La Prensa Medica Argentina

Research Article

Study of Fibrinogen Levels in Blood after Infused a Modified Plasmin in Rabbit

Agustín Joison[1*] and Federico Gallo[2]

[1]*Professor and chairman of Biological Chemistry, Cordoba Catholic University, Argentina*

[2]*Vascular centre, General Roca Rio Negro, Argentina*

[*]**Corresponding author:** Agustin N Joison, Dean Funes 1752 T1 Floor 9 Dep. C, X5000ESU Córdoba, Argentina, Tel: +54-351-4807421; E-mail: ajoison2001@yahoo.com.ar

Rec date: Dec 10, 2013 Acc date: Jan 03, 2014 Pub date: Jan 07, 2014

Abstract

Background: Plasmin is a two chains molecule connected by two Disulfide bridge, Plasmin results of plasminogen (MW 92 Kdal) activation by rupture of the union Arg560-Val-561. Plasminogen activation is produced by activators of plasminogen as t-PA (tissue - Plasminogen Activator) or Streptoquinase. Plasmin has interactions with substances, proteases and inhibitors as α2-antiplasmin with to a lesser activity α2-macroglobulin for its regulation in blood. Methods: ELISA Detection of Plasmin the test principle applied in this kit is Sandwich enzyme immunoassay. New Zealand white rabbits weighing 3 Kg were separated in two groups 10 (placebo) and 10 (Plasmin study). The determination of fibrinogen with thrombin clotting time is based on the Clauss method. Bleeding the ear artery after 30, 60 and 240 min of the infusion in both groups. Fibrinogen levels in plasma were analyzed in both groups. Native polyacrylamide gel electrophoresis was performed to run and compare the same protein in different temperature and pH precipitation conditions. SDS-PAGE was performed by the method of Laemmli using a 10% polyacrylamide gel. Results: The Fibrinogen levels in the Group of rabbits before and after the injection of saline solution had an average of 299.8 +/-1.8 mg/dl. The values of Fibrinogen in the group before and after the injection of modified plasmin had an average of 294.7 +/-1.04 mg/dl. Conclusion: a preparation of modified plasmin was developed by autocatalytic treatment, which being a truncated derivative of plasmin breaking Lys453-Cys454 sites, increasing affinity for fibrin and inhibited lesser by a2-antiplasmin.

Introduction

Plasmin is a two chains molecule connected by two Disulfide bridge, Plasmin results of plasminogen (MW 92 Kdal) activation by rupture of the union Arg560-Val-561. Plasminogen activation is produced by activators of plasminogen as t-PA (tissue - Plasminogen Activator) or Streptoquinase. Plasmin molecule has heavy chain (57 Kdal) and a light chain (26 Kdal) [1,2]. The extend of proteolysis to the plasminogen molecule, is important because the light chain derivate from the carboxyl-terminus of plasminogen, can be from 63 Kdal to 12 Kdal of molecular weight [3]. There are 5 kringles (triple loop) in the heavy chain of plasmin; kringles 1-4 has affinity to substances as ω-aminocarboxylic acid and fibrin binding sites [4]. Plasmin belongs to serine protease family with some properties as lysis to fibrin, activation of proteins of coagulation and complement system. Plasmin has interactions with substances, proteases and inhibitors as α2-antiplasmin with to a lesser activity α2-macroglobulin for its regulation in blood [5,6]. Some laboratories and pharmaceutical companies in the end of 1950s put special attention on the feasibility of applying plasmin as a thrombolytic agent in patients with thrombotic diseases. The results were good when plasmin was infused intra thrombus, but ineffective in systemic administration [7,8].

After some trials and studies with plasmin, their development and application in diseases thrombotic was abandoned. The reasons for this were its inhibition in blood by alpha 2-antiplasmin, degradation of coagulation factors and the cost of infusion locally by catheter device [9,10].

Derivatives or modifications of plasmin offer therapeutic alternatives as fibrinolytic agents. Plasmin as mini plasmin, and microplasmin forms are studied as potential thrombolytic. Although biochemical and molecular properties differ between them, there are not advantages in terms of fibrinolytic activity [11]. For many years they have been modifying and obtaining different variants of plasminogen activators, in order to improve the thrombolytic therapy and at the same time achieve greater safety in secondary Haemostatic disorders after used it, intracranial hemorrhage remains the worst manifestation of bleeding [12].

It is very important in a thrombolytic therapy with activators of plasminogen activators as t-PA (tissue- plasminogen activator), rt-PA (recombinant tissue- plasminogen activator), plasmin and others, perform a monitoring the levels of fibrinogen as predictive bleeding [13]. There is consensus and evidence in studies carried out with plasminogen activators, mainly in the thrombolytic therapy of acute myocardial infarction, the risk of complications of bleeding as a result of the rupture of the molecule of Fibrinogen [14]. News Researches on the use of plasmin as a thrombolytic agent, showed better haemostatic safety increased compared with plasminogen activators. Studies using a derivate plasmin (Δ K2-5) with the kringle 1 linked to serine protease plasmin domain, confirm it [15].The present study evaluates the effect of a new human modified plasmin infused intravenously in rabbits on fibrinogen levels in blood. This plasmin has two chains with different molecular weight and sites to link to fibrin respect to native plasmin, so this modified plasmin doesn´t inhibited by alpha 2-antiplasmin. The different cut in the molecule has the advantage do not develop hemorrhagic events.

Methods

ELISA Detection of Plasmin the test principle applied in this kit is Sandwich enzyme immunoassay. The microtiter plate provided in this kit has been pre-coated with an antibody specific to Plasmin/Antiplasmin Complex (Ref ahrps, Lot 29, Radimivd, substrate TMB Lot 03): The assay protocols were used: rabbit polyclonal to plasmin (Biotin) ab 48350). Standards or samples are then added to the appropriate microtiter plate wells with a biotin-conjugated antibody specific to Plasmin/Antiplasmin Complex (PAP). Next, Streptavidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. After TMB substrate solution is added, only those wells that contain Plasmin/Antiplasmin Complex (PAP), biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated

by the addition of sulphuric acid solution and the color change is measured spectrophotometrically (Hyperion (Organon Teknika Bélgica) at a wavelength of 450nm ± 10nm. The concentration of Plasmin/Antiplasmin Complex (PAP) in the samples is then determined by comparing the O.D. of the samples to the standard curve. 100 ul IgG (pcAb Capture) was added to the wells of a 96-well assay plate (Immulon 4, Dynatech Laboratory, Chantilly, VA). After incubation at 4°C Overnight, the capture solutions were removed by flicking the plates and the wells were blocked with blocking/diluent buffer (5% dry milk in PBS with 0.05% Tween 20), for 3 hours at room temperature. The wells were then washed five times with wash buffer (0.9% NaCl containing 0.05% Tween 20). Standard of plasmin and samples with modified plasmin were generally diluted 1/100 and 1/1000 in PBS. Then HRP was added to the plates and measured spectrophotometrically at a wavelength of 450nm.

Animal's Model:

New Zealand white rabbits weighing 3 Kg were separated in two groups 10 (placebo) and 10 (Plasmin study), both were anesthetized with 40 mg/kg ketamine HCl (Phoenix Pharmaceuticals, St Joseph, MO) and 5 mg/kg xylazine (Phoenix Pharmaceuticals; 2.7-3.25 mL total volume) by intramuscular injection. After anesthesia the left external ear vein was catheterized with a 24 G x 0.75 catheter (BD Angiocath,) to injected 10 ml of saline solution (placebo) and bolus 3 mg/Kg of plasmin. Anesthesia was maintained by intermittent injections of the ketamine HCI/xylazine mixture every 3 to 10 minutes as required. Bleeding the ear artery after 30, 60 and 240 min of the infusion in both groups. The blood was collected in a tube containing sodium citrate 0.25 M (Mallinckrodt, St. Louis). Fibrinogen levels in plasma were analyzed in both groups.

Fibrinogen Assay:

The determination of fibrinogen with thrombin clotting time is based on the Clauss method. An excess of thrombin, fibrinogen is transformed into fibrin and clot formation time is inversely proportional to the concentration of fibrinogen in the sample plasma. 200 ul of each sample of plasma was mixed with 100 ul of thrombin (0.1 mg/ml) (Wiener Lab. Argentine). The results of time (seconds) were analyzed in a calibration curve. The calibration curve was made using dilutions (1/5, 1/10, 1/15, 1/20, and 1/30 of plasma standard (260 mg/dl).

Native polyacrylamide gel electrophoresis was performed to run and compare the same protein in different temperature and pH precipitation conditions. Native 10% of polyacrylamide was prepared with Acryl amide and N'N'N'N' Bis-acrylamide (29 g/100 ml and 0,8 g/100 ml respectively), with Tris- HCl buffer, pH 8.8 as gel buffer and Tris- Gly pH 8 as running buffer, during 40 min at 120 volts. Sample buffer (5x): 15.5 ml of 1M Tris-HCl pH=6.8 2.5 ml of a 1% solution of bromophenol blue; 7.0 ml of water; and 25 ml of glycerol, staining: silver stain. SDS-PAGE was performed by the method of Laemmli using a 10% polyacrylamide gel. Samples were prepared with 10 % SDS. After PAGE the proteins were stained with silver stain.

Plasmin was obtained of human plasma with double isoelectric precipitation with citric acid in sodium borate as buffer. The purified by lysine affinity and ionic interchange chromatography. The modification of the molecule was done with different pH precipitation, changing pH 5 to pH 10 with sodium hydroxide.

Results

ELISA assay with variant human plasmin linked to plasmin antibody showed that plasmin and standard of plasmin present similar DO. According to the method of ELISA used, modified human plasmin was recognized by the antibody anti plasmin. The standard used in this assay (native plasmin) showed a value of OD was 168.2 with a mean of +/- 14.2. The OD of modified human plasmin was 402 with a mean of +/- 43.47 (Table 1).

Samples	OD (405 nm)
Plasmin Standard	168.2 +/- 14.2
Human modified plasmin	402.0 +/- 43047

Table 1: ELISA OD of Rabbit antibody antiplasmin and human modified plasmin.

Native gel electrophoresis revealed with silver stain a single band with apparent Mr of 80 kDal, corresponding to modified human plasmin before isolation of human plasma (Figure 1).

Figure 1: Native–polyacrylamide gel electrophoresis of modified plasmin. Lanes: 1 (0.01 mg), 2 (0.02mg) of modified plasmin.

SDS gel electrophoresis revealed a single band with apparent Mr of 56 kDal under non-reducing and two bands of 30 kDal, and 28 Kdal (Figure 2).

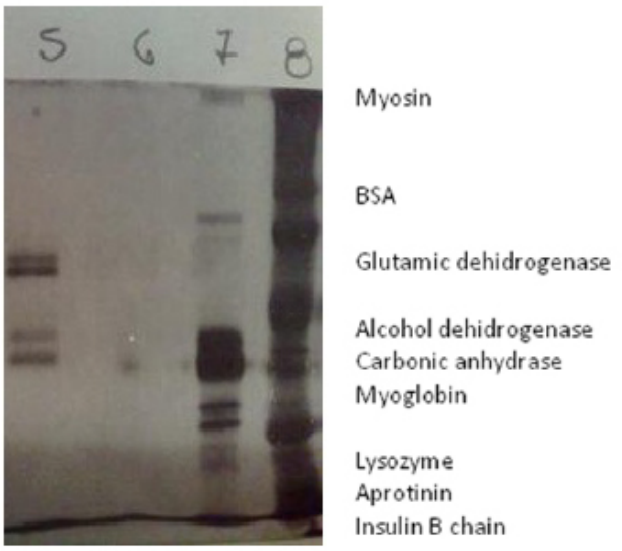

Figure 2: Sodium dodecyl sulphate-gel electrophoresis. (lane 5) modified plasmin chains; (lane 7) modified plasmin affinity chromatography; (lane 8) Standard sizing proteins, Myosin 188 KDa, BSA 62 KDa, Glutamic dehidrogenase 49 KDa, Alcohol dehidrogenase 38 KDa, Carbonic Anhydrase 28 KDa, Myoglobin 18 KDa, Lysozyme 14 KDa, Aprotinin 6 KDa, Insulin B chain 3 KDa)

The Fibrinogen levels in the Group of rabbits before and after the injection of saline solution had an average of 299.8 +/-1.8 mg/dl (Figure 3).

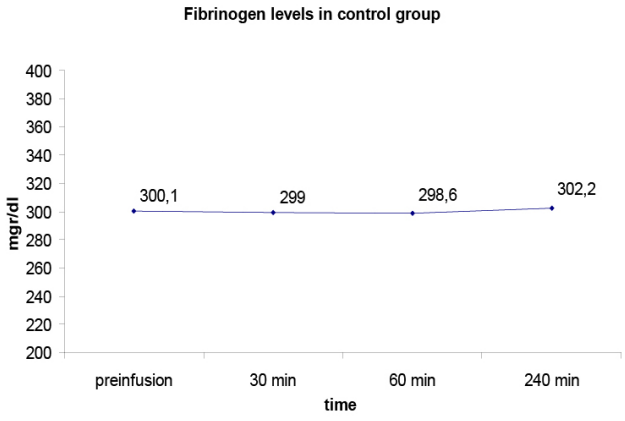

Figure 3: Levels of fibrinogen in blood after saline solution infusion in control rabbits.

The values of Fibrinogen in the group before and after the injection of modified plasmin had an average of 294.7 +/-1.04 mg/dl (Figure 4).

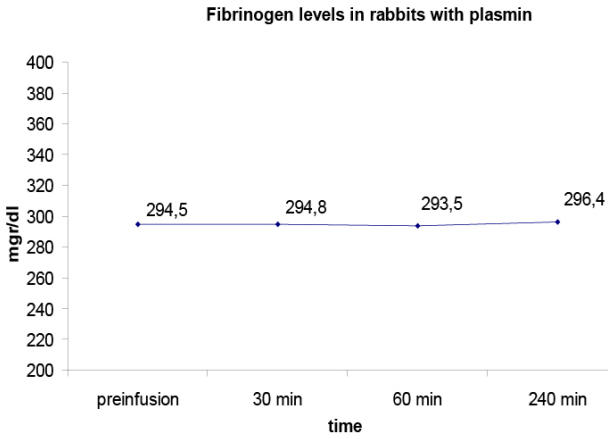

Figure 4: Fibrinogen levels in blood after modified plasmin infusion in rabbits

Studying correlation (Chi square, with 0.95 of statistical significance) between the two groups of rabbits and their values of Fibrinogen before and after infusion of saline and modified plasmin, we found that before the administration of saline control group values were 300 mg/dl and after subsequent media administration was 299.7 mg/dl average. In the group treated with modified plasmin value before the injection 294.5 mg/dl and the subsequent to its administration of 294.9 mg/dl ($p > 0.97$) (Table 2)

	Before Injection	After Injection		
		30 min	60 min	240 min
Control group	300,1 +/- 27.5 mg/dl	299 +/-26.1 mg/dl	298,6 +/-19.4 mg/dl	302,2 +/-20.4 mg/dl
Plasmin group	294,5 +/- 19.3 mg/dl	294,8 +/- 22.1 mg/dl	293,5 +/- 14.9 mg/dl	296,4 +/- 14.8 mg/dl

Table 2: Fibrinogen levels (mg/dl) before and after to injected saline solution and 3 mg/kg of modified human plasmin in rabbits.

Discussion

ELISA determination was important to confirmation that we were working with plasmin. So when it was positive subsequent studies were performed

Since the advent of thrombolytic agents in the treatment of diseases such as stroke and myocardial infarction, there is a consensus that has improved the results in terms of recanalization and after reperfusion of the ischemic areas. The development of new agents as plasmin and its derivatives made levels of security in terms of bleeding as severe adverse effect to increase [16].

At the same time, however, t-PA can dissolve haemostatic plugs at sites of vascular injury, and an unavoidable bleeding risk results. In contrast, plasmin administered systemically is rapidly (within seconds) neutralized by α2-antiplasmin, and does not dissolve thrombus, but, by the same token, systemic plasmin does not induce bleeding [17]. In our study modified plasmin isolated and purified of human plasma

was injected in rabbits in order to 3 mg/Kg of weight and the levels of fibrinogen doesn't suffer modification. Recent studies and investigations on thrombolytic drugs, have informed that can get plasmin without the activation of plasminogen by plasminogen activators. This requires cutting joints peptide stages by mechanisms such as changes in pH, ionic strength and specific chromatographic processes to obtain different forms of plasmin as mini plasmin, micro plasmin, delta plasmin [18]. In the case of our modified plasmin was obtained by sequential in different conditions of pH, specific ionic forces, resulting in a heavy chain of 56 Kdal with 4 Kringles and two chains of light molecular weight from 30 and 28 Kdal. These chains will be therapeutic properties as the non-modification of the levels of Fibrinogen in blood. When Plasmin is treated in a medium of pH close to 11, occurs autocatalysis losing the 5 kringles, by a rupture of the Arg530-Lys531 peptide bond, generating micro plasmin which can not bind to fibrin and is slowly inhibited by alpha 2 antiplasmin and by alpha 2 macroglobulin [19,20].

At respect using different steps and Catalysis conditions of purification, the retrieved cut is in Lys453-Cys454, which is different from those obtained previously. This cut generates a separation of the Kringle 5 that results in less-binding inhibitors of the plasmin and less adverse effects on fibrinogen, without losing its characteristic fibrin specific. Currently the development of these thrombolytic drugs is still in discussion, not only by the economic costs of producing them, but also by the relative and not absolute effectiveness of them. For many years all variants of fibrinolytic could not been able to reduce the adverse effects as bleeding by reduction in blood of clotting factors, including fibrinogen, and less accessibility to down social people. This modified plasmin has the advantage that can be administered many times as necessary, without bleeding complications since results showed that levels of fibrinogen are kept in similar values before and after a dose of 3 mg/Kg infusion and for its low cost of production too.

Conclusion

The preparation of modified plasmin was developed by autocatalytic treatment, which being a truncated derivative of plasmin breaking Lys453-Cys454 sites, increasing affinity for fibrin and inhibited lesser by a2-antiplasmin.

"The author(s) declare(s) that there is no conflict of interests regarding the publication of this article."

References:

1. Collen D (1980) On the regulation and control of fibrinolysis. Edward Kowalski Memorial Lecture. Thromb Haemost 43: 77-89.
2. Violand BN, Castellino FJ (1976) Mechanism of the urokinase-catalyzed activation of human plasminogen. J Biol Chem 251: 3906-3912.
3. Gately S, Twardowski P, Stack MS, Cundiff DL, Grella D, et al. (1997) The mechanism of cancer-mediated conversion of plasminogen to the angiogenesis inhibitor angiostatin. Proc Natl Acad Sci U S A 94: 10868-10872.
4. Zheng H, Chen Y, Ao W, Shen Y, Chen XW, et al. (2009) Antiphospholipid antibody profiles in lupus nephritis with glomerular microthrombosis: a prospective study of 124 cases. Arthritis Res Ther 11: R93.
5. Robbins KC (1987) Hemostasis and Thrombosis (2nd edtn) JP Lippincott Co., Philadelphia, USA.
6. Collen D (1986) Blood Coagulation, Elsevier, New York, USA.
7. Comerota AJ, Paolini D (2007) Treatment of acute iliofemoral deep venous thrombosis: a strategy of thrombus removal. Eur J Vasc Endovasc Surg 33: 351-360.
8. Ambrus JL, Ambrus CM, Back N, Sokal JE, Collins GL (1957) Clinical and experimental studies on fibrinolytic enzymes. Ann N Y Acad Sci 68: 97-136.
9. Douglas AS, Mcnicol GP (1964) Thrombolytic Therapy. Br Med Bull 20: 228-232.
10. Novokhatny V (2008) Structure and activity of plasmin and other direct thrombolytic agents. Thromb Res 122 Suppl 3: S3-8.
11. Swenson S, Markland FS Jr (2005) Snake venom fibrin(ogen)olytic enzymes. Toxicon 45: 1021-1039.
12. Gore JM, Sloan M, Price TR, Randall AM, Bovill E, et al. (1991) Intracerebral hemorrhage, cerebral infarction, and subdural hematoma after acute myocardial infarction and thrombolytic therapy in the Thrombolysis in Myocardial Infarction Study. Thrombolysis in Myocardial Infarction, Phase II, pilot and clinical trial. Circulation 83: 448-459.
13. Skeik N, Gits CC, Ehrenwald E, Cragg AH (2013) Fibrinogen level as a surrogate for the outcome of thrombolytic therapy using tissue plasminogen activator for acute lower extremity intravascular thrombosis. Vasc Endovascular Surg 47: 519-523.
14. Matosevic B, Knoflach M, Werner P, Pechlaner R, Zangerle A, et al. (2013) Fibrinogen degradation coagulopathy and bleeding complications after stroke thrombolysis. Neurology 80: 1216-1224.
15. Marder VJ, Manyak S, Gruber T, Goyal A, Moreno G, et al. (2010) Haemostatic safety of a unique recombinant plasmin molecule lacking kringles 2-5. Thromb Haemost 104: 780-787.
16. Lewandowski C1, Barsan W (2001) Treatment of acute ischemic stroke. Ann Emerg Med 37: 202-216.
17. Wiman B, Lijnen HR, Collen D (1979) On the specific interaction between the lysine-binding sites in plasmin and complementary sites in alpha2-antiplasmin and in fibrinogen. Biochim Biophys Acta 579: 142-154.
18. Marder VJ, Novokhatny V (2010) Direct fibrinolytic agents: biochemical attributes, preclinical foundation and clinical potential. J Thromb Haemost 8: 433-444.
19. Wang J, Brdar B, Reich E (1995) Structure and function of microplasminogen: I. Methionine shuffling, chemical proteolysis, and proenzyme activation. Protein Sci 4: 1758-1767.
20. Ma Z, Lu W, Wu S, Chen J, Sun Z, et al. (2007) Expression and characterization of recombinant human micro-plasminogen. Biotechnol Lett 29: 517-523.