrations as low as 1 ng/ml of PMA. Preincubation of endothelial cells with PMA for 5 to 60 min did not affect the thrombin-induced prostacyclin production (not shown), indicating that PMA had no inhibitory effect on prostacyclin production. No β-hexosaminidase could be detected in the medium after the different incubations, indicating that the integrity of the cells was not affected.

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>6-Keto-PGF₁α</th>
<th>von Willebrand factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>-EDTA</td>
<td>0.1 ± 0.1</td>
<td>11.6 ± 1.2</td>
</tr>
<tr>
<td>+EDTA</td>
<td>0.9 ± 0.1</td>
<td>10.9 ± 0.7</td>
</tr>
<tr>
<td>Thrombin</td>
<td></td>
<td>9.2 ± 1.1</td>
</tr>
<tr>
<td>PM A</td>
<td>0.1 ± 0.1</td>
<td>32.9 ± 18.5</td>
</tr>
<tr>
<td>A23187</td>
<td></td>
<td>9.0 ± 0.7</td>
</tr>
</tbody>
</table>

*ND, not detectable.

The thrombin- and ionophore-induced release of von Willebrand factor is dependent on extracellular Ca²⁺ concentration (8). Similarly, the PMA-induced release is dependent on the presence of extracellular Ca²⁺. When endothelial cells were preincubated 2 min with 5 mM EDTA, the stimulus-induced release of von Willebrand factor by thrombin, PMA, and A23187 was almost completely inhibited (Table I). On the other hand, the thrombin-stimulated prostaglandin production was not influenced by EDTA while the A23187-stimulated prostaglandin production was partly inhibited (Table I and Ref. 8).

Phospholipid Methylation and Ca²⁺ Influx—Enzymatic methylation and Ca²⁺ fluxes play an important role in transduction of receptor-mediated signals through membranes of a variety of cells (11). Consistent with these, an increase in methylation was noted when endothelial cells were incubated with thrombin (2 units/ml). A typical experiment is shown in Fig. 1A. The increase in methylation peaks between 10 and 20 s. Subsequent analysis of the phospholipids showed that the methyl group was predominantly incorporated in phosphatidylcholine (Fig. 1B). Addition of EDTA had no effect on
thrombin-induced methylation of the phospholipids (not shown).

Ca\(^{2+}\) influx is maximal within 20 s after the addition of thrombin and PMA (Fig. 2) and then, probably due to efflux, declines to control values. The ionophore A23187 induced Ca\(^{2+}\) influx comparable to the Ca\(^{2+}\) influx induced by thrombin without affecting phospholipid methylation (not shown).

Effect of Metabolic Inhibitors—The relationship between phospholipid methylation, Ca\(^{2+}\) flux, prostaglandin synthesis, and von Willebrand factor release was further investigated with metabolic inhibitors. When endothelial cells were preincubated for 2 h with 3-deazaadenosine (10 μM), an inhibitor of 3-adenosylmethionine-requiring methyltransferase, including phospholipid methyltransferase (23), the thrombin-induced increase in phospholipid methylation was inhibited (Fig. 1, A and B). Complete inhibition of the increase in methylation induced by thrombin occurred at 5 μM 3-deazaadenosine (not shown). Preincubation of endothelial cells with 2.5 or 5 mM EDTA did not influence the thrombin-induced phospholipid methylation (not shown).

Twenty s after addition of a stimulus there is a maximal Ca\(^{2+}\) influx in endothelial cells (Fig. 2). When endothelial cells were preincubated with 3-deazaadenosine (100 μM), the thrombin-induced increase in phospholipid methylation was inhibited (Fig. 3). On the other hand, the Ca\(^{2+}\) influx stimulated by PMA or A23187 was not affected. As expected, incubation of endothelial cells with 5 mM EDTA completely blocked the Ca\(^{2+}\) influx induced by thrombin, PMA, or A23187 (Fig. 3).

When endothelial cells were preincubated for 2 h with 10 μM 3-deazaadenosine, the thrombin-induced release of von Willebrand factor was completely inhibited whereas the thrombin-induced synthesis of 6-keto-PGF\(_{1\alpha}\) was not affected (Table II). Even in the presence of 100 μM 3-deazaadenosine no influence on 6-keto-PGF\(_{1\alpha}\) production was noted (not shown). The stimulation of von Willebrand factor release and prostacyclin synthesis by A23187 and PMA was not or only slightly influenced by 3-deazaadenosine (Table II).

Phospholipid methylation can also be blocked by homocysteine, another inhibitor of phospholipid methylation (15, 24). Preincubation of endothelial cells for 4 h with 5 mM homocysteine blocked the thrombin-induced release of von Willebrand factor (Table III) but had no inhibitory effect on the thrombin-induced synthesis of 6-keto-PGF\(_{1\alpha}\) (not shown).

To test whether the stimulus-induced release of von Willebrand factor was influenced by metabolic inhibitors, endothelial cells were incubated for 2 h with or without 10 μM 3-deazaadenosine (DAA) in serum-free medium and subsequently stimulated with thrombin (2 units/ml), PMA (100 ng/ml), or A23187 (10 μM). After 10 min the amount of von Willebrand factor and 6-keto-PGF\(_{1\alpha}\) was measured. Results are expressed as mean ± S.D. (3 separate incubations).

**Table II**

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>6-Keto-PGF(_{1\alpha})</th>
<th>von Willebrand factor</th>
</tr>
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<tbody>
<tr>
<td>-DAA +DAA</td>
<td>16 ± 1.3</td>
<td>2.3 ± 0.6</td>
</tr>
<tr>
<td>Thrombin</td>
<td>9.3 ± 1.2</td>
<td>14.8 ± 4.2</td>
</tr>
<tr>
<td>PMN</td>
<td>0.5 ± 0.5</td>
<td>14.6 ± 3.5</td>
</tr>
<tr>
<td>A23187</td>
<td>52.6 ± 3.0</td>
<td>33.0 ± 6.4</td>
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**Table III**

<table>
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<tr>
<th>Stimulus</th>
<th>Homocysteine von Willebrand factor</th>
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<tbody>
<tr>
<td>-</td>
<td>1.6 ± 1.5</td>
</tr>
<tr>
<td>+</td>
<td>10.1 ± 2.7</td>
</tr>
<tr>
<td>+</td>
<td>1.4 ± 1.3</td>
</tr>
<tr>
<td>+</td>
<td>3.9 ± 0.8</td>
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**Table IV**

<table>
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<tr>
<th>Stimulus</th>
<th>von Willebrand factor</th>
<th>6-Keto-PGF(_{1\alpha})</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>+ASA +Mepacrine</td>
<td>2.0 + 0.8</td>
</tr>
<tr>
<td>Thrombin</td>
<td>+ASA +Mepacrine</td>
<td>20.0 + 0.6</td>
</tr>
<tr>
<td>A23187</td>
<td>+ASA +Mepacrine</td>
<td>14.4 + 0.2</td>
</tr>
</tbody>
</table>

Results are expressed as an average of two separate incubations which agree within 10% of the mean.
Phospholipid Methylation, Thrombin, and Endothelial Cells

TABLE V
Resume of the effects of inhibitors on metabolic events in endothelial cells induced by thrombin, A23187, and PMA

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>PL methylation</th>
<th>Ca²⁺ influx</th>
<th>VWF release</th>
<th>PGI₂ synthesis</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>T</td>
<td>A</td>
<td>P</td>
<td>T</td>
</tr>
<tr>
<td>None</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Deazaadenosine</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EDTA</td>
<td>T</td>
<td>J</td>
<td>-</td>
<td>J</td>
</tr>
<tr>
<td>ASA/mepacrine</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
</tbody>
</table>

lebran factor is a consequence of prostaglandin formation, the effects of acetylsalicylic acid, an inhibitor of cyclooxygenase (25), and mepacrine, a phospholipase inhibitor, were examined. Acetylsalicylic acid (200 μM) and mepacrine (5 μg/ml) completely blocked the thrombin- and A23187-induced prostacyclin release, but no influence could be noted on the stimulus-induced release of von Willebrand factor (Table IV).

Table V summarizes the effects of the inhibitors on the metabolic events induced by the various stimuli.

DISCUSSION

In this study we for the first time have shown that in human endothelial cells thrombin induces a rapid increase in phospholipid methylation, associated with Ca²⁺ influx, prostaglandin production, and von Willebrand factor release. Various types of metabolic inhibitors were used to determine whether these thrombin-induced processes are linked as sequential events.

Addition of deazaadenosine or homocysteine, both inhibitors of the enzymatic methylation of phosphatidylethanolamine, markedly decreased thrombin-induced phospholipid methylation, Ca²⁺ influx into endothelial cells, and release of von Willebrand factor. Addition of EDTA inhibited Ca²⁺ influx and the thrombin-induced release of von Willebrand factor. However, no effect of EDTA on phospholipid methylation could be noted. From these findings we conclude that thrombin-stimulated phospholipid methylation is coupled to Ca²⁺ influx and subsequent release of von Willebrand factor from endothelial cells. PMA and the Ca²⁺ ionophore both seem to bypass the phospholipid methylation and directly stimulate Ca²⁺ influx and von Willebrand factor release. Patients with homocystinuria, an autosomal recessive inborn error of metabolism (26) which results in elevated levels of homocysteine in plasma, exhibit a progressive vascular disease with recurrent arterial and venous thrombosis. The finding that homocysteine blocks the stimulus-induced release of von Willebrand factor and possible other cellular components may be pertinent to the question of how homocysteine induces vascular dysfunction.

Hirata et al. (27) showed that the release of histamine from mast cells induced by concanavalin A is linked to methylation of phospholipids. In additional experiments, Ishizaka et al. (28) provided evidence that methylation of phospholipids is a requirement for Ca²⁺ entry. In basophils, however, a stimulus-induced release of arachidonic acid, parallel with the histamine release, is observed (14). Since inhibition of the arachidonic acid release by mepacrine, an inhibitor of phospholipase A₂, also inhibits the histamine release, a correlation between phospholipid methylation, histamine release, and arachidonic acid release has been suggested. Similar results have been obtained with human fibroblasts (13); stimulation of release of arachidonic acid by bradykinin also stimulates phospholipid methylation, and inhibition of methylation, at least in part, inhibits arachidonic acid release. A similar cascade has been found for receptor-mediated stimulation of rabbit neutrophils, phospholipid methylation, prostaglandin synthesis, and chemotactic activity (29). Hence, in this study, endothelial cells prostacyclin synthesis seems to be independent of phospholipid methylation and Ca²⁺ influx. This conclusion is based on the following observations. 1) PMA stimulates Ca²⁺ influx but has no immediate effect on endothelial prostacyclin synthesis. 2) Prostaglandin synthesis was not or only partly blocked by EDTA (see also Ref. 8). 3) Deazaadenosine has no effect on the stimulus-induced prostacyclin synthesis even at a dose which is 20-fold higher than the concentration required to block phospholipid methylation completely (5 μM). The reason for these differences between endothelial cells and basophils, fibroblasts, and neutrophils is at present not clear. It can be speculated that arachidonic acid used as precursor for prostaglandin synthesis in endothelial cells is esterified in different phospholipid classes. Therefore, different mechanisms for liberation of arachidonic acid in different cells might play a role (see also Ref. 11). In this respect it is interesting to note that in fibroblasts and basophils the prostaglandin synthesis can only be partially inhibited by 3-deazaadenosine which suggests more than one mechanism for the activation of prostaglandin synthesis (13, 14).

In blood platelets thrombin induces an immediate and sustained decrease in the rate and extent of N-methylation of platelet phospholipids (29). This thrombin effect correlates with serotonin release and is dissociated from platelet prostaglandin synthesis. This indicates that, similar to endothelial cells, also in platelets perturbation of the methylation pathway participates in signal transduction for release reactions but not for prostaglandin synthesis.

Another possibility to explain the deviate effect of 3-deazaadenosine on prostaglandin synthesis is the presence of more than one pool for phospholipid methylation, one pool coupled to secretion and relatively sensitive for 3-deazaadenosine, and another pool coupled to prostaglandin synthesis which is rather insensitive to 3-deazaadenosine.

Differences in experimental conditions (e.g., methylation studies were performed in serum-poor medium, whereas the study on the secretion of prostacyclin, von Willebrand factor, and the Ca²⁺ influx was carried out with cells maintained in 20% serum) could account for the differential effects on the metabolic inhibitions studied. This seems unlikely, however. Previously we have shown that endothelial cells grown and maintained in serum-free medium secrete normal amounts of von Willebrand factor (30). Similarly, maintaining the cells in 1% serum did not substantially affect the capacity of endothelial cells to produce von Willebrand factor and prostacyclin after addition of thrombin (not shown).

The Ca²⁺ ionophore A23187 induces both prostacyclin synthesis and von Willebrand factor release. Since Ca²⁺ influx into endothelial cells alone is not sufficient to stimulate prostacyclin production and EDTA only partly inhibits the A23187-induced prostacyclin production, one could wonder by which mechanism A23187 stimulates prostacyclin production. Pickett et al. (31) demonstrated that EDTA enhances the A23187-induced prostaglandin synthesis in blood platelets. One explanation could be that A23187 also elevates the Ca²⁺ concentration in certain intracellular compartments whereas PMA only raises the cytosolic Ca²⁺ concentration in endothelial cells. Another possibility could be that A23187 liberates Ca²⁺ stored in endothelial cells.

Acetylsalicylic acid, a cyclooxygenase inhibitor, completely
blocks the prostaglandin synthesis without affecting the release of von Willebrand factor (Table III). Comparable results were reported by Loesberg et al. (8) who showed that both dibutyryl cAMP (a cAMP analogue) and isobutylmethylxanthine (an inhibitor of cyclic nucleotide phosphodiesterase), substances known to increase cAMP levels, inhibited prostaglandin production by endothelial cells. This effect of prostaglandin synthesis completely but had no effect on the stimulus-induced release of von Willebrand factor. These results indicate that von Willebrand factor release is not a consequence of prostaglandin production by endothelial cells.

The model presented in Fig. 4 outlines possible pathways for receptor-mediated prostacyclin synthesis and von Willebrand factor release in cultured human endothelial cells. Both prostacyclin synthesis and stimulus-induced von Willebrand factor release require the presence of the active site of thrombin (3, 7, 8, 32). After binding of thrombin to endothelial cells at least two different responses occur. Thrombin stimulates the methylation of phosphatidylethanolamine to form phosphatidylycholine. This process has been found to increase membrane fluidity (33) and induce a Ca2+ influx. Due to an increase of the cytosolic Ca2+ concentration the release of a storage pool (7, 8, 34) of von Willebrand factor is triggered. The Ca2+ influx can also be achieved by PMA and A23187. Thrombin can also stimulate phospholipase activity which liberates arachidonate, a substrate for prostaglandin synthesis. This effect of thrombin could only be mimicked by A23187. This dual scheme explains the effects of the various inhibitors. However, the presence of two different thrombin receptors, one associated with phospholipid methylation and von Willebrand release and one coupled to prostacyclin synthesis, cannot be excluded.

REFERENCES