Caution in the interpretation of continuous thrombin generation assays: a rebuttal

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See also Butenas S, Mann KG. Caution in the interpretation of continuous thrombin generation assays. This issue, pp 1084–5.

We share the concern of Butenas and Mann [1] about the influence of the signaling substrate in continuous measurement of thrombin generation. Any substrate necessarily binds to thrombin and, moreover, is liable to bind to other clotting proteases. It therefore always inhibits thrombin and is potentially an inhibitor of the clotting mechanism at other sites as well. Given the importance of thrombin-driven feedback reactions, inhibition of thrombin will, theoretically, always influence prothrombin conversion. Whether and under what conditions this is of practical importance remains to be answered for each single substrate. In the articles in which we introduced continuous thrombin generation measurement with chromogenic [2] or with fluorogenic [3] substrates, we have shown that prothrombin conversion is not significantly affected by the proposed substrates but thrombin decay is slowed down in a predictable manner because plasmatic antithrombins cannot interact with thrombin that is occupied by the substrate. It might suffice to refer to these articles but in view of its accruing clinical relevance we readdress the subject for the currently most-used substrate: Z-Gly-Gly-Arg-aminomethylcoumarine (AMC).

From Table 1 it can be seen that Z-Gly-Gly-Arg-AMC indeed prolongs the initiation phase (lag time) of thrombin generation at low tissue factor (TF) concentrations, be it less in our experiments than in those of Butenas and Mann. We agree that this is likely to be due to inhibition of thrombin-driven feedback reactions. The effect on the lag time in TF-induced thrombin generation in plasma is weaker than that on the reconstituted 'coagulosome', which suggests that caution is in its place when extrapolating from reconstituted systems to the 'isolated organ' plasma. The information contained in the lag time is essentially the same as that in conventional clotting times. It is a key question whether the substrate interferes with the information that is unique for the thrombogram, i.e. that on the amount of prothrombin converted and on the time course of the thrombin production.

Plasma contains the natural antithrombin II2macroglobulin (II2M), the 'bait region' of which is split by thrombin after which thrombin remains bound to the II2 M molecule and, although biologically inactive, retains its activity towards the signal substrate. The residual amidolytic activity, after
Influence of substrate concentration on thrombin activity. (A) Amidolytic activity, i.e. thrombin and α2macroglobulin–thrombin activity; (B) thrombin activity only; (C) thrombin activity compensated for binding to substrate. Defibrinated pooled normal plasma. Reaction conditions as in [2]. Five pM tissue factor. Substrate concentrations from top to bottom: 832 μM (black), 416 μM (red), 208 μM (black), 104 μM (red).

Table 1 Effect of substrate on feedback phenomena

<table>
<thead>
<tr>
<th>Substrate (μM)</th>
<th>n</th>
<th>0</th>
<th>104</th>
<th>208</th>
<th>416</th>
<th>832</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lag time TF 5 pm (s)</td>
<td>16</td>
<td>105 ± 6</td>
<td>104 ± 6</td>
<td>107 ± 8</td>
<td>127 ± 7</td>
<td></td>
</tr>
<tr>
<td>Lag time TF 1 pm (s)</td>
<td>16</td>
<td>225 ± 13</td>
<td>226 ± 13</td>
<td>234 ± 11</td>
<td>253 ± 12</td>
<td></td>
</tr>
<tr>
<td>α2 M–IIa end-level 5 pm (nm)</td>
<td>8</td>
<td>92.2 ± 5.7</td>
<td>90.6 ± 6.5</td>
<td>93.9 ± 6.4</td>
<td>96.8 ± 5.9</td>
<td>94.8 ± 6.2</td>
</tr>
<tr>
<td>α2 M–IIa end-level 1 pm (nm)</td>
<td>8</td>
<td>74.7 ± 5.4</td>
<td>70.8 ± 6.3</td>
<td>79.8 ± 5.0</td>
<td>77.7 ± 4.9</td>
<td>77.2 ± 5.0</td>
</tr>
<tr>
<td>Inhib. by 10 nm TM TF 5 pm (%)</td>
<td>8</td>
<td>68 ± 5</td>
<td>69 ± 4</td>
<td>67 ± 5</td>
<td>62 ± 4</td>
<td>45 ± 3</td>
</tr>
<tr>
<td>Inhib. by 4 nm TM TF 1 pm (%)</td>
<td>8</td>
<td>81 ± 6</td>
<td>83 ± 7</td>
<td>78 ± 6</td>
<td>77 ± 5</td>
<td>63 ± 6</td>
</tr>
</tbody>
</table>

The lag time is defined as the moment that the thrombin concentration curve crosses the thrombin concentration value of 10 nM. The inhibition by thrombomodulin (TM) gives the α2macroglobulin–(α2 M–) thrombin end-level with TM as a percentage of the end-level without TM. Inhib., inhibition; TF, tissue factor.

Effects on the course of prothrombin conversion

In Fig. 1C, the effect of substrate binding on thrombin breakdown has been abolished. The resulting curves represent thrombin generation as if antithrombin action would have been normal. The curves are so similar as to be indistinguishable, except for the effect on the lag phase. We conclude that not only the amount of prothrombin converted is not influenced by the substrate but also the time course of prothrombin conversion is hardly or not influenced.

In conclusion, to probe a system means disturbing it, so when proposing a new technique one has to investigate what this disturbance amounts to. This we did in our original publications and here we show additional evidence that the extent and the time course of prothrombin conversion are minimally disturbed by the presence of Z-Gly-Gly-Arg-AMC at 416 μM. Thrombin inactivation is slowed down to an exactly predictable extent. The necessary effect on thrombin-driven feedback, in plasma, does not cause an important increase in the lag time but does diminish the action of added thrombomodulin by about 10%.
These conclusions pertain to Z-Gly-Gly-Arg-AMC at the usual concentration, i.e. at around twice its $K_m$. They should not be extrapolated to other substrates or to higher concentrations. They certainly should not be interpreted as a laissez passer for any other substrate. Thrombin generation experiments with high-affinity substrates, e.g. those performed in [6], are very likely to reflect a coagulation mechanism in which feedback mechanisms are severely disturbed.

Disclosure of Conflict of Interests

The authors state that they have no conflicts of interest.

References


Evidence that pre-existent variability in platelet response to ADP accounts for ‘clopidogrel resistance’: a rebuttal

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We read the article by Michelson et al. [1] with interest. We believe that several issues concerning the evaluation of clopidogrel resistance or response variability should be emphasized. Most importantly, in order to assess ‘resistance’ or response variability to an antiplatelet agent, it is mandatory to measure platelet function before and after therapy [2,3]. Therefore, in the study by Michelson et al., the complete absence of pretreatment and post-treatment measurements in patients and the absence of an analysis of platelet inhibition in the healthy control group precludes any conclusions related to clopidogrel resistance. Moreover, most studies addressing clopidogrel response variability have enrolled a homogeneous patient population, as platelet reactivity to adenosine diphosphate (ADP) varies with the disease state, and patients were treated with a uniform dosing regimen. Finally, the lack of serial prespecified measurements, we believe, is another important limitation of the study.

In 2001, by serially measuring ADP-induced platelet aggregation and expression of activation-dependent receptors in 100 patients undergoing coronary stenting treated with a 300-mg clopidogrel loading dose followed by a 75-mg daily maintenance dose, we demonstrated that clopidogrel treatment did not produce a uniform level of post-treatment platelet reactivity to ADP [4]. We had also previously demonstrated that there is variability in ADP-induced platelet aggregation in aspirin-treated patients not receiving clopidogrel [5]. In 2003, we reported a study of 96 patients undergoing elective stenting who were treated with a 300-mg clopidogrel loading dose in the catheterization laboratory followed by a 75-mg maintenance dose. ADP-induced platelet aggregation and activation-dependent platelet surface marker expression [P-selectin and activated glycoprotein (GP) IIb/IIIa] were assessed before clopidogrel therapy and serially for 30 days following treatment for stenting. In the latter study and a subsequent study, we again demonstrated non-uniform post-treatment platelet reactivity to ADP that followed a normal distribution [6,7].