Inhibition of In Vitro Platelet Aggregation and Release and Fibrinolysis

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ABSTRACT

Inhibition of in vitro platelet aggregation and release of contents of platelet granules is necessary in order to assess accurately platelet activation in vivo. This can be accomplished by using a variety of inhibitors added to blood collection containers. An additive mixture of citrate, theophylline, adenosine, and dipyridamole (CTAD) provides a practical alternative to a mixture of acid citrate dextrose (ACD), acetylsalicylic acid (aspirin), and prostaglandin E₁ (PGE₁) because of the stability problems associated with PGE₁. Inhibition of in vitro fibrinolysis is essential for the accurate measurement of fibrin degradation products (FDP). This can be accomplished by using a mixture of thrombin, soybean trypsin, or aprotinin into which blood is collected. However, in patients receiving heparin, the fibrinolysis inhibitor mixture is ineffective unless it is supplemented with reptilase. With increasing use of recombinant tissue-type plasminogen activator therapy (rt-PA), an inhibitor such as D-phenylalanine-proline-arginine-chloromethylketone (PPACK) used as a blood collection additive is superior to a conventional protease inhibitor, such as aprotinin.

Introduction

Minimization of preanalytical error in the accurate assessment of in vivo platelet aggregation and release of contents of the platelet granules can only be accomplished by use of inhibitors added to blood which will insure that in vitro platelet aggregation and release do not occur. This will permit accurate measurement of contents of platelet granules released in vivo and also minimization of inactivation of an administered anti-coagulant such as heparin by platelet factor IV (PF₄), released in vitro, and thus permit accurate heparin measurement by either chromogenic substrate (anti-Xa activity) or the conventional activated partial thromboplastin time (aPTT) methodology. Similar concerns in minimizing in vitro artifacts are the basis of utilization of inhibitors of fibrinolysis for the accurate measurement of fibrin degradation products (FDP) and in monitoring recombinant tissue-type plasminogen activator (rt-PA) therapy.
Inhibition of In-Vitro Platelet Aggregation and Release of Contents of Platelet Granules

To assess accurately platelet activation in vivo, one platelet activation in vitro must be prevented. Platelet activation can be assessed by the measurement of one of the many constituents within the platelet α-granule which is released upon activation of platelets. Thus, when platelets are activated, the contents of α-granules such as platelet Factor IV (PF₄), beta thromboglobulin (βTG), platelet derived growth factor (PDGF), thrombospondin (TSP) fibronectin, fibrinogen, albumin, and Factor VIII-related von Willebrand factor polymers (VIII:vWF) are released. Platelet activation also results in the release of contents of platelet dense granules such as adenosine diphosphate (ADP) and serotonin. Adenosine diphosphate induces absorption of fibrinogen to platelet membrane glycoprotein receptors: glycoprotein IIb-IIIa (GP IIb-IIIa). This triggers platelet aggregation by fibrinogen-bridged adjacent platelets, an event facilitated by release of adhesive proteins from the platelet α-granules such as fibronectin, TSP, and VIII:vWF. Continued generation of thromboxane A₂ which triggers release of contents of platelet granules is potentiated by increased cytoplasmic ionic calcium concentration which in turn activates phospholipase A₂ that generates substrates for subsequent reactions leading to thromboxane A₂ production. The biochemical events leading to release of contents of platelet granules which subsequently triggers platelet aggregation are summarized in figure 1.

In vitro release of these constituents must be prevented in order to permit accurate assessment of in vivo platelet activation. Among additive mixtures that have been proposed for inclusion in blood collection tube to prevent in vitro platelet activation, the following deserves mention: a mixture of acid citrate dextrose (ACD, 1 to 5 dilution), 30 micromolar acetylsalicylic acid (aspirin) and 1 micromolar prostaglandin E₁ (PGE₁). The rationale is that aspirin, by acetyllating and inhibiting fatty acid cyclooxygenase enzyme in platelets, will inhibit release of contents within the platelet α-granules and ADP-induced platelet aggregation by preventing formation of thromboxane A₂.

Because of the stability problems associated with PGE₁, an alternative inhibitor of platelet aggregation and release has found application. This additive mixture is called CTAD (citrate, theophylline, adenosine, and dipyridamole). The rationale for the utilization of CTAD is based on the maintenance of increased intra cellular levels of cyclic AMP (c-AMP) which is a potent inhibitor of platelet aggregation. Increased levels of c-AMP are maintained by activation of adenyl cyclase by the addition of free adenosine. Although red blood cells are a source of free adenosine formed from adenine nucleotides, they avidly take up the formed adenosine, as a result of which the level of plasma adenosine is low. The uptake of adenosine by the red cell is inhibited by dipyridamole, thus permitting increased activation of adenyl cyclase by adenosine resulting in increased c-AMP levels needed to prevent platelet aggregation and release. Degradation of c-AMP must be prevented by the enzyme phosphodiesterase. This is achieved by theophylline and, to a certain extent, by dipyridamole present in the CTAD mixture. By chelating calcium, citrate functions as an anticoagulant. In figure 2 are summarized the role of theophylline, adenosine, and dipyridamole in inhibiting release of contents of platelet granules which would otherwise trigger platelet aggregation.
The CTAD mixture can be used in the blood collection tube for monitoring heparin therapy by measurement of either heparin by chromogenic substrate assay or by using the aPTT determination. Since in vitro aggregation and release of contents of α-granules in platelets will be inhibited by CTAD, release of PF₄ present in the platelet α-granules will be minimized, thus permitting a reliable assessment of circulating heparin level in plasma. In addition, CTAD can be used to measure in vivo release, if any, of the contents within the platelet α-granules such as PF₄, fibronectin, βTG, and PDGF, by ensuring inhibition of in vitro platelet aggregation and release. The exact composition of CTAD mixture that have been used by investigators⁸,⁹ is as follows: 0.11 M citric acid, 15 mM theophylline, 3.7 mM adenosine, and 0.198 mM dipyridamole, with pH adjusted to 5.0. Nine volumes of blood are mixed with one volume of the CTAD additive anticoagulant mixture. The efficacy of CTAD additive in minimizing in vitro platelet aggregation and release of contents of α-granules was demonstrated by measurement of platelet factor (PF₄) levels in blood collected from healthy subjects in CTAD and the conventional buffered citrate anticoagulant. While typically PF₄ levels were in excess of 100 ng per ml in the conventional buffered citrate anticoagulant tube, levels measured in the CTAD tube were in the range of 12 to 14 ng per ml.¹³

The use of CTAD as a blood collection additive permits reliable monitoring of heparin therapy by either the chromogenic substrate assay or the aPTT.²,¹³
In Vitro Inhibition of Fibrinolysis

Assessment of fibrinolysis in the laboratory is made by the measurement of fibrin degradation products (FD): X, Y, D, and E that are formed as a result of the action of the enzyme plasmin on fibrin and fibrinogen. In figure 3 are summarized steps in fibrinolytic sequence.

To measure FDP, the in vitro action of plasmin must be inhibited by including in the blood collection tube an inhibitor of plasmin. Either aprotinin or soybean trypsin can be used as an inhibitor of plasmin.

A complete conversion must be made of any residual fibrinogen that is present to fibrin by the addition of thrombin to the blood collection tube. Otherwise a spurious increase might be seen in the FDP value. This is the rationale for the use of thrombin and either soybean trypsin or aprotinin in the blood collection tube intended for the measurement of FDP. However, in patients receiving heparin, the conversion of residual fibrinogen, even in the presence of thrombin, is slow, giving rise to spurious increases in FDP value owing to the remaining unconverted fibrinogen. This can be overcome by the addition of snake venom, also known as reptilase which can rapidly convert any residual fibrinogen to fibrin. Thus, for patients receiving heparin, the ideal blood collection additive mixture for the measurement of FDP is reptilase, aprotinin, or soybean trypsin and thrombin. Presence of residual fibrinogen or fibrin monomer and polymers (FFMP) in heparinized plasma can lead to false positive results of FDP which can be overcome with reptilase.1

Inhibition of In Vitro Recombinant Tissue-Type Plasminogen Activator (rt-PA)

Unlike streptokinase and urokinase which activate circulating plasminogen, rt-PA activates plasminogen-bound fibrin. Thus in contrast to streptokinase and urokinase where the resulting plasmin proteolyses in addition to fibrinogen, Factors V and VIII:C, rt-PA therapy appeared attractive. In theory, at least plasmin generated by rt-PA would limit itself to dissolving the fibrin mass.14

With the introduction of rt-PA therapy, it is important to inhibit the in vitro activation of plasminogen to plasmin and the resulting fibrinolysis. Although aprotinin inhibits plasminogen activation by urokinase, it is ineffective against inhibiting the rt-PA activation of plasminogen.4

Specific synthetic peptides of arginine chloromethyl ketone have found applica-
tion in the activation of trypsin-like enzymes. Of these, D-phenylalnine proline-arginine-chloromethyl ketone (PPACK) has been proven to be effective in inhibiting in vitro rt-PA activity. The inhibitor PPACK irreversibly inactivates rt-PA by alkylating the active center of amino acid histidine; PPACK is also a potent thrombin inhibitor. Five mM of PPACK added to either 10 mM of citrate or 4.2 mM of ethylene diamine tetraacetic acid (EDTA) was effective in inhibiting rt-PA in blood collected in these additive mixtures. Unlike aprotinin which inhibits kalikrein, coagulation factors in the preliminary phase of blood clotting and plasmin, PPACK apparently does not interfere with the potential active site of the proenzyme plasminogen as demonstrated by lack of interference in the measurement of plasminogen levels in anticoagulated blood containing PPACK. Aprotinin, in addition to interfering in the assay of plasminogen, will also interfere with the α2-antiplasmin assay since both these assays are dependent on the activity of plasmin which is inhibited by aprotinin. In contrast, the concentration of PPACK used (5 mM) in blood collection may be too low to inhibit the plasmin which is added to the α2-antiplasmin assay. Thus, a blood sample collected with the appropriate anticoagulant and 5 mM PPACK can be used for the accurate measurement of plasminogen, α2-antiplasmin, fibrinogen, FDP, and immunoreactive rt-PA.

Conclusion

Preanalytical errors in the assessment of in vivo platelet aggregation and release of contents of α-granules, can be minimized by inclusion of appropriate inhibitors during blood collection. Inhibition of in vitro fibrinolysis is para-

mount to accurate measurement of fibrin degradation products and monitoring of heparin therapy. Inclusion of inhibitor of recombinant tissue type plasminogen activator in blood collection system is a prerequisite not only to the inhibition of in vitro fibrinolysis, but also in minimizing error in specific coagulation tests.

References

INHIBITION OF IN VITRO PLATELET AGGREGATION


