Original Article

Synergism between Collagen — Adenosine Diphosphate and Collagen — Epinephrine in Platelets' Aggregation: Different dose response relationships

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Abstract

Objectives: To evaluate the possible synergistic interaction of collagen - adenosine diphosphate and collagen - epinephrine in aggregation of human platelets.

Methods: An experimental study was carried out at Armed Forces Instiute of Pathology, Rawalpindi, Pakistan, from June 2001 to December 2002. The platelet aggregation was determined by means of turbidometric method, which measures changes in optical density of platelet suspension. After determining the sub-threshold values of each agonist with the help of dose-response curve, these agonists were added in pairs to determine the synergism between them.

Results: The differences between means of threshold and sub-threshold concentrations of agonists were significant (Collagen: P <0.001, ADP: P <0.001, Epinephrine: P <0.002). The responses of Collagen and Epinephrine in sub-threshold concentrations were synergistic in causing platelet aggregation, whereas there were no potentiating effects in response to that of Collagen and Adenosine diphosphate.

Conclusion: The study reveals the synergistic potentiation of some of the agonists in circulation that might be responsible for the activated state of platelets and associated atherosclerotic complications (JPMA 59:368; 2009).

Introduction

Platelets play an essential role in haemostasis through a discrete series of steps involving platelet adhesion to the wounded area and platelet activation. The platelets then undergo rapid morphologic changes from normal discoid shape to spiny sphere with long thin filopodia, extending several micrometers out from the platelets and ending in points.[1] They also release the contents of their granules during activation. These factors stimulate the proliferation of smooth muscle cells in intima of arteries, promote the migration of fibroblasts from media to intima of the vessel wall, and aggregate the activated platelets to one another.[2]

In addition to their normal role, platelets play a central role in causing myocardial infarction, stroke, and other thrombotic disorders that are responsible for most of the deaths.[3] Recent studies have revealed a definite role of platelets in sheer stressed induced platelet aggregation.[4]

In conjunction with these, the cells often encounter agonists in the microenvironment that trigger platelet secretion and aggregation. Of particular physiological importance are platelet derived agonists (Adenosine diphosphate, serotonin, platelet activating factor, arachidonate metabolites), collagen present in subendothelium, thrombin, and circulating epinephrine.[5,6] These agonists differ in their intrinsic ability to produce the effects on physiological responses by the platelet.[7]

Platelet activation induced by multiple agonists simultaneously is not additive. Infact, two platelet agonists, together potentiate the effect of each other.[8] There have studies reporting increased platelet aggregation and increased response to agonists stimulation in vitro,[9] platelet activation in vivo probably involves a combination of agonists, with perhaps collagen more important at the beginning, thrombin more important later on, and with the other agonists in varying mixture throughout.[5]

This study was conducted to evaluate the possible synergistic interaction of subthreshold concentrations of collagen — adenosine diphosphate and collagen — epinephrine in aggregation of human platelets.

Subjects and Methods

The experimental study was approved by the ethics committee of Army Medical College, Rawalpindi, Pakistan and carried out at Armed Forces Institute of Pathology, Rawalpindi, Pakistan, from June 2001 to December 2002. Informed consent was obtained from all volunteers prior to their blood donations. 120 samples of platelets were isolated from blood samples of healthy non-obese, non-smoker, non-hypertensive and non-diabetic volunteers. These volunteers were in age group of 20-50 years and had not taken medications that interfere with platelet functions (Aspirin, Indomethacin, Promethazine, Amitriptyline) for 2 weeks prior to the study.

Subjects were evaluated by taking history, performing examination and laboratory investigations like bleeding time,

platelet count, blood glucose, serum urea, serum creatinine and urine for glucose and proteins.

Fasting venous blood was drawn with minimal venous occlusion, to avoid stasis and contamination with tissue fluids. After anticoagulation with sodium citrate 3.8% in a ratio of blood to anticoagulant of, 9:1, the blood was centrifuged at 150 revolutions per minute to get platelet rich plasma (PRP), which was carefully removed with the help of micropipette. The remaining anticoagulated blood was recentrifuged at 4000 revolutions per minute to get platelet poor plasma (PPP). A platelet count was performed on PRP and adjusted to 350,000 per microliter ± 50,000 with the help of PPP as needed.[10]

Platelet aggregometer and the chart recorder were switched on to warm up the heater block. 500 µL of PPP in a glass cuvette was placed in a well marked 'PPP' that represents 100% platelet aggregation and 450 µL of PRP in another glass cuvette was placed in a well labeled 'PRP' after adding magnetic stirring bar.

The platelet aggregation was determined by using a platelet aggregometer of Chronolog corporation - USA (Model no. 540), which works on turbidometric method originally developed by Born,[11] and the change in light transmittance was recorded on Omniscribe chart recorder, attached to the aggregometer.

Collagen, Adenosine diphosphate and Epinephrine (Sigma Diagnostic) were used in the study to determine the agonists induced platelet aggregation. Collagen used in concentrations of 0.5, 1.0, 2.0, 3.0, 4.0, 5.0 µg/ml, Adenosine diphosphate in concentrations of 0.9, 1.0, 1.5, 2.0, 3.0, 4.0 µmol/L and Epinephrine in concentrations of 0.1, 0.2, 0.3, 0.4, 0.5, 1.0 µmol/L. The aggregation response was recorded by adding 50 µL of each of the aggregating reagents to each cuvette containing 450 µL of PRP, and interpreted as intensity of aggregation using the technique explained by Roper et al.[12]

Statistical Analysis:

Observed differences between the means of threshold and subthreshold concentrations of agonists were statistically determined by using Student's t-test (unpaired). The difference between the two groups was considered significant when P value was <0.05.

Results

The blood samples were taken from randomly selected healthy non-smoker volunteers of 20-50 years of age (Mean = 30.50 ± 6.06). Mean platelet count 276,333.33 ± 51,406.87/cmm and mean bleeding time was 1.41 ± 0.37 minute. With the help of dose-response curve, the sub-threshold concentration of collagen was found to be 3 µg/ml, with P = 0.001 (5 g/ml-51%, 4 g/ml-42%, 3 g/ml-4%, 2 g/ml-2%, 1 g/ml-1%).

Similarly, sub-threshold concentration of epinephrine was found to be 0.4 µmol/L with P = 0.001 (1 mol/L-59%, 0.5 mol/L-50%, 0.4 mol/L-23%, 0.3 mol/L-10%, 0.2 mol/L-6%, 0.1 mol/L-3%), and that of adenosine diphosphate was found to be 1.5 µmol/L with P = 0.002 (4 mol/L-78%, 3 mol/L-63%, 2 mol/L-44%, 1.5 mol/L-9%, 1 mol/L-4%). The concentrations of agonists were the final concentrations when added to plasma.

The sub-threshold concentrations of collagen and epinephrine when added together, the dose-response curve showed the pattern of platelet aggregation in a dose dependant manner. As shown in Figure-1, dose response curve was obtained by keeping the collagen dose constant in sub-threshold concentration, i.e., 3 g/ml, and decreasing the sub-threshold concentrations of epinephrine. The result was reconfirmed by keeping the epinephrine concentration constant at sub-threshold value, i.e., 0.4 mol/L, and decreasing the sub-threshold concentrations of collagen, as shown in Figure-2.

Where as, when sub-threshold concentration of collagen (3 g/ml) was added with decreasing sub-threshold concentrations of ADP, the platelet aggregometer showed 5% of platelet aggregation which was less than the optimal platelet aggregation, i.e., the dose response curve showed no platelet aggregation. Similar result was obtained when reconfirmed by keeping the sub-threshold concentration of ADP (1.5 mol/L) constant and decreasing the sub-threshold values of collagen, as shown in Figure-3.

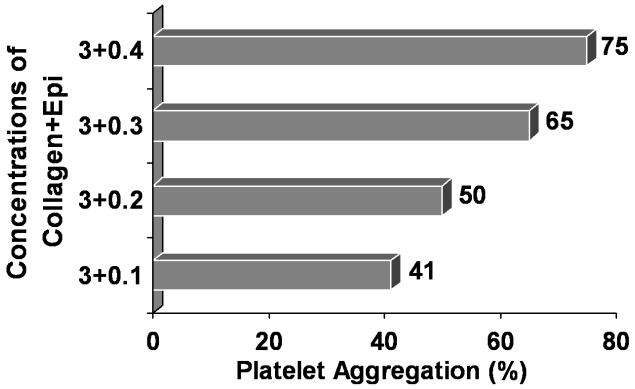

Figure 1: Platelet aggregation by sub-threshold concentration (3 g/ml) of collagen and sub-threshold concentrations (0.4, 0.3, 0.2 and 0.1 mol/L) of epinephrine.

Discussion

The natural agonist, collagen, is the most abundant protein of sub-endothelial matrix, which is also one of the most thrombogenic components of the atherosclerotic plaque.[13] It has multiple cell surface receptors, the best characterized receptors is an integrin Glycoprotein (GP) Ia/IIa ($\alpha 2\beta 1$). Another important one is GPVI and both of these are required for a full platelet response. GP IV, another platelet membrane

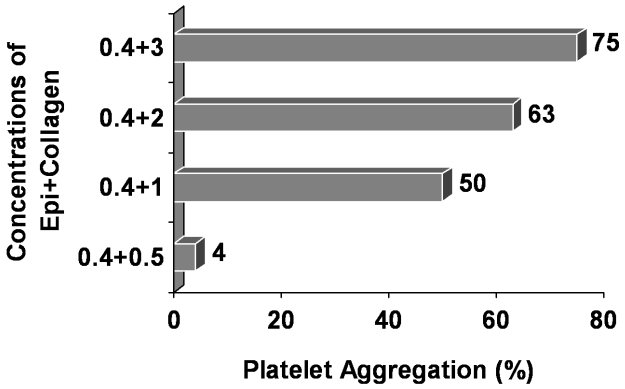

Figure 2: Platelet aggregation by sub-threshold concentration (0.4 mol/L) of epinephrine and sub-threshold concentrations (3, 2, 1 and 0.5 g/ml) of collagen.

glycoprotein, also has some stimulatory effect on this reaction.[14] The interaction of collagen with its receptors on platelets results in tyrosine phosphorylation and activation of phospholipase C (PLC) γ2. This enzyme in turn catalyzed the cleavage of phosphatidylinositol 4,5 diphophate (PIP2) into inositol triphosphate (IP3), which causes Ca^{2+} release in the

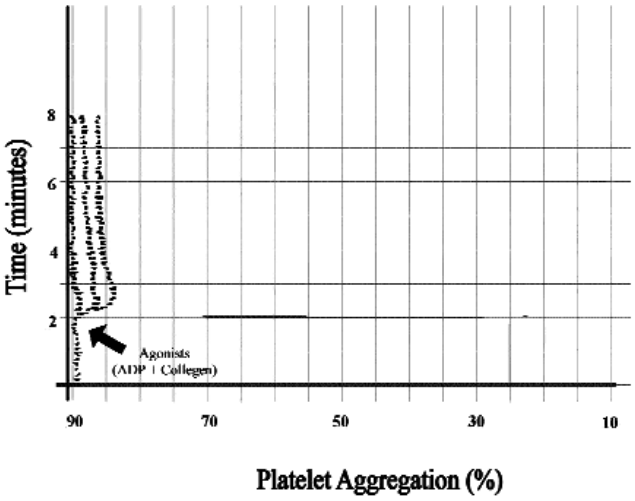

Figure 3: Platelet aggregation by sub-threshold concentration (1.5 mol/L) of ADP and sub-threshold concentrations (3, 2 and 1 g/L) of Collagen.

platelet and diacylglycerol (DAG), which activates protein kinase C (PKC).[15]

Epinephrine acts via seven-transmembrane, G-protein-coupled α2 adrenergic receptors, that activate Gα1 which in turn inhibits adenylyl cyclase and thus prevent the formation of cyclic adenosine monophosphate (cAMP). However, this alone is unlikely to be sufficient to mediate platelet aggregation, but might be sufficient to amplify the activation induced by other agonists.[10]

ADP is another natural agonist present in abundance in platelet dense granules. It is released in response to collagen and other agonists and amplifying the responses of them for platelet aggregation.[16] It has at least three purinergic receptors. Two of these, P2Y1 and P2TAC, are seven transmembrane G-protein-coupled receptors, responsible for most of the physiologic effects of ADP. P2Y1 activates Gαq which in turn activates PLCβ, whereas P2TAC are linked to the same intracellular pathway as epinephrine receptors. P2α1, third recptor of ADP is a ligand gated ion channel that cause rapid influx of calcium on its activation.[17] The synergism between the pairs of these agonsists occurs either at platelet surface receptors, or at intracellular signal processing pathway as proposed by Moroi and Brocchieri.[18,19]

The synergistic interaction in causing platelet aggregation between collagen, epinephrine and ADP were determined in the study by using dose response relationships. The true synergism was demonstrated by a parallel shift to the right of dose-response curve to one agonist in the presence of a sub-threshold concentration of the other.[20]

The concentrations of each agonist just below the threshold for aggregation was determined and it is reffered to as "sub-threshold'. The subthreshold value for collagen was found to be slightly higher than the established value, where as, the subthreshold value for epinephrine is consistent with the Grant et al.[8] It is also near to the findings of Venags et al[20] and Butt et al.[21] The subthreshold concentration of ADP is also comparable to the findings of Venags et al.[20]

Significant synergistic interaction is observed in the study by collagen in combination with epinephrine when added simultaneously in low concentration. This result is also in agreement with the result of Huang and Detwiler, who demonstrated the potentiated response to platelet aggregation in combination with collagen and epinephrine, and that the pattern of responses were intermediate between the typical for either agonist alone when neither agonist was in relatively higher concentration.[5]

On the other hand, a pair of collagen and ADP did not show the synergistic effect when one agonist at a concentration just below its threshold for induction of aggregation was added with second agonist at a concentration that ranged from far below the threshold to just below the threshold. This sequence was also reversed and even then it was found that no synergism existed between the aggregating effects of the two. Nunn's study also showed similar results and concluded that the collagen can aggregate platelets unresponsive to high concentrations of exogenous ADP. Also, ADP did not play an essential role in collagen induced aggregation.[22] On the other hand, Philippe Ohlmann et al studied the same agonists too and stated that ADP plays a key role in haemostasis as it stimulates

platetelet aggregation and potentiates the aggregation response induced by other agents, including collagen.[23] Carter and Heptinstall also stated the synergistic interaction of ADP with collagen in a dose dependant manner but whole blood was used rather than PRP.[24]

In conclusions this study brings to light the synergistic potentiation of some of the agonists in circulation in subthreshold concentrations. This agonist synergism may be responsible for the activated state of platelets and associated atherosclerotic complications as observed in different pathological states. However, the biochemical basis of these synergistic responses have not been investigated here and it needs further exploration.

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