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C-Reactive Protein binds to $\alpha_{IIb}\beta_3$.

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C-reactive protein (CRP) is a member of the pentraxin protein family which have a native pentameric ring structure made up of 5 non-covalently associated monomers. It is an acute phase protein produced predominantly in the liver with plasma levels increasing up to 10,000-fold in response to infection. CRP is widely used as a marker of cardiovascular disease [1] and has been implicated in its pathogenesis [2], although its exact role is unclear [3]. While circulating CRP exists in the native pentameric form (pCRP) it can also exist in a modified monomeric form (mCRP). These two distinct forms of CRP have been demonstrated to have opposing biological actions.

CRP has been shown to modulate the interaction between platelets and neutrophils [4, 5]. mCRP was demonstrated to enhance the interaction under shear via an interaction with P-selectin and CD18 while pCRP inhibited the interaction via an interaction with the Fc γ RIIIa [5]. Heuertz *et al.* demonstrated that mCRP interacted with CD16 (Fc γ RIIIb)

on neutrophils, but not with CD32 (Fc γ RIIIa) [6].

CRP has been associated with procoagulant [7] and anti-fibrinolytic activity [8] and has also been shown to have diverse effects on platelets. While heat-agglutinated CRP [9] and mCRP [10] have been shown to activate platelets pCRP has been shown to inhibit platelet aggregation by a range of agonists [11, 12, 13]. The mechanism by which pCRP inhibits platelet aggregation is unknown and this study was designed to elucidate the mechanism of platelet inhibition using platelet adhesion and purified protein ELISA based assays.

Platelet adhesion and aggregation studies were performed as previously described [14]. For this study we used healthy volunteers who had not taken any non-steroidal anti-inflammatories for a fortnight and did not discriminate based on age or sex. Ethical approval was obtained from the RCSI ethics committee. As with previous studies we found that recombinant pCRP (Calbiochem, Darmstadt, Germany, 10 μ g/ml) inhibited platelet aggregation induced by low-dose thrombin receptor

activating peptide, (TRAP, 2-4 μ M), collagen (38 μ g/ml) and ADP (1-5 μ M) by 72% \pm 10%, 95% \pm 2% and 91% \pm 4% respectively ($P < 0.001$ for all) indicating an agonist-independent effect of pCRP, similar to that seen with $\alpha_{IIb}\beta_3$ inhibitors. To determine if there was a direct interaction between CRP and platelets, we investigated the ability of washed platelets to bind to immobilised CRP. Resting platelets adhered strongly to fibrinogen and to a lesser extent to CRP (30% \pm 7% of the fibrinogen control, fig. 1A).

As $\alpha_{IIb}\beta_3$ is a key receptor in platelet aggregation and adhesion we investigated its ability to bind CRP. Mn^{2+} alters the conformation of $\alpha_{IIb}\beta_3$ increasing its affinity for RGD containing peptide ligands [15]. This is not as strong as other agonists and does not lead to signalling which would lead to granule release and platelet shape change. All other agonists signal into the platelet (outside-in signalling), and lead not only to granule release, but also an increase in P-selectin expression. Thus due to the reports that CRP interacts with P-selectin [4, 5], we might see increased platelet adhesion or at least $\alpha_{IIb}\beta_3$ -independent adhesion if platelets activated with soluble agonists were used. The use of $MnCl_2$ allows us to look at the $\alpha_{IIb}\beta_3$ interaction without the complications of the P-selectin interaction.

Direct activation of $\alpha_{IIb}\beta_3$, using $MnCl_2$ (1mM) had little effect on platelet adhesion to fibrinogen but increased adhesion to CRP approximately 4-fold (128 \pm 6% of fibrinogen control, $P < 0.001$). Tirofiban, a specific $\alpha_{IIb}\beta_3$ antagonist [16] (1 μ M) completely inhibited both resting and activated platelet adhesion to fibrinogen (from 100% to 3% \pm 6% (resting) and from 99% \pm 3% to 3% \pm 9% ($MnCl_2$

activated), $P < 0.001$ for both). Tirofiban inhibited resting platelet adhesion to CRP by 43% (from 30% \pm 7% to 17 \pm 2%), although this inhibition was not statistically significant due to the variation observed in this assay at low levels of adhesion. Tirofiban completely inhibited the adhesion of $MnCl_2$ -activated platelets to CRP (from 128 \pm 6% to 0 \pm 12%, $P < 0.001$).

To confirm a direct interaction with $\alpha_{IIb}\beta_3$, we utilized an ELISA to measure binding of purified $\alpha_{IIb}\beta_3$ to CRP (fig. 1B). These data directly mirrored the results from the platelet adhesion study with adhesion of resting $\alpha_{IIb}\beta_3$ to CRP at 36% \pm 6% of the adhesion to fibrinogen. This was also increased 4-fold to 133% \pm 25% with $MnCl_2$ activation of $\alpha_{IIb}\beta_3$ ($P < 0.001$). Tirofiban strongly inhibited purified $\alpha_{IIb}\beta_3$ adhesion to CRP (Resting: from 36% \pm 6% to 2% \pm 3%, $P = NS$; $MnCl_2$ activated $\alpha_{IIb}\beta_3$: from 133% \pm 25% to 14% \pm 6%, $P < 0.001$). We also investigated a role for the $Fc\gamma RIIa$ due to its role in immune-mediated platelet activation [14, 17] and previous reports of an interaction between the $Fc\gamma RIIa$ and pCRP. Neither adhesion to fibrinogen nor CRP was inhibited by the monoclonal antibody to the $Fc\gamma RIIa$, (IV.3) (data not shown), suggesting that this receptor does not play a role in platelet adhesion to CRP.

We have demonstrated that immobilized CRP can support platelet adhesion and that it has a higher affinity for manganese-treated platelets suggesting a greater affinity for the activated form of $\alpha_{IIb}\beta_3$. In support of this, we have demonstrated that immobilized CRP interacts directly with purified $\alpha_{IIb}\beta_3$, and also has increased binding to manganese activated $\alpha_{IIb}\beta_3$ in this system. This interaction was

inhibited by tirofiban, suggesting that CRP interacts with $\alpha_{IIb}\beta_3$ through the RGD binding site. Analysis of the surface exposed residues of CRP identified the RGD-like motif, $^{58}\text{RQD}^{60}$. Thus it is possible that this sequence could be responsible for the interaction with $\alpha_{IIb}\beta_3$ which we have demonstrated.

These data may explain previous reports on the platelet inhibitory activity of pCRP. Recently, pCRP was shown to decrease fibrinogen binding to platelets pre-activated with ADP [19] which our data would suggest is due to direct binding of pCRP to $\alpha_{IIb}\beta_3$. We suggest that soluble pCRP can interact with activated $\alpha_{IIb}\beta_3$, blocking fibrinogen binding thus acting as an $\alpha_{IIb}\beta_3$ antagonist in a soluble setting. This is likely to be relevant in conditions associated with infection [17] or inflammation where the high levels of pCRP may act to suppress platelet aggregation. Atherosclerosis is probably influenced more by mCRP interactions with the vessel wall. We cannot exclude the possibility of an interaction between mCRP and $\alpha_{IIb}\beta_3$. Previous reports have suggested that pCRP changes conformation upon binding to cationic surfaces [18] and pCRP bound to membranes has been shown to undergo a transition to mCRP_m and then to mCRP_s (REF). Taking into consideration the ability of soluble pCRP to inhibit aggregation induced by wide range of platelet agonists and its ability to support platelet adhesion when immobilised it is possible that both pCRP and mCRP can directly interact with $\alpha_{IIb}\beta_3$.

In conclusion we have shown that CRP at levels found during episodes of inflammation directly binds to the activated form of $\alpha_{IIb}\beta_3$ and inhibits platelet aggregation.

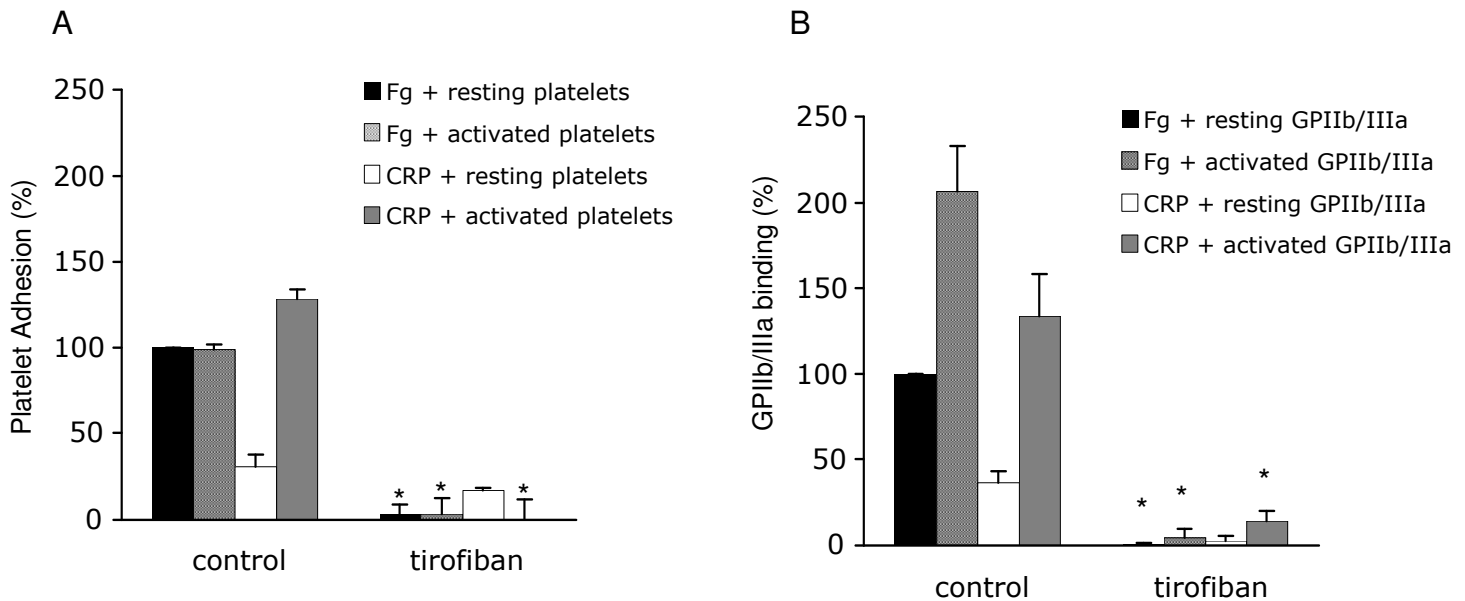
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Activation	Treatment	Platelet adhesion		GPIIb/IIIa ELISA	
		Fibrinogen	CRP	Fibrinogen	CRP
Resting	No drug	1.00 ± 0.14	0.32 ± 0.11	0.24 ± 0.04	0.09 ± 0.02
	Tirofiban	0.04 ± 0.07 <i>P</i> <0.001	0.17 ± 0.04 <i>NS</i>	0.00 ± 0.01 <i>P</i> <0.001	0.01 ± 0.01 <i>NS</i>
MnCl ₂ treated	No drug	0.99 ± 0.17	1.30 ± 0.24	0.47 ± 0.04	0.30 ± 0.04
	Tirofiban	0.05 ± 0.11 <i>P</i> <0.001	0.00 ± 0.12 <i>P</i> <0.001	0.01 ± 0.01 <i>P</i> <0.001	0.03 ± 0.02 <i>P</i> <0.001

Figure I: Platelets and purified $\alpha_{IIb}\beta_3$ adhere to immobilized pCRP. **A** Platelet adhesion to pCRP is potentiated by manganese activation of platelets and inhibited by $\alpha_{IIb}\beta_3$ antagonists. **B** Purified $\alpha_{IIb}\beta_3$ adhered to immobilized pCRP in an ELISA based assay. Plates were coated with fibrinogen or pCRP (50 $\mu\text{g}/\text{ml}$) by incubating protein solutions in the wells for 2h at 37 °C, and further blocked with 1% BSA. Resting (washed) platelets (A) or purified $\alpha_{IIb}\beta_3$ (B) were treated with 1 mM MnCl₂ for 10 min prior to adhesion. Where indicated, platelets or purified protein were incubated with tirofiban (1 μM) for 10 min prior to adhesion to immobilized pCRP or fibrinogen. For the ELISA assay, (B), 50 $\mu\text{g}/\text{ml}$ $\alpha_{IIb}\beta_3$ (Calbiochem, Darmstadt, Germany) was incubated with the plates for 1 hour. Anti-CD41 clone SZ22 (2 $\mu\text{g}/\text{ml}$) was incubated for 90 min and HRP-conjugated goat anti-mouse (Pierce, Rockford, IL) (1/15000) for 45 min. Values were normalized to the fibrinogen control (resting). Data is represented as the mean \pm SEM. Statistics were carried out prior to normalization using repeated measures ANOVA with Bonferroni's correction for multiple comparisons (* represents *P*<0.001, n=3). Absolute values for the platelet adhesion assay (absorbance 405 nm) and the GPIIb/IIIa ELISA assay (absorbance 450 nm) are tabulated below (C).