PAF-Acether–Induced Release of Tissue-Type Plasminogen Activator From Vessel Walls

By J.J. Emeis and C. Kluft

Platelet-activating factor (PAF-acether; 1-O-octadecyl-2-acetyl-sn-glyceryl-3-phosphorylcholine) induced the release of plasminogen activator in rat, both in vivo and in perfused hind legs. The released plasminogen activator was shown by immunogenic and functional criteria to be tissue-type plasminogen activator (t-PA). Release of t-PA by PAF-acether could be inhibited by phospholipase inhibitors and by lipoygenase inhibitors, but not by cyclooxygenase inhibitors. It is suggested that PAF-acether induces the release of t-PA from vascular endothelial cells by the (calcium-dependent) activation of a phospholipase–lipoygenase pathway.

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PLASMINOGEN ACTIVATORS can be defined as serine proteases that will convert the circulating proenzyme plasminogen into the fibrinolytically and thrombolytically active protease plasmin. Among the various functionally and immunologically distinct types of plasminogen activators, tissue-type plasminogen activator (t-PA) is thought to be of prime importance in initiating fibrinolysis and thrombolysis. In animal experiments, t-PA, in the induction of thrombolysis, is superior to urokinase-type plasminogen activator.

Normally, a low content of t-PA will be found in blood. However, its concentration will increase temporarily by exercise, venous occlusion, or the administration of drugs. The increase is due to the release of t-PA from vascular endothelial cells into the circulation. Indeed, in isolated perfused vascular beds, the release of t-PA from the vessel wall can be induced by various drugs. Knowledge of the mechanisms leading to t-PA release from the vessel wall is still wanting.

We show that, first, platelet-activating factor (PAF-acether; 1-O-octadecyl-2-acetyl-sn-glyceryl-3-phosphorylcholine) is a potent inducer of t-PA release from the vessel wall and, second, that said induction of t-PA release will be inhibited by compounds known to interfere with the lipoygenase pathway of fatty acid metabolism.

MATERIALS AND METHODS

Male Wistar rats (200 to 220 g) were used. All experiments were performed with animals anesthetized with pentobarbital (Nembutal, Sanofi, Paris) (60 mg/kg intraperitoneally).

Rat hind leg perfusion. Plasminogen activator (PA) release from a perfused vascular bed was studied in the perfused rat hind legs system described in detail elsewhere. Briefly, rat hind legs, by means of a roller pump at constant flow (10 mL/min), were perfused through the aorta with oxygenated Tyrode’s balanced salt solution containing 0.1 mg/mL of bovine serum albumin (Tyrode/BSA; pH 7.4; 37 °C). After a 30-minute preperfusion solely with Tyrode/BSA, a five-minute perfusion followed with Tyrode/BSA containing the indicated amount of PAF-acether (see Results). Perfusate fractions were collected from an outflow cannula in the inferior caval vein at one-minute intervals. The PA content of the fractions was determined on plasminogen-rich bovine fibrin plates. PA concentrations were calculated from a reference curve of Bowes melanoma cell-derived t-PA dissolved in Tyrode/BSA (0.05 to 5.0 U/mL). One unit of t-PA corresponds to about 10 ng of protein.

Spectrophotometric t-PA assay. In quenching experiments, PA activity of samples was determined by an indirect spectrophotometric parabolic rate assay. To wells of a 96-well microtiter plate were added 60 µL buffer (0.1 mol/L Tris HCl, pH 7.5, containing; 0.1% Tween 80), 20 µL soluble fibrin digest (1 mg/mL), 40 µL sample, and 0 to 10 µL antibody solution. After incubation for 15 minutes at 37 °C, 100 µL S-2251 (0.66 mmol/L) and 25 µL human plasminogen (1.11 µmol/L) were added, and the microtiter plate was incubated at 37 °C. After 60, 120, 180, and 240 minutes, absorption was measured at 405 nm in a Titertek Multiscan Spectrophotometer (Flow Laboratories, Irvine, Scotland). In some experiments, the soluble fibrin digest was omitted from the incubation mixture.

In vivo experiments. Rats were injected intravenously with PAF-acether (0.5 to 4.0 µg/kg), dissolved in Tyrode/BSA. Blood samples were obtained from a cannula in the carotid artery before and at 1, 2, 3, 5, and 10 minutes after injection of PAF-acether. Controls received Tyrode/BSA only.

In vitro blood clot lysis. Blood (0.2 mL) was diluted to 10% in 1.7 mL of 0.12 mol/L sodium acetate (pH 7.4), clotted with 0.1 mL of thrombin (20 NIH U/mL), and incubated at 37 °C. Lysis times were read in minutes. Where indicated, antibodies were added to the diluted blood five minutes before the addition of thrombin.

Measurement of t-PA inhibitor. The concentration of t-PA inhibitor in rat plasma was determined spectrophotometrically, as described by Verheijen et al, using 10 µL of citrated rat plasma.

Fibrin autography. Sodium dodecyl sulfate/8% polyacrylamide slab gels were prepared according to Laemmli. Fibrin autography was performed according to Loskutoff and Mussoni.

PAF-acether. Stock solutions of PAF-acether and of lyso-PAF-acether were prepared in 70% ethanol (600 µg/mL), diluted to 60 µg/mL with Tyrode/BSA, and stored at ~90 °C. Working solutions were prepared immediately before use. Fully synthetic PAF-acether and lyso-PAF-acether were provided by Prof J.J.Godfrind (Université de Paris VII, France).

Materials. The IgG-fraction (8.5 mg/mL) of a rabbit antiserum against human uroplasminogen t-PA was a gift from Dr D.C. Rijken of TNO, Health Research Division TNO, Herenstraat 5d, 2313 AD Leiden, The Netherlands.

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Supported by the “Präventiefonds” project No. 28-813. Submitted Sept 10, 1984; accepted Jan 3, 1985. Address reprint requests to Dr J.J. Emeis, Gaubius Institute TNO, Health Research Division TNO, Herenstraat 5d, 2313 AD Leiden, The Netherlands.

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0006-6497/85/6601-0012$03.00/0

Belgium). The sources of the other materials used have been detailed elsewhere.5,25

RESULTS

In the perfused rat hind legs system, after a 30-minute wash-out of the vascular bed, the addition of PAF-acether to the Tyrode/BSA resulted in a dose-dependent release of PA from the perfused vascular bed (Fig 1). The amount of PA released was maximal during the first two minutes after adding PAF-acether and decreased slightly during the subsequent minutes. Lyso-PAF-acether induced the release of only a small quantity of PA (Fig 1). The solvent for PAF-acether did not induce PA release.

Nature of PA released. The activator released by PAF-acether was identified as tissue-type by the following observations. First, the PA activity of the perfusate samples could be quenched by the IgG-fraction of an antisera raised against human uterine t-PA, although not by the IgG-fraction of a nonimmune rabbit serum (Fig 2). Second, the activation of plasminogen by t-PA is, as reported,23 enhanced by soluble fibrinogen fragments, whereas plasminogen activation by urokinase-type plasminogen activators is not. Using the spectrophotometric PA assay,19 plasminogen activation by perfusate samples was detectable in the presence of soluble fibrinogen fragments only. Third, by fibrin autography, perfusate samples showed a single lysis zone. This lysis band was found to be at about the same position (M₀ = 70,000) as the lysis band produced by human (Bowes melanoma cells-derived) t-PA, M₀ = 67,500 (Fig 3). Because all plasminogen activator present in perfusates is inhibited by antibodies against human t-PA, will comigrate with authentic human t-PA in SDS-gel electrophoresis, and, moreover, is stimulated by fibrin, the PA to be found in perfusates after stimulation with PAF-acether can be identified as rat tissue-type plasminogen activator.

Inhibition of t-PA release. Next, possible mechanisms involved in the induction of t-PA release by PAF-acether were studied, using inhibitors (Table 1). The inhibitors were added both during the last 20 minutes of the preperfusion period and during the stimulation of t-PA release by PAF-acether.

No release of t-PA was observed in the absence of extra-

![Fig 1. Induction of plasminogen activator release from perfused rat hind legs by PAF-acether. Shown are the means ± standard deviations (n = 4 to 12) of the amounts of plasminogen activator released by the indicated concentrations of PAF-acether (ng). Plasminogen activator concentrations were determined by the fibrin plate method. One unit of plasminogen activator equals 10 ng protein of t-PA. Also shown is the amount of plasminogen activator released by 40 nmol/L of lyso-PAF-acether (ng; n = 4).](image)

![Fig 2. Quenching of plasminogen activator activity in samples from perfused rat hind legs, stimulated by 20 nmol/L PAF-acether, by anti-human uterine t-PA IgG (•), and by control IgG (■). Shown are the means ± standard deviations of the percentage of residual activity, as determined in six separate experiments. The six samples averaged 0.43 U/mL of plasminogen activator. Activities were determined by the spectrophotometric parabolic rate assay.](image)

![Fig 3. Fibrin autography of samples from perfused rat hind legs stimulated with 20 nmol/L PAF-acether (A); of human melanoma cell-derived t-PA (B); and of human urokinase (C). The fibrin-agarose gel was incubated for 18 hours. The estimated molecular weights are 70,000 (A), 67,500 (B), and 55,000 and 33,000 (C).](image)
Table 1. Inhibition of t-PA Release Induced by PAF-Acether

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>t-PA Released (U/mL)</th>
<th>n</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>0.57 ± 0.15*</td>
<td>12</td>
</tr>
<tr>
<td>EDTA (1 mmol/L; no calcium)</td>
<td>0.02 ± 0.01†</td>
<td>4</td>
</tr>
<tr>
<td>Mepacrine (100 μmol/L)</td>
<td>0.11 ± 0.10†</td>
<td>5</td>
</tr>
<tr>
<td>p-Bromophenacylbromide (100 μmol/L)</td>
<td>0.17 ± 0.04†</td>
<td>4</td>
</tr>
<tr>
<td>Nor-dihydroguaiaretic acid (70 μmol/L)</td>
<td>0.08 ± 0.07†</td>
<td>7</td>
</tr>
<tr>
<td>AA-861 (10 μmol/L)</td>
<td>0.22 ± 0.09†</td>
<td>4</td>
</tr>
<tr>
<td>BW 755C (200 μmol/L)</td>
<td>0.54 ± 0.12</td>
<td>4</td>
</tr>
<tr>
<td>Diethylcarbamazine (10 mmol/L)</td>
<td>0.07 ± 0.01†</td>
<td>4</td>
</tr>
<tr>
<td>Aspirin (30 μmol/L)</td>
<td>0.44 ± 0.21</td>
<td>5</td>
</tr>
<tr>
<td>Indomethacin (30 μmol/L)</td>
<td>0.49 ± 0.10</td>
<td>5</td>
</tr>
</tbody>
</table>

t-PA release was induced by PAF-acether (20 nmol/L). Inhibitors were added to the perfusion fluid at the indicated concentrations during the last 20 minutes of the preperfusion period and during stimulation with PAF-acether. Concentrations of t-PA were measured by the fibrin plate method.

*Mean ± standard deviation.
†Significantly different from control (P < .005; Student's t test).

was an increase in perfusion pressure in the presence of mepacrine.

In vivo experiments. To see whether PAF-acether would also induce t-PA release in vivo, the compound was injected intravenously into rats. PAF-acether rapidly (Fig 4) and dose-dependently (Fig 5) increased blood fibrinolytic activity, as evidenced by decreased clot lysis times. This increase in blood fibrinolytic activity could be quenched by preincubation of the diluted blood with antibodies against human uterine t-PA (Fig 6).

The addition of PAF-acether to diluted blood in vitro did not reduce clot lysis times (data not shown). Also, the solvent did not induce increased fibrinolytic activity either in vivo or in vitro.

As the observed increase in blood fibrinolytic activity might have been caused by a decrease of the fast-acting t-PA inhibitor, t-PA inhibitor concentrations were measured 1, 3, 6, and 10 minutes after injection of PAF-acether (1 μg/kg). As shown in Fig 7, PAF-acether injection resulted in a decrease in inhibitor concentration of about 0.5 U/mL (eg, 17% of preinjection values) at three minutes. At ten minutes after injection, the inhibitor concentration had returned to preinjection values.

DISCUSSION

Platelet-activating factor has a wide spectrum of potent biological activities, including vasodilation, hypotension, increased vascular permeability, and platelet activation (although the latter effect is not observed in rats). Also, as shown here, PAF-acether induces increased blood fibrinolytic activity in vivo and the release of t-PA in a perfused vascular bed. The increase in blood fibrinolytic activity (Figs 4 and 5) can be quenched by antibodies against t-PA (Fig 6) and can therefore be ascribed to increased blood t-PA activity. Although increased t-PA activity might be due to decreased levels of t-PA inhibitor, this is unlikely. First, fibrinolytic activity was maximal at one minute after injection (Fig 4), while the largest decrease in inhibitor concentration occurred at three minutes (Fig 7). Second, at ten minutes, fibrinolytic activity was still increased, while the inhibitor level had returned to its original value.

Fig 4. Time course of dilute blood clot lysis times (10% blood clots) of rats injected intravenously with PAF-acether (1 μg/kg body weight). Shown are the means ± standard deviations of ten experiments.

Fig 5. Dose-dependent reduction of dilute blood clot lysis times of 10% blood clots from rat injected intravenously with PAF-acether (0.5 to 4.0 μg/kg body weight). Blood samples were obtained one minute after injection of PAF-acether. Means ± standard deviations of four to ten experiments.
preinjection value. The temporarily decreased t-PA inhibitor concentrations are therefore presumably a consequence, and not the cause, of the observed increase in blood fibrinolytic activity. The release of t-PA from a perfused blood-free vascular system suggests that PAF-acether has a direct effect on the vascular wall, presumably on the vascular endothelial cells, which are the only vascular cells to contain t-PA in vivo. That t-PA is, indeed, the activator released, can be deduced from the quenching of its activity by antibodies against human uterine t-PA, from its strict dependence on fibrin for plasminogen activation, and from its molecular weight of about 70,000, identical to that of human t-PA.

Similar perfusion experiments demonstrated that release of PA can be induced by a variety of compounds, including thrombin, heparinoids, bradykinin, acetylcholine, histamine, and (nor) adrenaline. Of these compounds, only thrombin and PAF-acether are active at nanomolar concentrations, whereas all other compounds mentioned are active only at higher (micromolar) concentrations. This makes PAF-acether one of the most potent inducers of t-PA release presently known. Whether PAF-acether, which can be detected in peripheral blood of both humans and rats, has a part in the release of t-PA under physiologic conditions cannot yet be decided. Experimental proof for this suggestion must stand over until the availability of specific antagonists of PAF-acether.

In perfused rat hind legs, the release of PAF-acether requires extra-cellular calcium and can be inhibited by mepacrine and p-bromophenacylbromide, by inhibitors of lipoxygenase (nor-dihydroguaiaretic acid and AA-861), and by diethylcarbamazine, not, however, by cyclo-oxygenase inhibitors and BW 755C. Mepacrine and p-bromophenacylbromide have been widely used as phospholipase inhibitors, but one should keep in mind that other effects of these compounds have been described; eg, on the incorporation of fatty acids into phospholipids, on membrane phospholipid composition, and on cyclo-oxygenase activity. In general, however, these effects occurred at higher concentrations than used in the present study. We therefore suggest that the release of t-PA involves a (calcium-dependent) activation of phospholipase(s), followed by the formation of a lipoxygenase product(s) from the liberated fatty acids. This suggestion would concur with recent observations that release of other cell products, eg, insulin from the pancreas or prolactin from the pituitary, is also mediated by lipoxygenase products. PAF-acether is, moreover, known to stimulate a phospholipase/lipoxygenase pathway in other cell types as well as in perfused lung. As far as we know, our observations are the first to suggest possible pathways leading to t-PA release from the vessel wall. Attempts to demonstrate these pathways in vitro using cultured endothelial cells have, as yet, proved impossible, as we have not been able to demonstrate the presence of a t-PA storage pool in such cells.

ACKNOWLEDGMENT

We are greatly indebted to Professor J.-J. Godfroid (Université de Paris VII) for providing synthetic PAF-acether and lyso-PAF-acether. The assistance of Mrs C.M. van den Hoogen, Mr. D. Jense, and Mr. W. Pieterse is gratefully acknowledged.

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