Chapter 1

The role of inhibition of nitric oxide synthesis in the aggregation of platelets due to the stimulated production of thromboxane A₂.
INTRODUCTION

The aggregation of platelets by different aggregating agents like ADP, l-epinephrine, collagen or thrombin is an essential life-saving physiologic event in the blood coagulation process [Furman et al., 1998]. Although, as mentioned here, there are several different aggregating agent as well as a recently discovered novel aggregating protein called dermcidin isoform 2 [Ghosh et al., 2011] all aggregating agents are however known so far to mediate their effect on platelet aggregation through ADP [Hamberg et al., 1975], suggesting thereby that this nucleotide might play the most critical role in the aggregation of platelets. Excessive platelet aggregation, on the other hand, has been reported to result in the development of ACS due to the aggregation of platelets on the site of atherosclerotic plaque rupture or fissuring on the coronary artery that may lead to the development of thrombus (a microaggregate of platelets embedded in fibrin mass) on the arterial wall that in consequence may result in the precipitation of the deadly condition due to the blockade of normal blood circulation in the heart muscles [Colman & Walsh, 1987; Fuster et al., 1996]. In the context of the development of ACS in human, the ADP induced platelet aggregation has been reported to be critically important [Chakroborty & Sinha, 2004]. And, as such, the ADP induced platelet aggregation can be considered to be important both in health and in disease.

The aggregation of platelets induced by ADP has been reported to be mediated through the activation of platelet COX [Hamberg et al., 1974] that catalysed the conversion of arachidonic acid to PGG$_2$, a potent inducer of platelet aggregation. PGG$_2$ subsequently converted to PGH$_2$ and finally to TXA$_2$ [Hamberg et al., 1974] which is a potent vasoconstrictor prostaglandin [Moncada & Vane, 1979]. In other words, the activation of COX leading to the synthesis of several prostaglandins that played a critically important role in the ADP induced platelet aggregation and to the genesis of ACS.

Under physiologic conditions, the excessive platelet aggregation, as described above, is counteracted by the inhibition of platelet aggregation by several humoral factors including prostacyclin [Whittle et al., 1978], insulin [Trovati et al., 1997] and interferon α [Bhattacharya et al., 2009]. While prostacyclin inhibits platelet aggregation through the increase of cAMP level [Dutta & Sinha, 1987], insulin and interferon α inhibits platelet aggregation by increasing the
cellular synthesis of NO, which is reported to increase both cAMP [Kanowitz, 1981] and cGMP level [Sinha, 1998] in platelets. However it has also been reported that NO was capable of inhibiting platelet aggregation through the lysis of intra platelets fibrinogen bridges which are reported to be essential for the aggregation of platelets. Furthermore it has also been reported that NO was capable of activating plasminogen to plasmin that dissolved intra platelet fibrinogen bridges [Bhattacharya et al., 2009] leading to disaggregation of the formed platelet aggregation without increasing either cAMP or cGMP [Acharya et al., 2001] level in platelets.

Taking all the above effects of NO on platelet aggregation, it might be concluded that NO synthesis in platelets played a critically important role in the inhibition of platelet aggregation. However, the role of ADP induced aggregation of platelets in the context of synthesis of NO in platelets remains obscure.

I herein report in Chapter 1 that ADP, the well known platelet aggregating agent that mediates its effect on platelet aggregation through the activation of COX [Hamberg et al., 1974], was also a potent inhibitor of NOS in platelets. I also report the consequences of the reduction of the basal NO levels in platelets.
RESULTS

ADP-induced platelet aggregation in PRP.

Incubation of PRP with different concentrations of ADP as shown in the Figure 21 resulted in the increased platelet aggregation with the increase of the concentrations of the aggregating agonist (from 0 to 8μM). It was found that even at 1.0μM ADP, the aggregation of platelets could be demonstrated. The extent of aggregation was gradually increased from 1.0μM to 8.0μM ADP. While the transmission of light at 1.0μM ADP was only 10%, at 8.0μM ADP the transmission of light was ≈90% (Figure 21). At 8.0μM ADP no distinction between the primary and secondary phases of the platelet aggregation could be made, at other concentrations of ADP, both the primary and secondary aggregation of platelets could however be seen.

![Figure 21: Aggregation of platelets in PRP induced by different concentrations of ADP](image)

PRP was prepared from the blood samples of normal female or male volunteers as described in Materials and Methods. The preparation of PRP was treated with different amounts of ADP as indicated in the Figure. The ADP induced aggregation at 37°C was carried for 5 min to achieve complete aggregation. The results shown here are typical of at least 10 other experiments using both male and female blood (M=5, F=5) from 10 different volunteers.

ADP-induced platelet aggregation and the synthesis of NO in platelets.

As discussed above, the ADP-induced platelet aggregation has been reported to be mediated by the activation of platelet membrane COX that catalysed the conversion of arachidonic acid to
prostaglandins [Hamberg et al., 1974], which in turn has been reported to aggregate platelets. Interestingly it was also found that the aggregation of platelets in PRP by ADP resulted in the reduction of basal platelet NO level in platelets (Figure 22). As described above, it was found that the increase of platelet aggregation by ADP was increased with the increased concentrations of ADP (0 to 8.0μM ADP) in the PRP (Figure 21). At 8.0μM ADP, the aggregation of platelet was maximally achieved where the first and second waves of platelet aggregation could not be identified and resulted in a uniform profile of aggregation as demonstrated by light transmission. When the basal NO levels in platelets after the completion of the ADP induced platelet aggregation (i.e. at 5 min) was determined, it was found that at all concentrations of ADP used to aggregate platelets, the basal NO level was found to decrease with the increase in the concentrations of the aggregating agonist, and at 8.0μM ADP, the basal platelet NO level in platelets was found to be reduced to 0nmol/10^8 platelets that contrasted 0.3 ± 0.10nmol/10^8 platelets (P< 0.0001; n=10) in the control experiment in the absence of added ADP in the PRP (Figure 22).

![Figure 22: Reduction of basal NO level in platelet in PRP incubated with different concentrations of ADP](image)

PRP was incubated with different amounts of ADP as indicated for 5min as described in Figure 21 and the NO level was determined as described in the Materials & Methods. Results shown here are mean ± S.D. of 6 different experiment each in triplicate using 5 different individuals (F=3, M=3).
Effect of ADP on the NOS activity of the cell free supernatant of the disrupted platelets.

As the above results demonstrated that ADP was capable of reducing the basal NO level in platelets, experiments were carried out to determine whether ADP could be an inhibitor of endogenous NOS(s) in platelets that was responsible for the reduction of the basal NO level in platelets (from 0.33 ± 0.10nmol/10^8 platelets in the control experiment to 0nmol/10^8 platelets in the presence of 8.0 μM ADP) in the first place.

Line weaver Burk plot of the NOS activities of the supernatant of the disrupted platelets in the presence of increasing concentrations of ADP demonstrated, that while the NOS activity of the supernatant in the absence of ADP had Km= 5.26μM L-arginine, with corresponding Vmax of 6.66nmol NO formed/h/mg protein (control, Fig not shown). The presence of 2.0μM ADP in the supernatant, reduced the Vmax to 2.22nmol NO formed/h/mg protein and Km= 10.0μM L-arginine. Further addition of 8.0μM ADP to the reaction mixture decreased the Vmax to 1.0 nmol NO formed/h/mg protein with Km= 20.0μM L-arginine. In other words, the presence of 8.0μM ADP in the reaction mixture reduced the Vmax of NOS by nearly 85% compared to control with subsequent increase of the Km for L-arginine, the substrate of NOS (Figure 23).

Figure 23: Line-weaver Burk plot of the NOS activity of the supernatant of the disrupted platelets in presence or absence of ADP
The supernatant of the disrupted platelet was prepared by centrifugation as described in Materials & Methods. The NOS activity of the cell free supernatant was determined by treating the supernatant with different concentrations of L-arginine in the presence of different concentrations of ADP as shown. Line-weaver Burk plot (i.e. double reciprocal) were subsequently constructed. The line A represents the formation of NO in the presence of 2.0μM ADP in PRP (●). The line B represents the formation of NO in the presence of 4.0μM ADP in PRP (■). The line C represents the formation of NO in the presence of 8.0μM ADP in PRP (○). Each point represents mean of 5 different experiments each in triplicate.

Effect of reduction of the basal nitric oxide level by l-NAME in platelets on the aggregation of platelets.

The above results taken together suggested that the ADP induced reduction of basal NO level could be related to the ADP induced platelet aggregation. As the basal NO level in platelets was the net cellular increase of the NO level over the breakdown of the compound in the cells, the inhibition of the basal NO synthesis by l-NAME [an inhibitor of NOS (Sakuma et al., 1988)] might result in the reduction of the NO level in platelets. And, as such, if the reduction of basal NO level in platelets itself was involved in the aggregation of platelets, the reduction of NO level in platelets by l-NAME an inhibitor of NOS [Sakuma et al., 1988], might result in the aggregation of platelets even in the absence of the added ADP to PRP. To determine whether ADP induced reduction of NO level in platelets was merely a “bystander” effect on the ADP induced platelet aggregation or the reduction of NO level in platelets was actually related to the aggregation of platelets, PRP was incubated with different amounts of l-NAME. It was found that the incubation of PRP with different concentrations of l-NAME, instead of ADP itself, resulted in the aggregation of platelets (Figure 24). It was found that 0.05mM l-NAME was found to be an as efficient aggregating agent as ADP, at 2.0μM ADP, as described in Figure 24 with the concomitant reduction of basal NO level in platelets. It was found, as described in the figure 24, that the reduction of NO level in platelets was related to the increase of platelet aggregation as determined by Pearson’s coefficient of correlation, “r” which was equal to -0.833 (P<0.0001; n=10). The negative Coefficient of correlation indicates that they are highly but negatively correlated as determined by Graph Pad Prism software.

These results also demonstrated that the reduction of NO level in platelets by l-NAME was at least qualitatively similar to the platelet aggregation induced by ADP itself (Figure 24 and Figure 21).
Figure 24: Aggregation of platelets in PRP induced in the presence of different amounts of l-NAME

The aggregation of platelets induced by l-NAME was carried out as in the case of ADP induced platelet aggregation except that the aggregation of platelets was carried for 10 min at 37°C. The aggregation of platelets in PRP at different concentrations of l-NAME (from 0.05mM-1.0mM) is shown. Numbers shown in the parenthesis are the amount of NO produced in the presence of l-NAME indicated in each case.

The role of prostaglandin synthesis in the aggregation of platelets due to reduction of NO level induced by l-NAME.

The results described under Figure 24, demonstrated that the use of different amounts of l-NAME instead of ADP resulted in the aggregation of platelets similar to that in the case of ADP, a well known aggregating agent of platelets, which is reported to mediate its effect through the synthesis of prostaglandins [Hamberg et al., 1974].

To find out whether the effect of l-NAME on the aggregation of platelets was necessarily mediated through the synthesis of prostaglandins, the PRP was incubated with different amounts of l-NAME and after incubation for 37°C for different times, the prostaglandin synthesis was determined by the assay of TXA₂ in its TXB₂ form. It was found that the increase in the synthesis of TXA₂ in the platelet (Figure 25) due to the presence of l-NAME was related to the reduction of basal NO level
induced by the NOS as determined by Pearson’s coefficient of correlation, “r” which was equal to -0.986 (P<0.001; n=10). The negative Coefficient of correlation indicates that the reduction of NO and the synthesis of TXB$_2$ were highly but negatively correlated as determined by Graph Pad Prism software.

Figure 25: Synthesis of TXA$_2$ (determined in TXB$_2$ form) and NO in platelets in PRP in the presence of l-NAME

PRP was incubated with different amounts of l-NAME as indicated for 10 min at 37°C and the synthesis of TXB$_2$ and NO in platelets were determined. Solid square (■) represents TXB$_2$ production and Open square (□) represents formation of NO. Results shown are mean±S.D. of 5 different experiments each in triplicate by using blood samples from 5 different volunteers (M=3, F=2).
These results demonstrated that the reduction of the basal NO level in platelets by ADP might be a critically important event in the aggregation of platelets. Although many studies before have established that the effect of ADP on the platelet aggregation was initiated through the activation of platelet membrane COX mediated through the synthesis of proaggregatory prostaglandins [Hamberg et al., 1974; Moncada et al., 1979]. The mechanism for the availability of arachidonic acid itself, an essential substrate for the COX [Hamberg et al., 1974] for the synthesis of the proaggregatory prostaglandin however remain obscure. Our results, on the other hand, demonstrated that the incubation of PRP with increasing concentrations of ADP resulted in the increased platelet aggregation (Figure 21) with simultaneous reduction of the basal NO level in platelets (Figure 22). It was also found that ADP was not only an activator of COX, but was also a potent inhibitor of platelet NOS (Figure 23). That the reduction of the basal NO level could be involved in the aggregation process was supported by the aggregation of platelets treated with l-NAME, an inhibitor of NOS in the absence of the added ADP to PRP (Figure 24).

More interestingly, the reduction of platelet NO level, like ADP (Figure 22) itself resulted in the synthesis of prostaglandin, the treatment of PRP with l-NAME also resulted in the liberation of arachidonic acid that resulted in the synthesis of prostaglandin as determined by the synthesis of TXA$_2$ (Figure 25) in its TXB$_2$ form due to the treatment of PRP with l-NAME. Thus the l-NAME induced platelet aggregation was in essence a mimicry of the well known ADP induced platelet aggregation through the synthesis of prostaglandins. However it must be pointed out in this context that while the aggregation of platelets induced by ADP could be demonstrated at μM ranges (Figure 21), the l-NAME induced platelet aggregation was found to occur in submM ranges. And the extent of platelet aggregation at 2.0μM was nearly similar to the platelet aggregation was 0.1mM. In other words, ADP was ≈50 fold more potent aggregating agent compared to l-NAME. However this comparison could be of lesser importance when the platelet aggregation induced by dermcidin isoform 2 was compared to that by ADP [Ghosh et al., 2011], in that dermcidin isoform 2 was >40 fold more potent inducer of platelet aggregation when compared to ADP through the activation of COX in both cases. Furthermore, while the optimum time of the l-NAME induced platelet...
aggregation was 10 min, the optimum time of ADP induced platelet aggregation was only 5 min
(Figure 21 and Figure 24). However these quantitative differences between the effects of l-NAME
and ADP might be expected as the former non-physiologic agent was merely a mimic to ADP.

It should however be mentioned here that no other man-made synthetic compound is currently
known, to the best to our knowledge, that like l-NAME can aggregate platelets mimicking ADP
through the activation of platelet COX. However, as reported before all known platelet aggregating
agents mediate their effect through ADP induced prostaglandin synthesis pathway [Hamberg et al.,
1974], the l-NAME induced aggregation of platelets in this respect also was not an exception in that
it too was found to mediate its effect through the prostaglandin synthesis. However, it is not
currently known whether the effect of l-NAME was indeed mediated through the same ADP
receptors on the platelet surface [Gachet, 2001].

These results nevertheless suggested that it is not the activation of platelet COX alone by ADP led
to the aggregation of platelets due to the subsequent synthesis of the proaggregatory prostaglandins.
In contrast, our result indicated that the inhibition of NOS in platelets that resulted in the reduction
of the basal NO level in platelets led to the release of arachidonic acid from the platelet membrane
for the synthesis of prostaglandin essential for platelet aggregation [Hamberg et al., 1974] (Figure
25). However it was not possible for us to determine whether the reduction of NO level by ADP in
platelets, which resulted in the release of arachidonic acid, was a priori event compared to the
activation of COX.

In the above context, it could be mentioned here that the inhibition of prostaglandin synthesis by
aspirin might not necessarily due to the inhibition of platelet COX alone but as aspirin has been
reported to increase platelet NO synthesis [Karmohapatra et al., 2007a]. And as such, the inhibition
of prostaglandin synthesis by aspirin might also be related to the increase of the basal NO level in
platelets leading to the unavailability of arachidonic acid for the subsequent synthesis of
prostaglandin by the COX. In other word, our result suggested that the ADP induced aggregation of
platelet could be related to the inhibition of platelet NOS leading to the reduction of basal NO level
in platelet that contrasted the currently held belief that ADP can directly activate platelet COX
leading to the prostaglandin synthesis in that the decrease of platelet NO level resulted in the availability of arachidonic acid for the subsequent synthesis of prostaglandin catalysed by COX.

These results also imply a central role of the basal NO level not only for the inhibition of platelet aggregation [Bhattacharya et al. 2009] but also for the aggregation of platelets as well as reported herein. The results presented above suggested that the reduction of NO synthesis in the platelets either by ADP or by l-NAME resulted in the increased platelet aggregation that has a critically important role in the development of coronary thrombosis prevalence leading to ACS. In the above context, studies by several investigators were reported, in that it was suggested that NO bioavailability is crucial for maintaining vascular endothelial health and there are evidences that the oxidative stress induced destruction of NO to the endothelial dysfunction accompanies a number of cardiovascular disease states including hypertension, diabetes, chronic heart failure and atherosclerosis [Steiner et al., 2002; Rush et al., 2005] which are the leading risk factors of ACS.

It is also reported that there is a positive association between superoxide dismutase (SOD) and urokinase type plasminogen activator (uPA) as well as plasmin/anti-plasmin (PAP) levels which suggests a link between enhanced oxidative stress (SOX) and the fibrinolytic activity where SOD is inhibited by NO [Steiner et al., 2002], that is the reduction of cellular NO might lead to the increase of SOD which in consequence may lead to increased fibrinolytic activity [Pawlak et al., 2006].