

In vitro Research of the Concentration Dependence of Effect of Adrenaline on Platelets Aggregation

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ABSTRACT: Purpose. Paper intended to present experimental evidences that adrenaline has a direct effect of inducing platelets aggregation in the concentration range 1-8 μ M. Material/ Methods. Platelet rich plasma from patients of Colentina Clinical Hospital, following an informed consent. The platelet rich plasma (PRP) was prepared by centrifuging the anticoagulated sample at 200 G for 10 minutes. Aggregation was evaluated by optical aggregometry, classical method of Born, using Helena PACKS-4 Aggregometer. Results. The curves transmission light-time followed the structure: a lag-time, a first phase aggregation, more or less linear, defined by a "Slope 1", a second wave of aggregation defined by "slope 2" and a "saturation" phase. Slope 1 increases with the concentration of adrenaline. The second slopes of the aggregation curves, maximum aggregation and areas under curves depended linear on adrenaline concentration. Conclusions. Adrenaline, in concentrations in the 1-8 μ M, induce aggregation of human platelets from platelet rich plasma. Linear regression models for slope and area were practically identical suggesting a rather unique than biphasic mechanism of action of adrenaline during the time course of aggregation.

KEYWORDS: Adrenaline, Platelet Aggregation, DLVO

Introduction

Platelets aggregate at sites of vascular lesion, leading to the formation of thrombi that stop bleedings but also might occlude atherosclerotic arteries, resulting in reduced blood flow and being strongly linked with cardiac and cerebrovascular diseases. Platelet aggregation is a fundamental mechanism for life saving from blood loss and alterations of its normal course and it is implicated in all cardiovascular diseases, mainly after stroke or transient ischaemic accidents [1,2].

Platelet aggregation, the process by which platelets adhere to each other in contact with denudated sub-endothelial tissue, has been considered a key factor in the physiopathological chain of hemostatic plug formation and thrombosis [3-7].

Several excellent reviews discuss the role of platelets in haemostasis and thrombosis [8-11].

Excessive accumulation of platelets at sites of atherosclerotic plaque ulcerations is one of the main events accelerating arterial thrombus formation, and resulting in acute myocardial infarction, ischemic stroke and sudden death [12]. Since thrombosis leads to a higher morbidity and mortality than any other pathology, the platelets are a major target for therapeutic intervention. Many factors contribute to the platelet-activating properties of ulcerated

atheromatous plaques, including its high concentration of fibrillar collagens [13-15], the presence of tissue factor [16], as well as the direct platelet-activating effects of high shear stress caused by arterial narrowing [17-20].

Many epidemiologic studies have demonstrated that coronary atherosclerosis and acute myocardial infarction occur more frequently in men who are subject to acute and recurrent stress [21-23]. Such individuals have been found to produce high levels of sympathetic catecholamines [24].

In vitro a number of investigators have found that sympathetic catecholamines will increase the "stickiness" of platelets and will cause platelet aggregation [25-27]. A number of investigators have postulated that the precipitating event in acute myocardial infarction may be the formation of a platelet thrombus intravascularly, that forms at or travels to, and occludes a segment of a coronary artery already narrowed by atherosclerosis [28-29].

Not much progress has been made in the understanding of platelet aggregation until the development of the platelet aggregometer in 1962 by Born [30] and independently by O'Brien [31], which measures platelet aggregation as a function of optical density of platelet suspension, as determined by means of a turbidometric method.

After this, optical aggregometry has been used worldwide in fundamental, clinical and epidemiological applications [32]. It was shown that adrenaline stimulated a cyclooxygenase independent pathway resulting in potentiation of platelet aggregation. Contrary to other reports, adrenaline also stimulated the cyclooxygenase pathway to an extent sufficient to generate TXB₂ when platelets were under the inhibitory influence of acetylsalicylic acid [33].

Other studies suggest that adrenaline does not modify platelet membrane fluidity, as studied with the lipophilic fluorescent probe trimethylammonium-diphenylhexatriene. It has no direct effect on fibrinogen binding to intact platelets, intracellular Ca²⁺ levels measured by quin2, or protein phosphorylation. Adrenaline potentiates the action of all types of aggregating agents on aggregation, secretion, intracellular Ca²⁺ levels, membrane fluidity, fibrinogen binding, or protein phosphorylation. These effects are mediated by alpha 2-adrenergic agonists and inhibited by alpha 2-adrenergic antagonists. This study shows that adrenaline alone does not induce modifications of morphology, metabolism, or function of intact and functional washed human platelets and that it cannot be considered per se as an aggregating agent. However, adrenaline interacts with alpha 2-adrenergic receptors on human platelets and potentiates biochemical and aggregatory responses induced by other platelet agonists [34].

The aim of this study was to determine the dependence between the concentration of adrenaline and the extent of the platelet aggregation process.

Material and Methods

Reagents

Adrenaline reagent: was purchased from Helena Laboratories and was of analytical grade.

Preparation for use: it was prepared a stock solution by reconstituting one vial with 1.0mL of distilled water. It was stirred gently until completely dissolved. After reconstitution it was obtained a stock solution of adrenaline bitartrate 3mM.

Storage and stability: The Adrenaline Reagent was stored in dry form at 2 to 8⁰ C and was stable until the expiration date on the vial. The reconstituted reagent was stable for 1 week at 2-8⁰ C.

Signs of deterioration: when dry, unreconstituted reagent was not uniformly white in appearance, it was not been used.

Specimen Collection and Handling

Specimen. Plasma obtained from whole blood collected with 3.2% sodium citrate as an anticoagulant was the *specimen of choice*.

Specimen collection. The blood was purchased from patients from internal medicine ward of Colentina Clinical Hospital, who gave informed consent before participating in this study. Blood was collected in evacuated test tubes.

Specimen preparation

1. The platelet rich plasma (PRP) was prepared by centrifuging the anticoagulated sample at 200 G for 10 minutes. The PRP was removed from the cells with a plastic Pasteur pipette and placed in a plastic tube labelled "PRP". The tube was capped and maintained at room temperature. 30 minutes after PRP was removed the test begun.

2. The platelet poor plasma (PPP) was prepared by centrifuging the remaining blood samples at 3000 G for 15 minutes at room temperature. The PPP was removed, placed in a plastic tube, labelled PPP and covered. It was stored at room temperature.

Storage and stability

Plasma as well as whole blood was stored at room temperature (15-25⁰ C). The samples were covered to maintain the pH. Test were performed within maximum three hours after sample collection.

Method

Platelet aggregation test was performed using Born turbidimetric light transmission method [30] using Helena PACKS-4 Aggregometer. The initial absorbance is caused by light scattered by the floating platelets in the solution. This absorbance is nearly proportional to the number of platelets. Platelet poor plasma (PPP) made from the same sample simulated 100% aggregation. Absorbance caused by factors other than platelets was determined by measuring the absorbance of PPP.

The aggregation capacity of the platelets was determined by the amount of aggregation induced when a known amount of reagent is added to PRP. The absorbance of the unreacted PRP mixed with the aggregation reagent represented 0% aggregation and the absorbance of the PPP control represented 100% aggregation (no floating platelets). As platelets aggregated, the number of floating platelets decreased, reducing the light absorbed by the PRP. It was measured human platelet

aggregation by the absorbance method using up to four channels simultaneously. The absorbance curve for each channel was displayed during data acquisition. The absorbance data was displayed and stored at the conclusions of the measurements, and was printed for permanent records.

The employed Helena PACKS-4 Aggregometer and the tests were done at 1000rpm. Patient results were compared to normal ranges run under the same conditions.

The following steps were employed:

1. The blood specimens were collected and prepared according to directions in "Specimen Collection and Handling" section.

2. The aggregation agents were reconstituted according to the directions in "Reagent" section.

3. The Adrenaline Reagent used was obtained by diluting the 3 mM stock solution with saline solution (0.9% NaCl). Final concentrations in sample when using diluted reconstituted reagent: 1-8 μ M.

4. The employed aggregometer was prepared for use as recommended in the Operator's Manual.

5. A volume of 450 μ L PPP was pipetted in a cuvette. This was the blank used to set the 100% aggregation.

6. A volume of 450 μ L PRP was pipetted in a cuvette with a stir bar. The cuvette was incubated at 37⁰C for one to three minutes.

7. The PPP cuvette was inserted into the appropriate channel and the instrument was set to 100% aggregation.

8. The PRP cuvette was inserted into the appropriate channel.

9. A volume of 50 μ L of aggregating reagent dilutions were added to de PRP cuvette and the aggregation percent was recorded (instrument set 0% when aggregating agent is added and the channel activated).

Results and Discussions

First question addressed by the present paper was to evaluate the direct effect of adrenaline alone, not in association with other aggregants. Some authors considered that adrenaline does not induce modifications of morphology, metabolism, or function of intact and functional washed human platelets and that it cannot be considered per se as an aggregating agent [35].

It was reported for example the potentiation of adrenaline by ADP [36]. In this respect, our result was clear: adrenaline had a proagregant activity. This was an expected result since adrenaline was proved to have also an effect on

the sedimentation of erythrocytes, most probable following an effect on their aggregation [37].

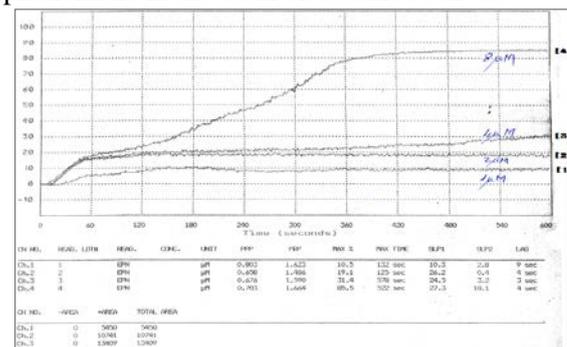
The second problem concerned concentrations required for obtaining aggregation. In a study on a large interval of adrenaline concentrations (0.25-16 μ M), was found that there are under-threshold concentrations of adrenaline (0.03-1 μ M) where antagregant effect appears only after association with collagen or serotonin [38]. At higher concentrations adrenaline alone is sufficient to promote aggregation. It is to underline that threshold is modified in different pathologic situations. For example it was found a decreased threshold of aggregation to low-dose adrenaline, which effect was interpreted as sign of platelet hyperaggregability in patients with thrombosis [39], mainly in sticky platelet syndrome (SPS), an autosomal dominant platelet disorder associated with arterial and venous thromboembolic events [40-45].

Concentrations used in this study were 1-8 μ mol/l which means concentrations greater than the threshold. So that it was expected a correlation of our results with previous reported results, for adrenaline or combinations of adrenaline with other antiaggregants at concentrations higher than 1 μ mol/l.

Form of curves.

The curves transmission light-time followed as a rule, the structure: a lag-time, a first phase aggregation, more or less linear, defined by a "slope 1", a second wave of aggregation, following the release of ADP from platelets and "enhancement" of aggregation, defined by "slope 2" and a "saturation" phase.

This is a possible standard behavior. As it can be seen for example in Fig. 1 these components are not clear separated or even are not present in some curves. The soft of aggregometer is identifying these phases in the curves and calculates associated parameters but the results have to be validated each time by visual inspections and considerations concerning phenomena in the back of obtained curves.



regression lines were practically parallel (slopes 3.86 and 3.69) which means a common mechanism in both linear and saturation parts of the sedimentation curves.

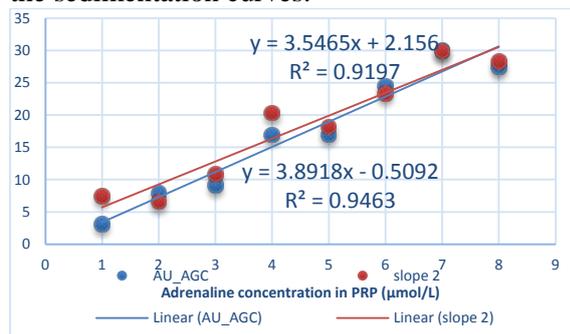


Fig. 4. Variation of AU-AGC and slope 2 as a function of adrenaline concentration in PRP, following normalization

Linear dependence on concentration was not reported as a characteristic until now but such result was obtained also in other studies though not observed by authors [48]. We represented the maximum platelet aggregation (MPA) estimated from curves obtained at different concentrations and obtained, in domain similar with our concentrations, an excellent linear regression.

On other hand a „saturation” or even a reverse of effect can appear with farther increasing of adrenaline concentration. If we consider that adrenaline influences the aggregation at the level of repulsion electric forces [37], the biphasic dependence [49] of platelet electrophoretic mobility response to ADP or noradrenaline have to lead finally to a significant change in the dependence of aggregation on adrenaline concentration.

Conclusions

Adrenaline, in concentrations in the 1-8µM, induce aggregation of human platelets from platelet rich plasma.

The second slopes of the aggregation curves and areas under curves depended linear on adrenaline concentration.

Linear regression models for slope and area were practically identical suggesting a rather unique than biphasic mechanism of action of adrenaline during the time course of aggregation.

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