Inhibition of Tissue Plasminogen Activator Activity by Aspirin In Vivo and Its Relationship to Levels of Tissue Plasminogen Activator Antigen, Plasminogen Activator Inhibitor, and Their Complexes

By Richard I. Levin, Peter C. Harpel, John G. Harpel, and Phoebe A. Recht

The observation that aspirin inhibits the increment in tissue plasminogen activator (t-PA) activity induced by venous occlusion of the forearm became controversial with the publication of several nonconfirmatory studies. The current study was performed to confirm the original observation and determine the mechanism by which aspirin suppresses the incremental t-PA activity induced by venous occlusion. Aspirin (650 mg/d x 2) caused no change in resting levels of t-PA antigen (t-PA:Ag) or activity, plasminogen activator inhibitor 1 antigen (PAI-1:Ag) or activity or t-PA-PAI-1 complexes. In contrast, aspirin reduced the increments induced by venous occlusion as follows: t-PA:Ag by 45% (P < .001); t-PA activity (euglobulin lysis time, ELT) by 43% (P < .006); and t-PA activity (alpha2-plasmin inhibitor-plasmin complexes, PIPC) by 41% (P = .003). The inhibition of incremental t-PA activity measured as ELT or PIPC was linearly correlated with the inhibition of incremental t-PA:Ag (respectively, r = .76, P < .02; r = .87, P < .05). Aspirin had no effect on the increment in PAI-1:Ag induced by venous occlusion, but similar to the effect on t-PA:Ag, aspirin induced a 51% inhibition of the increment in t-PA–PAI-1 complex formation. Aspirin did not alter the ability of alpha2-plasmin inhibitor to bind plasmin, nor the ability of plasma to support the fibrin-catalyzed generation of plasmin by t-PA, nor the subsequent formation of PIPC. Aspirin inhibits the t-PA activity induced by venous occlusion primarily by inhibiting the release of t-PA antigen.

PHYSIOLOGIC FIBRINOLYSIS is controlled by both the quantities and activities of tissue plasminogen activator (t-PA) and its inhibitors in the circulation and at the site of thrombosis.1-4 The factors modulating the synthesis and release of t-PA and its inhibitors by vascular endothelium are largely unknown but may include eicosanoids,5-10 vasoactive amines,11-15 thrombin,16-18 thyroid hormone,19 endotoxin,18 and insulin.19 In a previous publication1 we demonstrated that aspirin inhibited the increase in t-PA activity induced by venous occlusion of the forearm and speculated that such inhibition might be mediated by suppression of eicosanoid synthesis and t-PA antigen release from the vessel wall.

Our initial observations were based on a novel assay for plasminogen activator activity, which utilized an enzyme-linked immunosorbent assay (ELISA) for the detection of alpha2-plasmin inhibitor-plasmin complexes (PIPC) generated in vitro in the presence of subjects' plasma and reptilase.1 Since the presentation of our work, there have been several studies published in which a variety of techniques were used in an attempt to confirm our results and in which the authors claimed that aspirin did not alter t-PA activity.20-24; in contrast, three papers were confirmatory.25-27 The current study was performed, therefore, with several objectives: to confirm our initial observations; to compare different techniques for the measurement of t-PA activity; to correlate changes in t-PA activity with changes in t-PA antigen concentration, t-PA inhibitor antigen and activity; and to attempt to define some of the potential causes of variability in the use of forearm venous occlusion as a stimulus for the vascular release of t-PA. The findings confirm our original observations and demonstrate that inhibition of t-PA activity by aspirin is directly related to suppression of t-PA antigen release.

MATERIALS AND METHODS

Protocol. The protocol and methods are as previously described. After obtaining informed consent for participation in this study approved by the Institutional Board of Research Associates, subjects underwent venous occlusion of the forearm after random assignment to control or aspirin, 650 mg, 18 and 2 hours before blood collection. Venous blood was drawn into plastic syringes, added to 3.8% sodium citrate (9:1, vol/vol) in plastic tubes, and plasma obtained as described.1

Determination of t-PA activity using an assay for alpha2-plasmin inhibitor-plasmin complexes generated in vitro. The methods have been described in detail. The concentration of PIPC in each sample was determined by the differential antibody ELISA previously described.1-3 In each assay a standard curve was generated by adding exogenous t-PA (0 to 8 U/mL) to pooled plasma samples. Curves of best fit were determined by linear regression and the activity of unknown samples determined by interpolation.

Determination of t-PA activity by euglobulin lysis time. Immediately after centrifugation of venous blood, 1 mL of each subject's
plasma was added to 19 mL of distilled water at 0°C in an ice bath. CO₂ was bubbled through the diluted plasma for 2 minutes at 0°C and the euglobulin precipitate was removed by centrifugation. The precipitate was resuspended in 1 mL of phosphate-buffered saline at 0°C and clotted by the addition of 0.005 mL of a solution containing bovine thrombin 10 U/mL. The clot was incubated at 37°C, examined visually every 30 seconds, and the end point was the appearance of bubbles. A standard curve was developed in which t-PA (0 to 4 U/mL) was added to normal plasma prior to euglobulin precipitation. The relationship between the common logarithm of minutes to the initiation of clot lysis and the common logarithm of t-PA concentration was linear as described by Thorsen, and euglobulin lysis times of subjects' plasmas were therefore converted to units of t-PA activity after logarithmic transformation of the data.

**Analysis of t-PA antigen.** Antigenic t-PA concentrations were determined by ELISA with minor modifications of the method of Bergdorf et al using a commercially available kit (American Diagnostica). The manufacturer's recommendations were followed except that a development time of 40 minutes was utilized after it was determined to yield optimal results. Further, acidification of plasma was not utilized after it was determined that (1) recovery of authentic t-PA in the range of 0.1 to 2.4 ng/mL from unacidified plasma was essentially 100% (102.4% ± 6.8%; mean ± SD, n = 24) and (2) the shape of the curve of absorbance versus concentration of t-PA was virtually identical whether t-PA was added to buffer or to plasma (P > .5).

Plasma samples, which had been maintained at −70°C, of the 13 subjects who had been studied previously were also analyzed for antigenic t-PA concentrations by the method described.

**Analysis of plasminogen activator inhibitor-1 activity.** Functional PAI-1 activity was assayed by a two-stage indirect enzymatic assay using reagents supplied by Biopool AB (Umeå, Sweden). The assay was performed as detailed in the assay kit. t-PA was added to the plasma sample for 15 minutes at room temperature, following which the sample was acidified and incubated for 20 minutes at 37°C to destroy antiplasmin activity. The diluted sample was then added to a mixture of Glu-plasminogen, poly-lysine, and chromogenic substrate at neutral pH. The PAI concentration of the plasma sample was measured as the difference between the amount of t-PA added and the amount of t-PA found.

**Analysis of PAI-1 antigen.** Plasma levels of PAI-1:Ag were determined using a commercially available enzyme immunoassay kit (generously provided as a gift by CTXRX/Biopool AB, Umeå, Sweden), based on the double-antibody sandwich method described by Declerck et al. Samples were analyzed as per the instructions provided with the kit after determining that the presence of sodium citrate in plasma samples had no effect on the generation of the standard curve (Abs (492 nm) citrated sample = 0.963 X Abs (492 nm) noncitrated sample + 6.4, r = .999, P < .0001). The procedure was automated with the use of a robotic pipetting and plate washing machine (Perkin-Elmer).

**Analysis of t-PA–PAI-1 complexes.** The levels of complexes present in test plasma samples were quantified using a commercially available enzyme immunoassay kit (recently described kindly provided as a gift from Dr Jean Amirad (Diagnostica Stago, Asnieres-sur-Seine, France). An automated pipettor/plate washer (Perkin-Elmer) was used to facilitate the procedure. Diluted plasma samples were added (0.2 mL each) to a 96-well microtest plate coated with mouse monoclonal anti–PAI-1 antibodies and incubated for 2 hours at room temperature. The plate was washed five times with the provided isotonic solution, and 0.2 mL of a solubilized mouse anti-t-PA antibody conjugated to peroxidase was added to each well. After a 2-hour incubation at room temperature, the plate was washed as before, and 0.2 mL of OPD substrate (orthophenylene-
ediamine, 0.4 mg/mL) was added in the presence of hydrogen peroxide. The optical density of the developed yellow color was measured at 492 nm after 6 minutes at room temperature using a microtest plate reader. Unknown samples were compared with a standard curve included with each assay.

**Effect of aspirin on t-PA release during venous occlusion of the forearm.** The method is identical to that used previously. The formation of PIPC was measured by ELISA as described, and standard curves were prepared using the donor's pre-occlusion plasma from each day of the test by the addition of 0 to 8 U/mL t-PA and the activity of t-PA present in precocclusion and postocclusion samples determined by interpolation. Postocclusion values were corrected for hemoconcentration.

**Effect of aspirin on the ability of plasma to support the generation by t-PA of alpha-2-antiplasmin inhibitor-plasmin complexes in vitro.** To determine whether aspirin through acetylation or other reaction altered the ability of the components of plasma to support t-PA–directed generation of PIPC, aspirin (150 μmol/L), or its vehicle (ethanol, 0.1% final concentration) was incubated with normal plasma for 2 hours at 37°C. The plasma was dialyzed overnight at 4°C with three changes of a buffer containing 0.15 mol/L NaCl and 0.05 mol/L Tris HCl, pH 7.4. Similarity of the diazoyzed plasma to plasma was determined spectrophotometrically by comparing the optical densities of the two plasmas at 280 nm. The diazoyzed plasmas were clotted with either reptilase (0.1 mL/mL plasma) or human thrombin (0.1 U/sample) and incubated as described above with either buffer alone or t-PA (2 U/mL). Supernates were subsequently analyzed for PIPC by the differential antibody ELISA as described.

**Analysis of plasma salicylate.** Salicylate concentrations in the subjects' plasma were determined by high performance liquid chromatography using minor modifications of the method of Amick and Mason as described.

**Statistical analysis.** Data were entered into an IBM-XT computer (IBM) and transformed using Supercalc 3 and 4 (Computer Associates). Statistical analyses were performed with programs in Statpak (NW Analytic) or PC Statistician/PC Anova (Human Systems Dynamics). The significance of differences noted in single comparisons was determined by the paired or unpaired t-test, and the significance of the influence of multiple factors and their interactions was determined by analysis of variance. The influence of single and multiple independent variables on dependent variables was determined by linear regression using least-squares. Correlation was determined by computing the Pearson r or the Spearman rho. All data are presented as mean ± SD unless noted otherwise.

**RESULTS**

**Study population.** The subjects in this series of experiments were similar to those of the first series. Five men with a mean age of 33.2 ± 3.3 years and five women with a mean age of 30.6 ± 6.6 years were enrolled. The mean age of the group was 31.9 ± 5.1 years. Three volunteers were subjects in both series of experiments.

**Resting levels of t-PA:Ag and activity, PAI-1:Ag and activity, and t-PA–PAI-1 complexes.** Resting levels of each of these variables are shown in Table 1. There were small differences in the activity of t-PA detected by the assay for euglobulin lysis time (ELT) and the ELISA for the generation of PIPC, but these were not significant (P = .254). Similar to our previous results, women had twice as much t-PA activity as men (0.4 ± 0.2 v 0.19 ± 0.4 U/mL) when t-PA activity was measured with our ELISA for PIPC; no such difference was noted when t-PA activity
was measured by ELT. Of interest is the observation that the activity of PAI was nearly exactly opposite to the activity of t-PA described above: women had approximately one half as much as men (7.7 ± 4.0 v 13.9 ± 7.4 U/mL) similar to the recent results of Krishnamurti et al.24 The effects of gender, although numerically strong, must be considered trends only because none reached statistical significance, perhaps due to the small number of subjects and the marked intersubject variability for these measurements.1,13,19

A formal analysis of the data from all subjects using Spearman rank-order correlation revealed an inverse relationship between the level of PAI-1 activity and t-PA activity measured either with our ELISA for PIPC (rho = −.6322, P < .05) or with ELT (rho = −.6565, P < .05). The lines of best fit describing the relationship between t-PA activity and PAI are t-PA_{PIPC} = −0.0270 × PAI + 0.585 and t-PA_{ELT} = −0.0167 × PAI + 0.5483.

Effect of venous occlusion on t-PA activity and antigen. Figure 1 shows the effect of venous occlusion on t-PA activity and antigen in the control period during which no drug was ingested. Venous occlusion induced a 5.4-fold rise in mean t-PA activity assessed by ELT from 0.37 ± 0.22 to 2.36 ± 1.97 U/mL (P < .004), whereas the increase in mean t-PA activity assessed by ELISA for PIPC was nearly eightfold from 0.29 ± 0.3 to 1.97 ± 0.93 U/mL (P < .0006).

In contrast, the rise in mean t-PA antigen was only 1.6-fold from 11.8 ± 10.3 to 19.0 ± 10 ng/mL (P < .002). Marked differences in the resting and postocclusion ratios of t-PA activity and t-PA antigen have been noted previously.10,40

Correlation between the two measures of t-PA activity. Figure 2 demonstrates the relationship between t-PA activity measured by ELT and t-PA activity measured by ELISA for PIPC in each of the 10 subjects. Strong correlations were present and the two methods yielded similar results. The line of best fit describing the relationship for preocclusion values is t-PA_{PIPC} = 1.08 × t-PA_{ELT} + 0.104 (r = .77, P < .01); the line of best fit describing the relationship for postocclusion values is t-PA_{ELT} = 0.4 × t-PA_{ELT} + 1.02 (r = .85, P < .01).

Correlation between t-PA activity and antigen levels. Unlike the relationship between the two techniques for assessing t-PA activity, there appeared to be no relationship between t-PA activity measured by ELT and t-PA antigen level (r = .03, P > .1) nor for t-PA activity measured by ELISA for PIPC (r = −.2, P > .1). Similarly, there were no correlations noted when the preocclusion values, the increments from preocclusion to postocclusion or their logarithmic transforms were analyzed. Thus, neither static levels of t-PA activity (resting or stimulated) nor the change in t-PA activity induced by venous occlusion can be related directly to, respectively, the static levels of t-PA antigen or the change in level of t-PA antibody induced by venous occlusion.

Effect of venous occlusion on PAI-1:Ag and t-PA–PAI-1 complexes. Figure 3 shows the effect of venous occlusion on PAI-1:Ag and t-PA–PAI-1 complexes in the control period during which no drug was ingested. Venous occlusion induced a small 44% increase in the level of PAI-1:Ag from 93.0 ± 45 to 134.3 ± 50.8 ng/mL (P = .007). t-PA–PAI-1 complexes were also increased by venous occlusion by 82.2% from 7.3 ± 4.2 to 13.3 ± 8.7 ng/mL (P = .024). These increments are similar to the 60% increment noted for t-PA:Ag (Fig 1). The resting levels of PAI-1 activity are shown in Table 1; postocclusion levels were not obtained.

Effect of aspirin on resting levels of t-PA:Ag and PAI-1:Ag and activity, PAI-1:Ag and activity, and t-PA–PAI-1 complexes. We have previously demonstrated that aspirin as administered had no effect on the resting levels of t-PA activity as measured by ELISA for the generation in vitro of PIPC. Table 2 demonstrates that in this series of experiments, aspirin has no effect on the resting levels of t-PA activity measured by either of the techniques used, or on the resting levels of t-PA:Ag, PAI-1:Ag or activity, or t-PA–PAI-1 complexes.

Effect of aspirin on the increments in t-PA:Ag and

---

### Table 1. Resting Levels of t-PA:Ag and Activity, PAI-1:Ag and Activity, and t-PA–PAI-1 Complexes in Male and Female Subjects

<table>
<thead>
<tr>
<th>N</th>
<th>t-PA:Ag (ng/mL)</th>
<th>t-PA Activity</th>
<th>PIPC (U/mL)</th>
<th>PAI-1 (U/mL)</th>
<th>PAI-1:Ag (ng/mL)</th>
<th>t-PA–PAI-1 Complexes (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Men</td>
<td>5</td>
<td>13.7 ± 8.1</td>
<td>0.35 ± 0.3</td>
<td>0.19 ± 0.4</td>
<td>13.9 ± 7.4</td>
<td>79.0 ± 57</td>
</tr>
<tr>
<td>Women</td>
<td>5</td>
<td>9.7 ± 11.4</td>
<td>0.38 ± 0.1</td>
<td>0.4 ± 0.2</td>
<td>7.7 ± 4.0</td>
<td>107.0 ± 29</td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
<td>11.8 ± 10.3</td>
<td>0.37 ± 0.2</td>
<td>0.29 ± 0.3</td>
<td>10.8 ± 6.5</td>
<td>93.0 ± 45</td>
</tr>
</tbody>
</table>

---

**Fig 1.** Effect of venous occlusion on t-PA antigen and activity. The individual responses of the 10 subjects and the mean ± SEM are shown. Panel A shows that venous occlusion significantly enhanced t-PA activity measured by euglobulin lysis time (P = .004); panel B shows a similar enhancement of t-PA activity measured by ELISA for the generation of alpha2-plasmin inhibitor-plasmin complexes (P = .0006); and panel C shows that venous occlusion significantly enhanced the concentration of t-PA antigen (P = .002). Abbreviations: ELT, euglobulin lysis time; PIPC, ELISA for the generation of alpha2-plasmin inhibitor-plasmin complexes; AG, t-PA antigen measured by ELISA as described.
activity, PAI-1:Ag and t-PA-PAI-1 complexes induced by venous occlusion. We have previously reported that aspirin induces a marked inhibition of the increment in t-PA activity induced by venous occlusion of the forearm and assessed by ELISA for PIPC. Figure 4 demonstrates that aspirin inhibits the increase induced by venous occlusion of t-PA activity measured by either technique as well as the increase in t-PA antigen. Aspirin (650 mg administered 18 and 2 hours prior to the study) inhibited the rise in t-PA activity assessed by ELT by 43% (1.99 ± 1.84 U/mL during the control study versus 1.14 ± 1.04 U/mL during the aspirin study, \( P = .006 \)). Aspirin inhibited the rise in t-PA activity assessed by ELISA for PIPC by 40.5% (1.67 ± 0.9 U/mL during the control study versus 1.04 ± 0.6 U/mL during the aspirin study, \( P = .003 \)). Further, there was a strong correlation between the amounts of inhibition of t-PA activity determined by the two measures in each subject (t-PA\_ELT = 0.81 X t-PA\_ELISA + 3.7216; \( r = .81, P = .004 \)). Thus, the enhancement of t-PA activity induced by venous occlusion and determined by either of two different techniques is significantly and similarly inhibited by aspirin.

Also, as shown in Fig 4, aspirin inhibited the rise in t-PA antigen by 46% (7.1 ± 4.7 ng/mL during the control study versus 3.8 ± 3.5 ng/mL during the aspirin study, \( P = .006 \)). Further, as shown in Fig 5, the aspirin-induced inhibition of incremental t-PA activity after venous occlusion, measured either by ELT or PIPC, correlated linearly with the aspirin-induced inhibition of incremental t-PA:Ag release (\( r = .749, P < .02 \) and \( r = .667, P < .05 \), respectively). Although these correlations do not demonstrate causality, the similar extent of inhibition of t-PA:Ag and activity coupled with the correlations noted are suggestive of such a relationship.

Figure 4 also demonstrates the effect of aspirin on PAI-1:Ag and t-PA-PAI-1 complexes. Aspirin did not significantly alter the increments in PAI-1:Ag levels induced by venous occlusion (25% inhibition from 43.7 ± 35.9 to 32.8 ± 31.6 ng/mL, \( P > .5 \)). Aspirin caused a greater reduction in the increments of t-PA-PAI-1 complexes induced by venous occlusion (51%, from 6.0 ± 7.1 to 2.9 ± 4.5 ng/mL), but this change also was not significant (\( P = .099 \)).

In contrast to the correlations noted above between the inhibition by aspirin of incremental t-PA:Ag and activity, no additional correlations of significance were found between the effect of aspirin on t-PA activity and the effect of aspirin on PAI-1:Ag or t-PA-PAI-1 complexes. Thus, of the many variables tested, only changes in t-PA:Ag induced by aspirin predicted changes in t-PA activity induced by aspirin.

The relation of plasma salicylate concentration to t-PA antigen and activity. To determine that aspirin had been ingested correctly and explore whether any of the changes noted in the aspirin study were related to salicylate concentration, salicylate levels were measured in all preocclusion plasma samples from both control and aspirin studies. Salicylate levels in all control samples were below the level of detection of the method (< 1 µg/mL). The mean salicylate concentration was 41.8 ± 9.8 µg/mL, and as observed previously, the concentration was higher in women (44.8 ± 9.8 µg/mL) than in men (35.4 ± 6.0 µg/mL). There were no significant correlations between salicylate level and either the actual levels or percentage inhibition by aspirin of any of the parameters noted.

The effect of aspirin on the ability of plasma to support the generation by t-PA of alpha-plasmin inhibitor-plasmin complexes in vitro. Because both assays for t-PA activity depend on multiple components of plasma, we sought to determine whether aspirin altered the ability of plasma to support plasmin generation and the subsequent formation of PIPC. Table 3 demonstrates that when plasma is incubated at 37°C for 2 hours with a concentration of aspirin, which represents the maximal reported plasma concentration after the ingestion of 650 mg aspirin (150 µmol/L),44 and the aspirin is then removed, there is no effect on the generation of PIPC. This was the case when either reptilase, the enzyme used to clot plasma in our assay, or thrombin, the physiologic enzyme of clot formation, was utilized to clot plasma. Thus, exposure to aspirin does not alter the ability of plasma to support the fibrin-catalyzed generation of PIPC.

### Table 3

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>Aspirin</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAI-1:Ag</td>
<td>7.1 ± 4.7</td>
<td>3.8 ± 3.5</td>
</tr>
<tr>
<td>t-PA-PAI-1</td>
<td>43.7 ± 35.9</td>
<td>32.8 ± 31.6</td>
</tr>
</tbody>
</table>

### Table 4

<table>
<thead>
<tr>
<th>Study 1</th>
<th>Study 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre</td>
<td>Post</td>
</tr>
<tr>
<td>Pre</td>
<td>Post</td>
</tr>
<tr>
<td>PAI-1:Ag</td>
<td>150</td>
</tr>
<tr>
<td>t-PA-PAI-1</td>
<td>35</td>
</tr>
</tbody>
</table>

#### Comparison of series 1 and series 2.

Table 4 shows a comparison of the results of the current study with the results of the previously reported series and includes the new...
analysis of t-PA:Ag levels in the original 13 subjects. Aspirin had no effect on the preoclusion levels of t-PA activity or antigen. In contrast, aspirin significantly inhibited the levels of t-PA activity and antigen in postocclusion plasma in both series of subjects. Further, and in large measure because of the effect of aspirin on absolute, postocclusion levels, aspirin significantly inhibited the increments in t-PA activity and t-PA antigen induced by venous occlusion in each series and in the entire group of 23 subjects formed by combining both series. (all, P ≤ .001).

Of note is the difference in the inhibitory effect of aspirin on both the absolute, postocclusion and incremental t-PA activity induced by venous occlusion in the two studies. There was significantly less inhibition in the second series compared with the first (29.1% ± 58.3% and 40.5% ± 66.4%, respectively, both P = .03). These reductions in the inhibitory effect of aspirin in the second study (50% and 39%, respectively) were paralleled by a 44% reduction in the effectiveness of venous occlusion in enhancing t-PA activity in the control phase of the second study (increment tPA (U/mL): 1.67 ± 0.9 v 3.0 ± 2.1, P = .08). These differences are unlikely to be due to sampling error because a comparison of the three subjects who were included in both studies shows an identical pattern: the increment in t-PA activity in the control phase of the current study in these three subjects was only 30% of that seen in the first study; the inhibition by aspirin of the increment in activity induced by venous occlusion was only 67% of that seen in the first study. Expressed formally, there is an extremely strong correlation between the extent of inhibition by aspirin of incremental t-PA activity and the degree of enhancement of t-PA activity induced by venous occlusion in the control state: the greater the increment of t-PA activity induced by venous occlusion, the greater the inhibition of this increment by aspirin (r = .956, P < .001).

**DISCUSSION**

In this study we have confirmed that aspirin inhibits the incremental t-PA activity induced by venous occlusion measured either by a classic technique, the lysis of a euglobulin clot, or by our previously described technique, which uses an ELISA for the fibrin-catalyzed generation of PIPC in vitro. We have shown that the two techniques are similar in their
ability to assess t-PA activity and reveal similar inhibitory activity of aspirin, thus assuring that the novel assay\textsuperscript{2,28} is in fact a specific measure of t-PA activity as described.

The cause for the varying conclusions in the literature\textsuperscript{1,16-27} concerning the ability of aspirin to inhibit the rise in t-PA activity induced by venous occlusion is not obvious. As shown in Table 5, all of the studies we could identify that used an aspirin dose and schedule of administration similar or identical to ours\textsuperscript{20,21,23,26} showed some inhibition of incremental t-PA activity after venous occlusion. The magnitude of inhibition ranged from 30% to 87%, and of the four additional studies published, only those of de Gaetano et al\textsuperscript{23} and Keber et al\textsuperscript{26} found the inhibition to be statistically significant. Additional studies that used substantially different doses and schedules of aspirin administration also reported varying but essentially negative results.\textsuperscript{24,42,43} It is important to note that the number of subjects enrolled in each of the studies that followed our protocol is small, the interindividual range of responses to venous occlusion is large,\textsuperscript{18,39,44,45} and, therefore, the chance of not finding a statistically significant effect when one is actually present (type II error) is also large. Further, because we have demonstrated a correlation between the magnitude of the rise in t-PA due to venous occlusion and the magnitude of inhibition of that rise by aspirin, techniques of venous occlusion that result in lesser increases of t-PA will be less likely to detect a significant effect of aspirin.

The differences cannot be attributed to the possibility that the different assays are measuring different functions of t-PA because euglobulin lysis time has now been used to assess the effect of aspirin on t-PA activity in four studies, including the current report, and the magnitude of inhibition of incremental t-PA activity varied from 30%,\textsuperscript{21} to 87%,\textsuperscript{25}

Table 3. Effect of Aspirin on the Ability of Plasma to Generate Alpha2-Plasmin Inhibitor-Plasmin Complexes (PIPC) In Vitro

<table>
<thead>
<tr>
<th>PIPC Formation (10\textsuperscript{-8} x A 405 nm min\textsuperscript{-1})</th>
<th>Rapplase</th>
<th>Thrombin</th>
</tr>
</thead>
<tbody>
<tr>
<td>t-PA</td>
<td>+t-PA</td>
<td>t-PA</td>
</tr>
<tr>
<td>Control</td>
<td>25</td>
<td>307</td>
</tr>
<tr>
<td>Aspirin</td>
<td>24</td>
<td>296</td>
</tr>
</tbody>
</table>

Aspirin (150 μmol/L) was incubated with normal plasma for 2 hours at 37°C, and the plasma was then dialyzed extensively against TRIS-buffered saline. The dialyzed plasma was clotted with the agents shown in the presence of 0 or 2 U/mL t-PA and the generation of PIPC determined by ELISA. Control plasma was incubated with ethanol (0.1%, final concentration), the vehicle for aspirin.

Percent inhibition was calculated as (control activity - aspirin activity)/control activity x 100. Methods utilized for the analysis of t-PA activity in the order in which they appear in the table were as follows: PIPC, enzyme-linked immunosorbent assay for in vitro generation of alpha2-plasmin inhibitor-plasmin complexes expressed as U/mL; EFA, euglobulin fibrinolytic activity measured by the fibrin plate method expressed as U/mL; ELT, euglobulin lysis time, expressed as U/mL (Hammouda and Keber) or minutes to lysis (de Gaetano); PFA, plasma fibrinolytic activity measured by a radiometric assay for degradation of \textsuperscript{125I}-fibrin expressed as ng fibrin lysed per hour; ELA, area of a fibrin clot lysed in 18 hours expressed as mm\textsuperscript{2}; FP, fibrin plate method expressed as U/mL.

*The differences between aspirin and control studies were found to be significant at least to the 0.05 level.

Table 4. Comparison of the Effect of Aspirin on t-PA Antigen and Activity, Before and After Venous Occlusion in the First and Second Series of Subjects

<table>
<thead>
<tr>
<th>Series 1 (n = 13)</th>
<th>C</th>
<th>A</th>
<th>C - A</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prevenous occlusion</td>
<td>0.3 ± 0.3</td>
<td>0.3 ± 0.3</td>
<td>0.02</td>
<td>NS</td>
</tr>
<tr>
<td>Postvenous occlusion</td>
<td>3.3 ± 2.3</td>
<td>1.2 ± 1.0</td>
<td>2.1</td>
<td>.002</td>
</tr>
<tr>
<td>Increment</td>
<td>3.0 ± 2.1</td>
<td>0.9 ± 0.7</td>
<td>2.1</td>
<td>.001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Series 2 (n = 10)</th>
<th>C</th>
<th>A</th>
<th>C - A</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prevenous occlusion</td>
<td>0.3 ± 0.3</td>
<td>0.4 ± 0.3</td>
<td>-0.1</td>
<td>NS</td>
</tr>
<tr>
<td>Postvenous occlusion</td>
<td>2.0 ± 0.9</td>
<td>1.5 ± 0.9</td>
<td>0.5†</td>
<td>.013</td>
</tr>
<tr>
<td>Increment</td>
<td>1.7 ± 0.9</td>
<td>1.0 ± 0.6</td>
<td>0.7†</td>
<td>.001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ALL (n = 23)</th>
<th>C</th>
<th>A</th>
<th>C - A</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prevenous occlusion</td>
<td>0.3 ± 0.4</td>
<td>0.4 ± 0.4</td>
<td>-0.1</td>
<td>NS</td>
</tr>
<tr>
<td>Postvenous occlusion</td>
<td>2.7 ± 1.9</td>
<td>1.3 ± 0.9</td>
<td>1.4</td>
<td>.001</td>
</tr>
<tr>
<td>Increment</td>
<td>2.4 ± 3.2</td>
<td>1.0 ± 0.5</td>
<td>1.4</td>
<td>.001</td>
</tr>
</tbody>
</table>

Table 5. Comparison of the Inhibitory Effect of Aspirin on t-PA Activity Measured in Different Studies by Various Techniques

<table>
<thead>
<tr>
<th>Study</th>
<th>N</th>
<th>Method</th>
<th>Increment in t-PA Activity Induced by Venous Occlusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ASA</td>
<td>+ASA</td>
<td>% Inhibition</td>
</tr>
<tr>
<td>Levin\textsuperscript{1}</td>
<td>13</td>
<td>PIPC</td>
<td>2.97</td>
</tr>
<tr>
<td>Bounameux\textsuperscript{26}</td>
<td>9</td>
<td>EFA</td>
<td>2.1</td>
</tr>
<tr>
<td>Hammouda\textsuperscript{21}</td>
<td>3</td>
<td>ELT</td>
<td>1.32</td>
</tr>
<tr>
<td>de Gaetano\textsuperscript{26}</td>
<td>6</td>
<td>PFA</td>
<td>34</td>
</tr>
<tr>
<td>Keber\textsuperscript{26}</td>
<td>10</td>
<td>ELA</td>
<td>319.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FP</td>
<td>1.4</td>
</tr>
</tbody>
</table>

Abbreviations: PIPC, t-PA activity; ASA, aspirin; ELA, area of a fibrin clot lysed in 18 hours expressed as mm\textsuperscript{2}; FP, fibrin plate method expressed as U/mL.
EFFECT OF ASPIRIN ON TISSUE PLASMINOGEN ACTIVATOR

with three of the four studies reporting significant results. Other than the explanations of statistical deficiencies or technical differences as discussed, there is no basis we could identify for explaining the apparent discrepancies between studies.

The mechanism by which aspirin inhibits the incremental t-PA activity induced by venous occlusion must in large part be related to the inhibition of t-PA:Ag release into the circulation that we have demonstrated. The activity of t-PA in the circulation is controlled by at least four independent factors: (1) the level of t-PA antigen; (2) the level and (3) the activity of two different plasminogen activator inhibitors, PAI-1 (that found in endothelial cells, platelets and other sites) and PAI-2 (that found in placenta and other sites); and (4) the activity of an as yet undefined plasminatic factor, PA-binding protein. Although aspirin had no effect on the resting level of t-PA antigen, it did inhibit the increment in t-PA antigen induced by venous occlusion. The mean magnitude of this inhibition was similar to the mean magnitude of the inhibition of t-PA activity, and the two phenomena were linearly correlated. In contrast, aspirin did not alter the increments in PAI-1:Ag induced by venous occlusion, and there were no correlations between the diminution in t-PA activity induced by aspirin and changes in the levels of PAI-1:Ag or t-PA–PAI-1 complexes. Thus, as far as we can determine, the aspirin-induced inhibition of t-PA activity can best be ascribed to inhibiting the release of t-PA antigen from the vessel wall.

The mechanism by which venous occlusion of the forearm induces t-PA release is unknown but may include both enhanced release and diminished clearance. The occlusion causes a generalized "stimulation" of the endothelium with release of factor VIII related antigen and prostacyclin as well as t-PA. However, the release of these factors may not be a physiologically coupled event but rather evidence of destruction of endothelium. Venous occlusion is described by subjects experiencing it as uncomfortable to frankly painful and is associated with capillary rupture as evidenced commonly by the formation of petechiae. Death of endothelial cells is known to cause the release of both factor VIII and prostacyclin, and, therefore, the appearance of these factors in plasma after venous occlusion may simply be an indication of trauma and endothelial disruption. Nonetheless, the release of factor VIII is not inhibited by aspirin, suggesting that the inhibitory effect of aspirin on incremental t-PA activity is selective. Regardless of the exact mechanism, inhibition of prostaglandin synthesis may be important to the inhibitory effect of aspirin on t-PA activity. We have confirmed our original observation that aspirin inhibits the incremental t-PA activity induced by venous occlusion of the forearm and shown that this inhibition is related to alterations in t-PA antigen. The antifibrinolytic effect of aspirin persists for some time, and "low-dose" aspirin does not exhibit inhibition of t-PA activity. Recent evidence has shown aspirin to be beneficial in the prevention of a variety of complications of atherosclerosis, and it is possible that utilization of aspirin doses that do not inhibit stimulated, t-PA-directed fibrinolysis may contribute to or augment this benefit.

ACKNOWLEDGMENT

The authors thank Dr John W. Fenton, III, New York State Department of Health, Albany, NY, for his gift of human thrombin, Dr Mats Ranby, Biopool AB, for reagents for the PAI assay, Dr Jean Amiral, Diagnostica Stago, for reagents for the t-PA–PAI-1 complex assay, Wendi Lovenvirth and Regina Sullivan for technical assistance, and to Margo Nikitas for skillful preparation of the manuscript.

REFERENCES

17. Graunger W, Pirich KR, Speiser W, Deutsch E, Waldhausl...

glyceride, body endothelial cells.

ogen activator and plasminogen activator inhibitor activities in a aspirin on fibrinolysis. Fibrinolysis 1:202, 1986 (suppl) (abstr)

occlusion. Thromb Haemost 54:175, 1985 (abstr)

glandin synthetase by aspirin. Proc Natl Acad Sci USA 72:3073, 1986


1642


51. Schleef RR, Sinha M, Loskutoff DJ: Immunoradiometric assay to measure the binding of a specific inhibitor to tissue-type plasminogen activator. J Lab Clin Med 106:408, 1985


55. Erickson LA, Hekman CM, Loskutoff DJ: The primary
plasminogen-activator inhibitors in endothelial cells, platelets, serum and plasma are immunologically related. Proc Natl Acad Sci USA 82:8710, 1985


Inhibition of tissue plasminogen activator activity by aspirin in vivo and its relationship to levels of tissue plasminogen activator inhibitor antigen, plasminogen activator and their complexes

RI Levin, PC Harpel, JG Harpel and PA Recht