ADP RELEASE AND THROMBOXANE A2 GENERATION FROM WHOLE BLOOD UNDER LOW-SHEAR

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ABSTRACT

This is an experimental study which was designed to study the relation between adenosine diphosphate (ADP) shear-induced release and thromboxane A2 generation. Their effects on platelet aggregation and adhesion were also studied. The results obtained suggest that acetylsalicylic acid (ASA) completely inhibits shear-induced thromboxane A2 generation when treated in-vitro, while it does not inhibit its generation from platelets when treated in-vivo. ADP inhibition does not affect shear-induced thromboxane A2 generation levels, which suggests that there is no correlation between shear-induced ADP release and thromboxane A2 generation, however similar mechanism of ADP and AA shear-induced release can be suggested. ADP is shown to be the major platelet agonist in shear-induced platelet aggregation and adhesion phenomena. Moreover, no synergism seems to exist between ADP and Thromboxane A2 shear-induced platelet aggregation and adhesion.

Key words: Shear-induced release, Shear-induced generation, Adenosine diphosphate, Thromboxane, Single platelet reduction.

INTRODUCTION

Blood usually undergoes hydrodynamic stresses when it flows through artificial blood pumping equipment such as blood oxygenators and blood dialysis devices [1]. It was indicated by Rajagopalan *et al.* 1] that physical forces may indicate different pathways for eicosanoid metabolism than most commonly used stimuli.

Substantial evidence has been found to suggest that chemicals, such as adenosine diphosphate (ADP), which are released from red blood cells (RBCs) exposed to low shear stress (below 200 dynes/cm^2) potentiate platelet adhesion to other platelets (aggregation) and to artificial surfaces [2-9]. Moreover, it has been reported that RBCs, by a physical mechanism, catalyze platelet aggregation and adhesion through augmentation of platelet convective diffusion and thereby increases the likelihood of platelet-surface interaction [10].

Thromboxane A2 is a chemical produced as a result of arachidonic acid (AA) release from platelets and its consequent metabolism by an enzymatic pathway. It is considered as a potent stimulator of platelet aggregation [11-16]. AA also exists within the membranes of RBCs and undergoes release when shear fields are imposed. Moreover, shear-induced effects of thromboxane A2 stimulator are not completely understood, and even some controversy exists on whether it contributes to platelet aggregation or not [10,17,18]. Furthermore, it is not completely understood whether thromboxane A2, generated as a result of imposing shear fields, acts as a direct aggregating agent or whether its synergistic interaction with platelets culminates in the release of intracellular ADP followed by platelet aggregation and adhesion.

This study was planned to investigate the relation between shear-induced ADP released and shear-induced thromboxane A2 generated. Moreover, the effect of those two

platelet stimulators on single platelet reduction through aggregation and adhesion was also planned to be examined. Finally, the possibility of a correlation existence between ADP and thromboxane A2 actions or release and formation was planned to be examined. Experimental procedure designed in this study depends on isolation of the action of the two different stimuli, through utilization of known inhibitors, such as apyrase (ADP scavenger), and acetylsalicylic acid (aspirin (ASA): thromboxane A2 formation inhibitor).

APPARATUS, MATERIALS AND METHODS

Apparatus

Blood samples were sheared for two minutes in a cone-and-plate compartment of a Weissenberg rheogoniometer model R16/R18 (Sangamo Weston Controls Ltd., Sussex, England) that was described elsewhere [2,10]. Physical parameters such as shear rate and shear stress as well as schematic diagram of the test compartment were also reported previously, as well as justification to why such a device was suitable for the application of shear levels employed [2,10].

Phase contrast microscope and hemacytometer (Fisher Scientific Company, Itasca – IL, U.S.A.) were used for microscopic studies and platelet reduction determination.

Materials

Blood: Two blood donor groups, presons 25 in each group were used, one group was not taking any medication for at least 14 days prior to donation, while the other group was taking ASA for one week prior to donation at a dosage of 500 mg/day. Both groups were of 25-40 years of age. The latter group was considered as an in-vivo testing for aspirin on whole blood (WHB) samples. A volume of 100 - 150 ml blood samples were withdrawn from every subject in tubes containing ACD/formula B (Fisher Scientific, Itasca, IL, U.S.A.).

Apyrase: Apyrase of 500 units/98 mg was obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). This material was used to inhibit the action of ADP on platelets. Activity of such scavenger on ADP involved neutralization of 1μmole of ADP with 5 units of apyrase used.

Acetylsalicylic acid: ASA was obtained from Sigma Chemical Company (St Louis, MO, U.S.A.) and was used for in-vitro studies.

Methods

Apyrase - WHB samples: 40 mg of apyrase were dissolved in 1 ml of isotonic buffer, and 0.2 ml of this solution was mixed with 6 ml of blood. This amount of apyrase was enough to neutralize ADP content of WHB, based on the above mentioned activity.

ASA-WHB samples: 1300 mg of ASA were dissolved in 10 ml of isotonic buffer and 0.2 ml of this solution was mixed with 6 ml of WHB for complete inhibition of thromboxane A2 formation.

Thromboxane B2 determination: a radioimmunoassay in a kit form (Amersham Corporation, Arlington Heights, IL, U.S.A.) was used for this purpose. Thromboxane B2 was used as an index for thromboxane A2 formation, because the latter is less stable since it is readily converted to its B2 stable form [10].

Single platelet reduction percent: Platelet count was determined for unsheared and sheared samples. The method involved using 0.02 ml sample which have been mixed with 1.98 ml of 1% ammonium oxalate. Platelets, then, were counted using phase - contrast microscope and hemacytometer. Single platelet reduction was determined by subtracting the sheared samples count from unsheared ones, and the percentages were recorded.

Experimental approach

The first set of experiments involved shearing of WHB samples treated with ASA in-vitro. The second set of experiments involved shearing WHB samples treated with apyrase in-vitro. The third set of experiments involved shearing of WHB samples treated in-vivo with ASA. Concentration of thromboxane B2 generated and single platelet reduction were recorded and analyzed for the purpose of answering questions related to the objectives of this study. The temperature at which all experiments were carried out was room temperature (21 ± 2) °C .

RESULTS AND DISCUSSIONS

The results obtained in this study indicate that ADP is the major, if not the sole shear-induced potentiator to platelet activation. Moreover, the connection between shear-induced ADP release and thromboxane A2 formation was also ruled out. The hypothesis that suggests existence of synergism between ADP and thromboxane A2 actions seems to be invalid and their actions on platelets, as platelet agonist, seem to be independent of each other.

Table 1 shows data on shear-induced thromboxane B2 (index of thromboxane A2 formation) generation from samples of WHB treated with aspirin (ASA) in-vitro. Thromboxane B2 was not detected, which may indicate that ASA totally blocked formation of thromboxane A2 via inhibition of AA metabolism. Moreover, visual observation of the plasma portion of sheared samples indicated darker pink color for samples treated with aspirin than non-treated samples due to more of shear-induced hemoglobin release. Furthermore, microscopic observation to RBCs indicated a shape change which supports possible change of the nature of their membranes.

Table 1 Thromboxane B2 generation from whole blood samples in-vitro

Shear Rate (s^{-1})	Thromboxane B2 Concentration (ng/100 ml)
0	0
542	0
2712	0
5424	0

Figure 1 presents data on thromboxane B2 shear-induced generation from WHB samples that were treated with apyrase (ADP scavenger) in-vitro. At the lowest shear rate (542 s^{-1}) the value is 1.51 ng/100 ml, which is lower than similar data obtained without treatment with apyrase, which was about 4 ng/100 ml. However, at higher shear rate levels, concentrations of samples with apyrase were significantly larger than those without apyrase treatment. At 2712 s^{-1} thromboxane A2 concentration was about 23 ng/100 ml and at 5424 s^{-1} it was about 38 ng/100 ml compared to 9 and 26 ng/100 ml for non treated samples at the same shear rates respectively. This may indicate that apyrase affected the membranes of RBCs and may increased rigidity of such membranes which resulted in a possible irreversible shear-induced damage at higher shear rate levels, and consequently increased AA release and hence increased thromboxane A2 formation. Moreover, hardening of RBCs (alteration of natural characteristics: elasticity) usually augment platelet diffusion substantially [2]. This fact is supported by visual observation of the color of plasma portion which indicated more of hemoglobin release. Hemoglobin molecules are larger than those molecules in consideration (ADP and AA) and the leakage of such large molecule is usually due to high degree of alteration to RBC membrane [19,20].

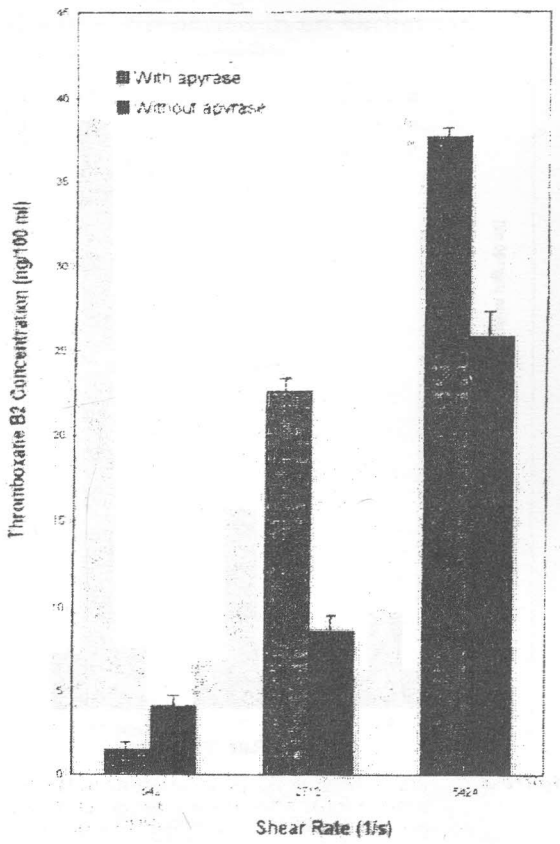

Figure 1 Thromboxane B2 generation from WHB samples with and without apyrase

Figure 2 represents data on thromboxane B2 generation from WHB samples with aspirin in-vivo. Data suggest that shear-induced AA release and consequently, thromboxane A2 generation were not inhibited completely. Dosage of ASA used (500 mg/day) may not be high enough for complete inhibition of AA metabolism. Concentration levels of thromboxane B2 generation were comparable to those from platelet rich plasma (PRP) samples in phosphate buffered saline (PBS). Therefore, possibility still exists to suggest that shear-induced thromboxane A2 generation originated from platelets is not inhibited by in-vivo treatment with ASA. This may suggest that platelet AA metabolism occurs within platelets membrane or in their vicinity before it is transported to the bulk. Moreover, it may suggest that AA released from RBCs is metabolized in the bulk where it is in this case inhibited by ASA.

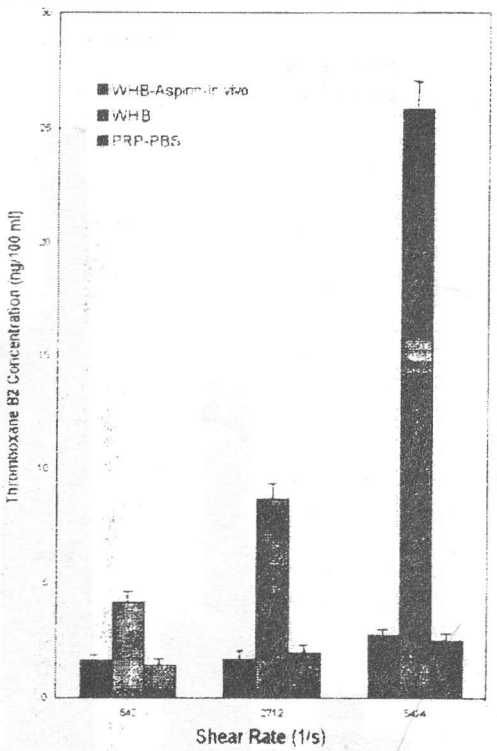

Figure 2 Thrombozane B2 generation from WHB treated with ASA in-vivo as compared to un treated WHB and PRP-PBS samples

Comparison between the data presented above (Table 1 and Figures 1 and 2) may suggest that there is no correlation between thromboxane A2 shear-induced formation and ADP shear-induced release. Both platelet agonists seem to be released or formed through different mechanism under the influence of low shear fields used in this study. However, since the same trend was seen earlier for ADP [2], therefore, thromboxane A2 generation, as a result of shear-induced AA release, may be suggested to correlate with ADP shear-induced release, or at least similar mechanisms, if not the same one, are responsible for the two phenomena. Therefore, only shear-induced release of both ADP and AA seems to be correlated but not with the formation of thromboxane A2.

Figure 3 shows single platelet reduction (SPR) for WHB samples that are free of any treatment and used as control for other experiments as compared to SPR from WHB treated with ASA in vivo. SPR was about 12% at the lower shear value (542 s^{-1}) and it was about 32% at the moderate level (2712 s^{-1}), while it was about 56% at the highest shear rate level (5424 s^{-1}). The average value for SPR over the whole shear rate range was about 33%. However, for in-vivo treatment with ASA, at the lowest shear rate value SPR was about 11% followed by 37% at the moderate shear rate and 53% at the highest. These values are not significantly different from those results associated with untreated WHB samples. The average value was about 34% which is very close to that for untreated samples (33%). In samples associated with in-vivo ASA treatment the action of thromboxane A2 was inhibited, therefore it is suggested that shear-induced thromboxane A2 generation does not affect or contribute to shear-induced single platelet reduction, or it can be said that it is not the potent platelet agonist in shear-induced platelet aggregation-adhesion phenomena.

Moreover, this result can be seen evident for other WHB samples treated with aspirin in-vitro. Not much of a difference is shown for such samples which are presented in Figure 4 for WHB treated with ASA in-vitro and untreated WHB samples. The average value of about 34% also obtained, which is the same as in-vivo treatment.

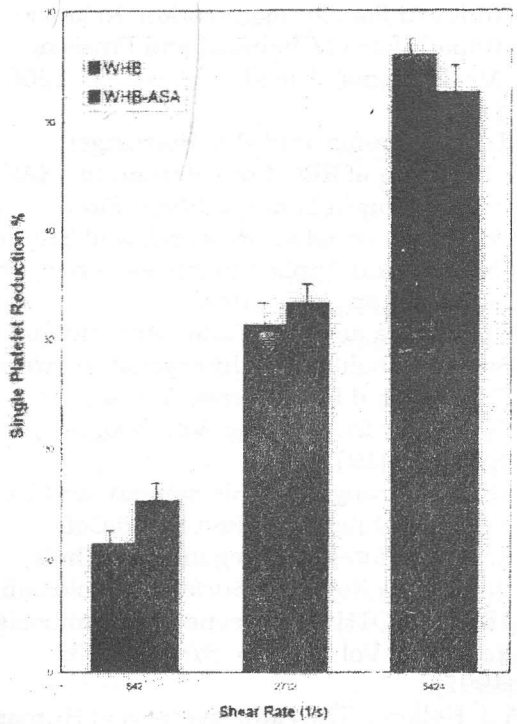

Figure 3 Single platelet reduction from WHB and WHB-ASA in-vivo

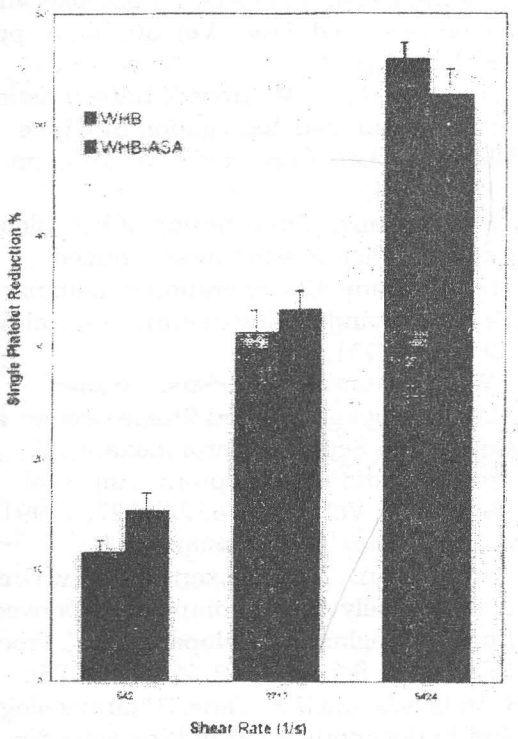

Figure 4 Single platelet reduction from WHB and WHB-ASA in-vitro

However, Figure 5 which represents SPR data for samples treated with apyrase (ADP scavenger) compared to untreated WHB samples, shows significant reduction in SPR values, associated with apyrase treatment. At the lower shear rate level (542 s^{-1}) SPR was about 4% and at the moderate shear rate (2712 s^{-1}) it was 7%, while at the highest shear rate level (5424 s^{-1}) it was about 10%. The average value for such samples was about 7% which was significantly lower than the average value for the untreated WHB samples (33%). In fact, about 80% decrease in SPR is observed when ADP is inhibited. Therefore, ADP can be considered as the major mediator for shear-induced single platelet reduction through adhesion and aggregation. The observed contribution in Figure 5, which is about 20% of the total was probably due to other platelet agonists such as thrombin which was reported in an earlier study [21].

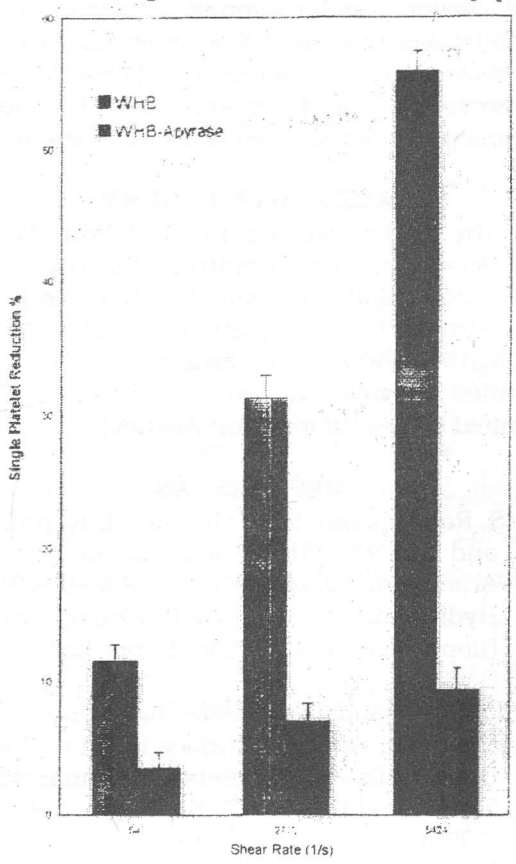

Figure 5 Single platelet reduction from WHB and WHB-apyrase

The actions of both thromboxane A2 and ADP in low shear fields seem to be not related and the possibility of synergism between their two mechanisms of action is not highly weighted. Therefore, future studies concerning the two agonists with respect to shear-induced platelet adhesion and aggregation are suggested to be separate. Correlations in this direction seem to be highly unlikely.

CONCLUSION

ASA is found to completely inhibit shear-induced thromboxane A2 generation when treated in-vitro, while it does not inhibit its generation from platelets when treated in-vivo. ADP inhibition does not affect shear-induced thromboxane A2 generation levels, which suggests that there is no correlation between shear-induced ADP release and thromboxane A2 generation, however a similar mechanism of ADP and AA shear-induced release can be suggested. ADP is shown to be the major platelet agonist in shear-induced platelet aggregation and adhesion phenomena. Moreover, no synergism seems to exist between ADP and Thromboxane A2 shear - induced platelet aggregation and adhesion.

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تحرير ادينوسين ثنائى الفوسفات وتكوين ثرمبوكسين أ٢ من الدم تحت ظروف اجهاد القص المنخفض

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ملخص البحث

صممت هذه الدراسة العملية لمعرفة العلاقة بين تحول أدينوسين ثنائي الفوسفات وتكوين ثرمبوكسين أ٢ بالإضافة إلى دراسة تأثيريهما على تجمع الصفائح الدموية والتصاقها . تشير النتائج إلى أن تكوين ثرمبوكسين أ٢ المحفز بالإجهاد يمنع كاملا عند معاملة الدم بالأسبرين بعد استخراج عينات الدم ولكنه لا يمنع كليا عند معاملة الدم بالأسبرين داخل الجسم وذلك بتناول الشخص المتبرع لتلك المادة . كما إن منع تحرير أدينوسين ثنائي الفوسفات لم يمنع تكوين ثرمبوكسين أ٢ المحفز بالإجهاد مما يدل على انه لا يوجد علاقة بين تحرير الأول وتكوين الثاني بينما يمكن أقتراح خطوات طريقة مشابهة للتحرير والتكوين تحت تأثير الاجهادات المنخفضة. وقد تم التحقق من أن أدينوسين ثنائي الفوسفات هو المسبب الرئيسي لتجمع الصفائح الدموية والتصاقها تحت تأثير الإجهاد ، كما تبين عدم وجود علاقة بين تأثير أي من المادتين على تجمع الصفائح الدموية والتصاقها تحت تأثير الإجهاد .