DISCUSSION

These results indicated that aspirin, a well-known inhibitor of platelet aggregation both in vivo and ex vivo, is also a potent fibrinolytic agent at least in vitro. However, since the oral ingestion of aspirin also resulted in thrombolysis ex vivo, it is possible that the compound might act as a thrombolytic agent also in vivo (Fig. 1e). This effect of aspirin was found to be dependent on the presence of platelets in the "thrombus" produced by recalcification of PRP, since no aspirin-dependent fibrinolysis could be demonstrated in the case of platelet-free plasma clot (Fig. 1c). These differential effects of aspirin on fibrinolysis in the presence and absence of platelet indicated a critical role of aspirin on platelets, since it was the aggregation of platelets themselves that initiated the thrombus formation in the first place. Since it has been reported that platelet membrane contains fibrinolytic activity [Miles, Plow, 1985; Park, et al, 1989], it is possible that the aspirin-induced thrombolytic effect was due to the platelet themselves and mediated through the inhibition of platelet aggregation. However, neither aspirin at 4μM inhibited platelet aggregation [Chakraborthy, et al, 2003] nor the presence of platelets alone in PRP clot show any thrombolysis (Fig. 1b). These results indicated that the platelet-associated fibrinolytic activity or the inhibitory effect of aspirin was not responsible for the observed thrombolysis (Fig. 1a, 1b). Furthermore, as described in the results, it was also found that NO itself was capable of fibrinolysis even in the absence of platelets, indicating that the lysis of clot was not mediated through the fibrinolytic activity of platelets themselves. On the other hand, the addition of NAME to PRP, which did not inhibit platelet aggregation, but inhibit NO production in platelets, resulted in the inhibition of aspirin-induced thrombolysis (Fig. 1d) supporting the conclusion that the aspirin effect was not a manifestation of platelet thrombolytic activity. It was also found that the lytic effect of aspirin could be demonstrated in the case of whole blood clot or in PFP treated with washed erythrocytes (not shown). Since the fibrinolytic effect of aspirin in the presence of platelets could be demonstrated even in the crude clotted plasma, the effect of the compound in platelets could be of pathophysiologic importance. This conclusion was drawn from the fact that, although various proteinases including trypsin, thrombin, kalikrein or plasmin can activate plasminogen to plasmin in purified system, all of them including the platelet-associated fibrinolytic activity itself
failed to do so in the clotted plasma (Fig. 1c). The failure of these proteinases has been related to the presence of potent plasmin inhibitors in the plasma [Rimon, et al, 1966. Angles-Cano, 1994]. Although plasmin could be formed from plasminogen through the activation of Hageman factor in the intrinsic pathway [Angles-Cano, 1994] the activation process is very complex and slow. Its significance in the dissolution of formed thrombus in CAD remains an unsettled issue. In this context tissue plasminogen activator and prourokinase, both produced in the endothelial cells are believed to play important role in the dissolution of formed thrombus in the system. These plasminogen activators, including urokinase (a product of renal tubular epithelial cells), tissue plasminogen activator (produced in the endothelial cells), streptokinase and staphylokinase (bacterial exoproteins without proteolytic activity) are used for their fibrinolytic activity to recanalize occluded coronary artery due to the thrombus formation in CAD [Fuster, et al, 1996. Antman, Braunwald, 1998]. On the other hand aspirin a small organic molecule was not only found to activate fibrinolytic pathway even in plasma, the lack of fibrinolysis of the clotted plasma in the absence of platelets or other cells indicated that the compound did not directly mediate its fibrinolytic activity but conjugation with plasminogen present in the plasma. The proteolytic activity was found to be mediated through the production of NO, which is known to be an important "biologic messenger molecule" with regulatory effects on diversified physiologic and pathologic events [Ignarro, 1990]. Our results on the effect of NO on plasminogen activation indicated the existence of a novel pathway for systemic fibrinolysis. The activation of platelet AANOS by aspirin would be critically important in the production of NO in platelets for the initiation of the pathway leading to the activation of plasminogen. Since NO is reported to be a potent inhibitor of platelet aggregation [Kanowitz, 1981. Sinha, et al, 1998], and as reported here the compound can also act as a direct activator of plasminogen even in crude plasma, it could be concluded that the platelet AANOS may have important role in the prevention of thrombosis through this COX independent pathway. The effect of sodium nitroprusside and insulin on the dissolution of PRP clot demonstrated that the fibrinolysis was not a unique effect of the aspirin in the presence of platelets but others agents which stimulate NO synthesis was capable of inducing fibrinolysis similar to aspirin. The effect of insulin in fibrinolysis could not only be demonstrated in vitro but we have previously reported that platelets from type-I diabetes mellitus patients, who have a systemic
insulin deficiency, showed an impaired fibrinolytic activity [Kahn, et al, 1995]. From these results it was concluded that, NO once available in the milieu either from cellular or external source was capable of activating plasminogen through the proteolytic excision of 14 kDa preactivation peptide from its zymogen (Fig. 11). This unexpected proteolytic effect of NO, a small inorganic molecule, has not been realized before. The NO effect is also unique in that no cells or cofactors were needed for this proteolytic cleavage. Furthermore this effect of NO was not dependant on NO induced cyclic GMP formation, which has been shown to be the “second messenger” in various NO induced biologic effects [Murad, et al, 1992]. Although in vivo increase of NO has been reported to induce thrombolysis [Sinha, et al, 1998. Yao, et al, 1995. Sinha, et al, 1999], our result provided an explanation of the NO effect in vivo.

The mechanism of NO induced production of plasmin from plasminogen is not currently known. However since the zymogen contains 2 interchain disulfide bonds [Robbins, et al, 1967] at [cys\(^90\) (plasmin heavy chain A) -- cys\(^208\) (plasmin light chain B) and at cys\(^100\) (plasmin heavy chain A) -- cys\(^108\) (plasmin light chain B)] it might be possible that the fragmentation of the zymogen to plasmin by NO involved the breakage of cross strand disulfide bonds (S-S bond dissociation energy is 214 / kJ / mol, which is one of the lowest in common bio molecules) in the precursor molecule due to high torsional and deformation energy stored in its structure [Miles, Plow, 1985]. That the breakage of cross – strand disulfide results in the generation of proteolytic activity in protein is not unknown. Indeed cleavage of cross – strand disulfide bond has been reported to generate endopeptidase activity in neurotoxin, a polypeptide composed of two subunits containing cross – strand disulfide bonds [Wouters, et al, 2003]. We have demonstrated here that aspirin induced NO formation activated plasminogen to plasmin leading to enhanced fibrinolysis. However it should be mentioned here that other mechanisms of pro-fibrinolytic activity of aspirin have also been reported. Acetylation of fibrinogen by aspirin has been reported to enhance fibrinolysis withought the activation of plasminogen (Bjornsson, Buzcko). We have found that aspirin itself unlike NO, (Fig.11) failed to activate plasminogen to plasmin through the excision of 14 kDa pre activation peptide. As such in our experiment the absence of fibrinolysis in cell-free plasma by aspirin (Fig.1c) was probably related to the methodological differences.
One of the major problems encountered in use of excess plasminogen activators in CAD is related to bleeding in patients receiving these proteins. It is generally accepted that the administration of excessive quantities of these proteolytic activators in the circulation, results in uncontrolled production of plasmin, which consequently overwhelms the amounts of plasmin inhibitors available in the system [Rimon, et al, 1966. Angles-Cano, 1994] and leads to the bleeding episodes due to hyperplasminemia [Rimon A, et al, 1966. Angles-Cano, 1994]. In contrast to these activators, it was noted that the activation of plasminogen by aspirin or by NO was an autoregulated event at least partly by the inhibitory effect of these compounds themselves (Fig.2, & Fig.6).

It was further noted that while the optimal concentration of aspirin for the maximal fibrinolysis of PRP clot was 4.0\(\mu\)M, the optimal concentration of the compound for the maximal inhibition of ADP (4\(\mu\)M) induced platelet aggregation has been reported to be \(\approx 80 \mu\)M [Chakraborty, et al, 2003]. These results indicated the possibility that on mole/mole basis aspirin could be a more potent thrombolytic agent than as an inhibitor of platelet aggregation at least in vitro.

It has been reported before that the development AIHD is the consequence of thrombus formation in the coronary artery [Falk, 1992. Fuster, et al, 1996. Antman, Braunwald, 1998]. On the other hand it is also well-known that the ingestion of aspirin improves all acute syndromes associated with AIHD which results from the abrupt reduction of blood flow to the myocardium due to thrombus formation. And as such it can be speculated that the observed improvement of the condition in AIHD by aspirin was at least partly due to the thrombolytic effect of aspirin. However no direct evidence of aspirin as a thrombolytic agent through NO production is currently available.

As presented in this study, the use of aspirin instead of streptokinase whose fibrinolytic action is well established as a thrombolytic agent did not prove to be less effective in the prevention of death due to thrombosis, albeit induced by ADP, at least in this animal model. It should however be mentioned that among all platelet aggregating agents, ADP is believed to be the most important agonist in the development of thrombosis in AIHD in men [Mills, 1996].

We have reported before that although both prostacyclin and prostaglandin E\(_1\) are more potent inhibitor of platelet aggregation compared to aspirin, these prostaglandins do not stimulate NO synthesis in platelets and when either of the prostaglandins was injected in the circulation it failed to protect these animal from death due to ADP induced
thrombosis [Sinha, et al, 1999]. On the other hand insulin or aspirin not only inhibit platelet aggregation they also stimulated NO synthesis in platelets [Trovati, et al, 1997. Kahn, et al, 2000. Sinha, et al, 1999. Chakraborty, et al, 2003]. It is also reported that NO donating non steroidal anti inflammatory agents also inhibit platelet aggregation [Lorenzo Fernandez, et al, 1977. Kalguutkar, et al, 2003]. Furthermore aspirin has been reported to promote vasodilatation through nitric oxide formation for the resumption of normal circulation [Bednar, Gross, 1999]. The systemic injection of either insulin as reported before [Sinha, et al, 1999] or aspirin, as reported here, was capable of preventing death of the animals from the ADP induced thrombosis. From these results it could be concluded that the inhibition of platelet aggregation alone by any agent might not be adequate for the protection from death due to thrombosis, but simultaneous stimulation of NO synthesis in platelets might be helpful for the prevention of death through the NO induced thrombolysis. It should be mentioned here that although aspirin induced fibrinolysis was mediated through the synthesis of NO, the mechanism of fibrinolysis by NO itself remains obscure, at present. However our preliminary studies indicated that NO may induce the breakage of cross-strand disulphide bridges [Wouters, et al, 2003] in the plasminogen molecule leading to plasmin formation. Finally, if the aspirin effect in this animal model could be reproduced in AIHD in men, it could be then suggested that the direct injection of aspirin in the circulation, instead of oral administration, could be more advantageous in this condition due to dose related combined antiplatelet (through the inhibition of prostaglandin synthesis) and thrombolytic (through NO synthesis) effects of the compound in the circulation. It should be noted here that although aspirin is known to reduce mortality of patients with MI, in the ISIS-2 study it was reported that the compound did not leads to significant thrombolysis in angiographic studies [Baigent, et al, 1998]. In contrast we have found that direct addition of aspirin solution (4µM) on the top of the clot or ingestion of aspirin (150 mg) by normal volunteers resulted in the spontaneous clot lysis in vitro [Karmohapatra, et al, 2007]. However it was also noted that the extent of clot lysis in the case of oral ingestion of aspirin was less than that in the case of the direct addition of aspirin solution to the clot.

As such, whether the failure of ISIS-2 study to detect thrombolysis by aspirin in AMI was related to the sub optimal dose of the compound in the circulation achieved through oral administration of aspirin is not known. In the initial phase of the study anesthetic
agents are not used for their well known antiplatelet and other hematologic effects [Holbrook, Coker, 1989. Mizobe, 1999], in latter part of the study it was found that to minimize the pain and suffering, the use of morphine sulfate (200µg / kg body weight) in these animal model did not interfere with the effect of aspirin in thrombolysis.

Our results indicated that aspirin could be used as a potent fibrinolytic agent through the stimulation of nitric oxide in vitro and in vivo [Karmohaputra et al, 2007], independent of its well-known inhibitory effect of platelet aggregation. And the injection of neutralized aspirin solution could be a new novel way for the prevention of CAD immediate after the precipitation as compared with other fibrinolytic agents in animal model.