

Original Research

Pravastatin and C reactive protein modulate protease- activated receptor-1 expression in vitro blood platelets

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Abstract: Protease-activated receptor-1 (PAR-1) plays an important role in mediating activation of human platelets by thrombin. However, mechanism of statin in ADP-induced platelet PAR-1 expression is also unknown. Aggregometry, flow cytometry, immunoblotting and ELISA were used to determine role of pravastatin participating in ADP-induced platelet activation and PAR-1 expression. ADP stimulation significantly increased PAR-1 expression on platelets. PAR-1 antagonist SCH-79797 inhibited platelet aggregation as well as decreased platelet P-selectin expression induced by ADP. CRP inhibited PAR-1 expression induced by ADP in a concentration-dependent manner. Pravastatin treatment reduced PAR-1 expression in a concentration-dependent manner. Combination treatment of CRP and Pravastatin significantly reduced platelet PAR-1 expression induced by ADP. By western-blot analysis, pravastatin treatment did not influence total PAR-1 after ADP treatment. CRP decreased platelet total PAR-1 expression induced by ADP. Pravastatin and CRP reduced TXB2 formation by ADP significantly. CRP decreased thrombin fragment F1+2 level with ADP treatment. Pravastatin, in contrast, did not influence F1+2 level. Upon treatment with Pravastatin reduced platelet LOX-1 expression induced by ADP. In conclusion, PAR-1 served as a critical mechanism to relay platelet activation process induced by ADP. CRP and pravastatin reduce PAR-1 expression in platelet by ADP pathway.

Key words: Protease-activated receptor-1, thrombin; pravastatin, Statins, platelet.

Introduction

Thrombin is the most effective activators of platelets. Thrombin receptors have been cloned and sequenced from human platelets (1) and are known as protease-activated receptors (PARs), protease-activated receptor-1 and -4 (PAR-1, PAR-4) (2,3). PAR1, the prototype for this family, mediates activation of human platelets at low thrombin concentrations. PAR-1 antagonist vorapaxar effectively reduced the occurrence of periprocedural myocardial infarction when added to the dual-antiplatelet therapy (4).

ADP, secreted from dense granules, is important positive feedback activator of platelets that amplify thrombus growth and increase thrombus stability (5). Cuong et al. reported that ADP could induce the HSP27 phosphorylation in the human platelets. Schweigel et al. also found that ADP could induce the phosphotyrosine-dependent signaling networks in human platelets by Src-homology 2 region profiling. Furthermore, there are also some of other mechanisms for the ADP activated platelets.

Statins favorably affect a number of diverse clinical events in addition to low-density lipoprotein cholesterol lowering, such as improved vascular relaxation, promotion of angiogenesis, and stabilization of vulnerable plaques (6). Studies showed that the statins is associated with a reduced thrombosis burden and diminished platelet activity in vitro and vivo. Evidence suggests that statins possess anti-inflammatory properties (7). C-reactive protein (CRP), an inflammatory marker, has emerged as a relevant predictor of future cardiovascular events (8). More recently, experimental studies have revealed that CRP also exert proatherogenic effects, elicits the activation of inflammatory and coagulation pathways (9).

However, the effect of CRP on platelet aggregation is not consistent with enhanced thrombosis. When applied to platelet, CRP suppresses platelets aggregation (10). The previous study showed that statins inhibits the activity and antigen level of the platelet PAR-1 thrombin receptor (11). This suggested that the statins has a major role in regulating platelet activity and thrombin formation. However, the mechanism of statin in ADP-induced platelet PAR-1 expression, especially under inflammation state, is unknown. This study aims to determine the regulation of PAR1 on human platelets by pravastatin.

Materials and Methods

Reagents

ADP, C reactive protein (CRP), Pravastatin sodium and bovine serum albumin (BSA) were purchased from Aldrich-Sigma (USA). PAR-1 antagonist SCH-79797 dihydrochloride was purchased from TOCRIS (UK). R-phycoerythrin (RPE)-conjugated anti-CD62P antibody was purchased from BD Biosciences (San Jose, CA), and PE-conjugated anti-thrombin receptor antibody SPAN-12 was purchased from Beckman Coulter (Marseille, France). Primary antibodies for LOX-1, primary antibodies for PAR1 were purchased from Santa Cruz

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Biotechnology (Santa Cruz, CA). Fluorescein isothiocyanate-conjugated secondary antibody and peroxidase coupled secondary antibodies were purchased from BD Biosciences (San Jose, CA). TXB2 EIA kit was purchased from Cayman Chemical Co. (USA), prothrombin fragment F1+2 kit was purchased from Behring Diagnostics.

Preparation of human PRP

Platelet-rich plasma (PRP) was isolated from peripheral blood collected in 3.8% sodium citrate (1/9 vol/vol) from healthy donors. All blood donors denied taking any medication during the 2 weeks preceding veni-puncture. The blood was centrifuged at 150 g for 20 min to obtain PRP. Platelet-poor plasma was isolated by centrifuging the plasma after PRP collection for another 10 min at 500 ×g at room temperature. The protocol was approved by the Institutional Review Board, and informed consent was obtained from each subject.

Platelet aggregation

Platelets aggregation was recorded in real time in a Chrono-Loglumi-aggregometer. Platelet count in PRP was adjusted at 300,000/mm³ by adding PPP to PRP. Then, the PRP was incubated at 37 °C for 5 min before treatment. In the first set of experiments, platelets were incubated with ADP at 1, 5, 10 μM. For the second set of experiments, platelets were pretreated with 100 μM SCH-79797 at 37 °C for 5 min, then, 5 μM ADP was added. Platelet aggregation was determined by measuring changes in light transmission.

Flow cytometric analysis

In ADP-stimulated platelets, the expression of P-selectin, PAR-1 and LOX-1 was determined by flow cytometric analysis. After 5 μM ADP stimulation for 5 min, platelets were washed and resuspended in FACS buffer containing phosphate-buffered saline, 2% fetal calf serum, and 0.1% sodium azide. Then, 5 μl PRP was then extracted and labeled with antibodies, respectively. The samples were incubated for 10 min. After fixation by adding 1% paraformaldehyde, samples were processed on a FC-500flow cytometer (Beckman Coulter, Fullerton, CA). Platelets P-selectin, PAR-1 and LOX-1 expression were reported as the percentages of positive cells or the mean fluorescent intensity (MFI).

Immunoblotting

Five minutes after ADP stimulation in PRP, platelets were collected by the addition of an ice-cold EDTA (10 mM) solution. The mixture was centrifuged at 10,000 ×g at 4 °C for 2 min. The pellet was washed twice with PBS and then lysed by sodiumdodecylsulfate (SDS) sample buffer (62.5 mM Tris-HCl, 2% SDS, 10% glycerol, 50 mM dithiothreitol, 0.1% bromophenol blue; pH 6.8) for 30 min at 4 °C. Lysates were boiled for 5 min at 95 °C before being stored at -20 °C. Platelet lysates were separated by 10% SDS-PAGE gel and then electrotransferred to apolyvinylidenedifluoride membrane and subjected to western blotting. Primary antibodies for PAR1, peroxidase coupled secondary antibodies and a chemiluminescent substrate were used for detection. Primary antibodies for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as loading and

staining control. Densitometric band scanning was performed using an Odyssey infrared imaging system.

Immunoassay

The TXA2 concentration in each example was determined by TXB2, given that TXB2 was the inactive metabolite of TXA2. Measurement of TXB2 production was conducted using aTXB2 EIA kit (Cayman Chemical Co, USA). Concentrations of prothrombin fragment F1 + 2, reflecting thrombin generation, were determined by immunoassay (Enzygnost F1 + 2 micro Behring Diagnostics). PRP was incubated with CRP (50 μg/ml) or pravastatin (10 μM) for 10 min. ADP (5 μM) was added and the mixture was incubated at 37°C for 5 min with stirring. The test was terminated by addition of EDTA (10 mM) before detection. After centrifugation at 12,000 ×g for 3 min, the amount of TXB2 and prothrombin fragment F1+2 were measured according to the manufacturer's instructions.

Results

Up-regulation of PAR-1 expression on platelets by ADP

We firstly studied platelet aggregation by ADP. 1 μM ADP stimulation provoked only a primary wave of aggregation which was followed by dis-aggregation. 5 μM ADP induced both the primary and secondary waves of aggregation (Fig. 1A). 10 μM ADP induced the two waves of aggregation fused and showed a sustained aggregation curve (the maximal aggregation ratio was 61%). We used 5 μM ADP as the agonist to active platelet in our study. We confirmed that PAR-1 is expressed on human platelets by using SPAN-12 by flow cytometry. 5 μM ADP stimulation significantly increased PAR-1 expression on platelets within 5 min. Mean fluorescence intensity (MFI) of PAR-1 expression was found to increase about 35% versus basal PAR-1 expression (Fig.1B).

PAR-1 are involved in ADP-induced platelet aggregation and activation

To determine whether PAR-1 with the role in platelet aggregation, we used PAR-1 antagonist SCH-79797. We have checked the integrity of the platelets under the 100 μM SCH-79797. The results illustrated the integrity of platelets under the 100 μM SCH-79797 (data not shown). We also found that pretreatment with SCH-79797 (100 μM) abolished second wave of platelet aggregation induced by ADP (5 μM) and caused partial

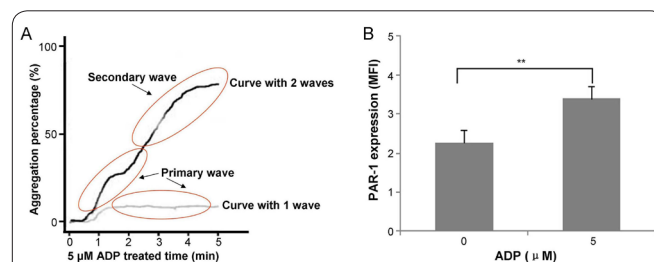


Figure 1. ADP increases PAR-1 expression on platelets. A. 5 μM ADP induced both the primary and secondary waves of aggregation. B. PAR-1 expression was determined in the PRP that had been treated with or without 5 μM ADP, using the flow cytometric analysis. Data were mean ± SEM in the panel, n = 5. **P < 0.01.

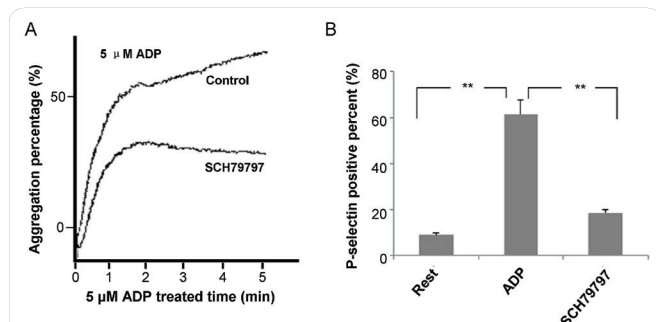


Figure 2. PAR-1 antagonist are involved in ADP-induced platelet aggregation and activation. PRP was preincubated with 100 μ M SCH-79797 at 37 °C for 10 min. 5 μ M ADP was then added with stirring at 1200 rpm in an aggregometer at 37 °C to induce platelet aggregation (A). Another study 5 μ M ADP stimulation for 5 min late, 5 μ l PRP was then extracted and labeled with FITC-conjugated anti-P-selectin antibodies in hepes solution for 10 min. P-selectin expression (B) of positive percent (%) on platelets were monitored by flow cytometry. Data plotted are Mean \pm SEM; n = 5, ** P < 0.01.

disaggregation. Platelet aggregation rate was reduced from $56.40 \pm 4.36\%$ to $28.30 \pm 3.48\%$ (Fig. 2A).

P-selectin expression, which reflects platelet α granule secretion during activation. We confirmed that P-selectin is expressed on human platelets by flow cytometry. ADP (5 μ M) stimulation significantly increased P-selectin expression on platelets. The positive percentage of P-selectin expression was significantly elevated from $8.76 \pm 0.94\%$ in rest platelets to $61.42 \pm 6.38\%$ at 5 min after ADP stimulation. Whereas, pretreatment with SCH-79797 (100 μ M) inhibited the P-selectin up-regulation ($18.45 \pm 1.36\%$) induced by ADP (Fig. 2B).

Modulation of ADP-Induced PAR-1 expression on platelet by CRP

PAR-1 is expressed on human rest platelets. ADP stimulation significantly increases PAR-1 expression on platelet detected by flow cytometry. It was still unclear whether CRP influences PAR-1 expression. Platelets were treated with CRP (25 μ g/ml, 50 μ g/ml) incubated for 10 min at room temperature. This was followed by the addition of 5 μ M ADP, and the sample was incubated at 37 °C for 15 min. PAR-1 on the platelets was determined by using the flow cytometry. Upon treatment with 25 μ g/ml, 50 μ g/ml CRP, the PAR-1 expression on platelets was found to be reduced significantly. CRP inhibited PAR-1 expression induced by ADP in a concentration-dependent manner. MFI of PAR-1 was reduced from the basal ADP treatment of 3.33 ± 0.11 to 3.06 ± 0.15 at 25 μ g/ml CRP, and further decreased to 2.82 ± 0.1 at 50 μ g/ml CRP treatment (Fig. 3).

Modulation of ADP-induced PAR-1 expression on platelet by pravastatin

Platelets were treated with Pravastatin (1 μ M, 10 μ M) incubated for 10 min at room temperature. This was followed by the addition of 5 μ M ADP, and the sample was incubated at 37 °C for 15 min. PAR-1 expression on platelet was detected by flow cytometry. 1 to 10 μ M pravastatin treatment reduced PAR-1 expression in a concentration-dependent manner (MFI were 2.72 ± 0.14 and 3.11 ± 0.18 , respectively). To test whether pravastatin influence PAR-1 expression in the existence

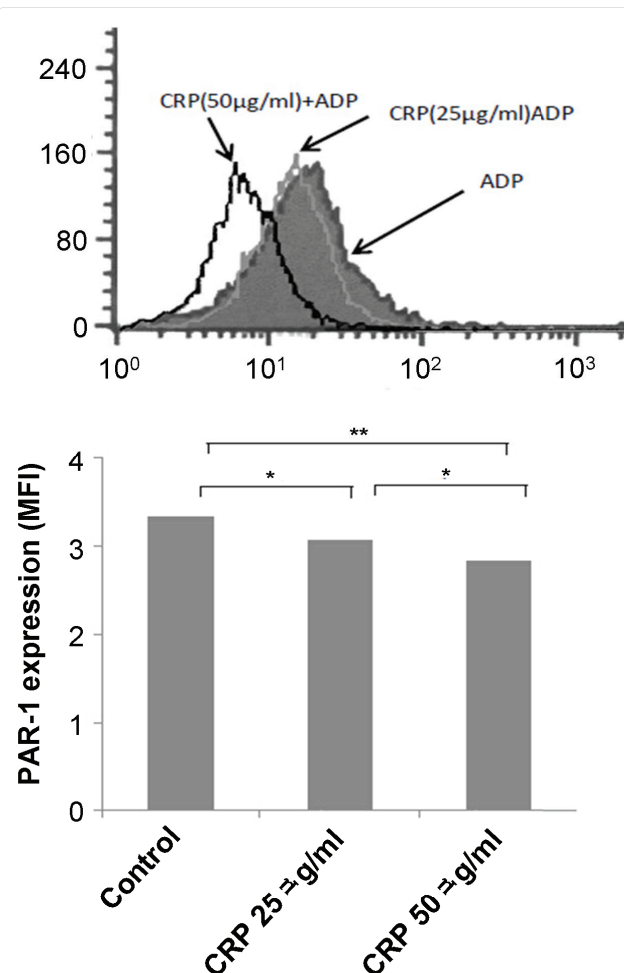


Figure 3. CRP induce platelets PAR-1 expression. Citrated PRP were preincubated with 25 μ g/ml CRP and 50 μ g/ml CRP for 15 min, then stimulated by 5 μ M ADP to induce PAR-1 expression on platelets. Data were mean \pm SEM. n = 5. ** P < 0.01, * P < 0.05.

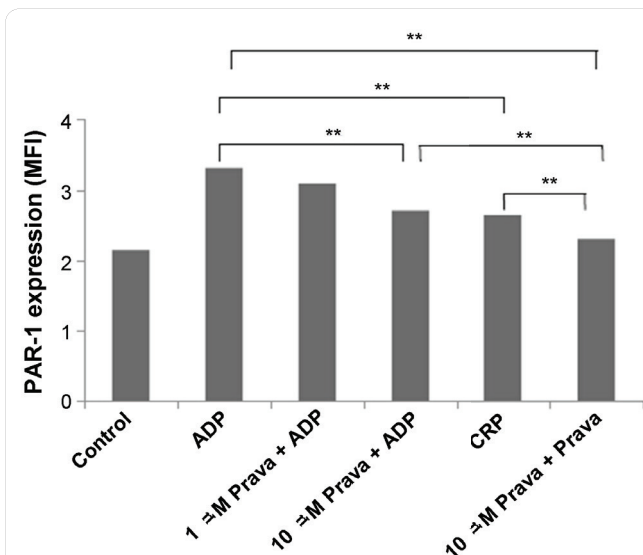


Figure 4. Modulation of ADP-Induced PAR-1 expression on platelet detected by flow cytometry. Citrated PRP were preincubated with vehicle, 1 μ M Pravastatin, 10 μ M Pravastatin, 50 μ g/ml CRP, combination of 50 μ g/ml CRP and 10 μ M Pravastatin for 15 min, then stimulated by 5 μ M ADP. PAR-1 expression on platelets were determined by flow cytometry. Data were mean \pm SEM. n = 5. ** P < 0.01, * P < 0.05.

of CRP, we combine 50 μ g/ml CRP and 10 μ M pravastatin incubation for 10 min before ADP treatment. Combination treatment significantly reduced PAR-1 expres-

sion level detected by flow cytometry (MFI 2.32 ± 0.11 , $p < 0.05$ versus CRP or Pravastatin alone). These results were listed in Fig. 4.

We also detected the total PAR-1 on platelets by western-blot. Platelets were incubated with 10 $\mu\text{g}/\text{mL}$ CRP and/or 10 μM Pravastatin for 10 min before ADP treatment. The results obtained by western-blot were different from that acquired from flow cytometry. We found that the total PAR-1 on platelets did not change significantly before and after ADP treatment. Pravastatin treatment also did not influence the total PAR-1 after ADP treatment. However, CRP alone or combination of CRP and pravastatin decreased total PAR-1 expression by ADP (Fig. 5).

Modulation of TXA2 activity of platelet by CRP and pravastatin

In platelets, TXA2 synthase catalyzes the conversion of PGH2 to TXA2. Because TXA2 is highly unstable, we measured the concentration of the stable metabolite TXB2 in platelets supernatant. In order to assess the effect of CRP on platelet thromboxane generation, PRP was treated with CRP (50 $\mu\text{g}/\text{mL}$) and/or pravastatin (10 μM) for 10 min before addition of 5 μM ADP. TXB2 concentration in the supernatant was measured using an enzyme immunoassay kit. TXB2 was apparently elevated from 88.26 ± 23.38 pg/ml in rest platelets

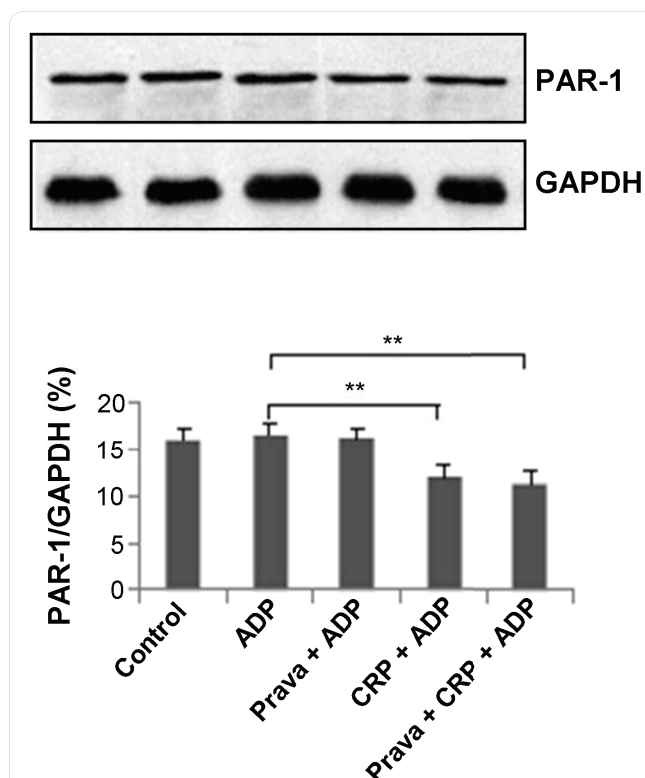


Figure 5. Modulation of ADP-Induced PAR-1 expression on platelet detected by western-blot. Platelets collected from PRP are stimulated with vehicle, 1 μM Pravastatin, 10 μM Pravastatin, 50 $\mu\text{g}/\text{mL}$ CRP, combination of 50 $\mu\text{g}/\text{mL}$ CRP and 10 μM Pravastatin for 15 min, then stimulated by 5 μM ADP for 10 min. The reaction was terminated by the addition of ice-cold EDTA solution and the platelet lysates were immunoblotted with an antibody specific for PAR-1. Representative blots from at least five independent experiments are shown in the upper panel, quantification of the ratio of PAR-1 to GAPDH are shown in the lower panel. **, $p < 0.01$ versus ADP stimulation alone.

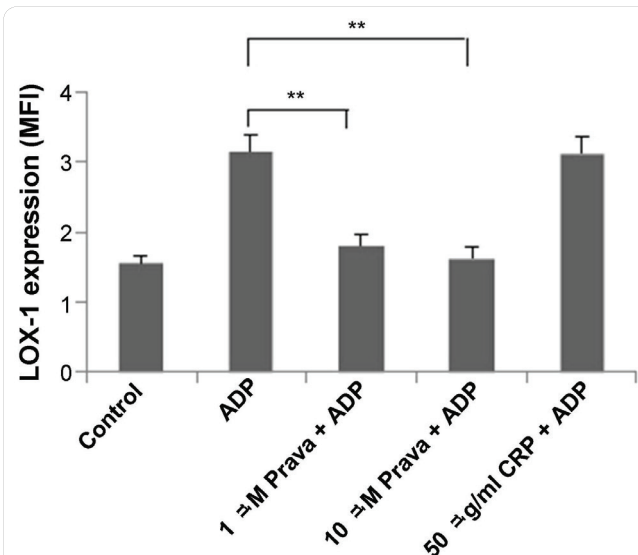


Figure 6. Modulation of ADP-Induced LOX-1 expression on platelet. LOX-1 express demonstrated as graph bars. Platelets collected from PRP are stimulated with 5 μM ADP in the presence of, 1, 10 μM pravastatin, and 50 $\mu\text{g}/\text{mL}$ CRP. LOX-1 level is expressed as the MFI with data from five different healthy donors. 1, 10 μM pravastatin reduce the expression level of LOX-1 significantly, **, $p < 0.01$ versus ADP stimulation alone.

to 185.3 ± 41.82 pg/ml after ADP stimulation. ADP-stimulated TXB2 expression in platelets supernatant was significantly suppressed to 112.68 ± 24.48 pg/ml by CRP. Likewise, 10 μM Pravastatin treatment also significantly reduced TXB2 concentration to 146.48 ± 46.54 pg/ml in platelets supernatant.

Inhibition of thrombin generation by CRP and Pravastatin

Concentrations of prothrombin fragment F1+2, reflecting thrombin generation as a potent initiator of thrombocyte activation, were determined by enzyme immunoassays using ELISA kits. Citrated PRP (3×10^8 platelets/ml) were stimulated by CRP (50 $\mu\text{g}/\text{mL}$) or pravastatin (10 μM) for 10 min, and the supernatant was collected before and after treatment with ADP (5 μM). The levels in samples were determined after centrifugation. ADP treatment increased F1+2 levels significantly (0.65 ± 0.2 vs 1.18 ± 0.8 nmol/l, $p < 0.05$), upon treatment with 50 $\mu\text{g}/\text{mL}$ CRP. Levels of F1+2 was found to be increased significantly with ADP treatment (2.26 ± 0.8 vs 1.18 ± 0.6 nmol/l, $p < 0.05$). For the pravastatin, in contrast, did not influence F1+2 level.

Role of LOX-1 in the down-regulation of PAR-1 on platelet induced by CRP and pravastatin

LOX-1 has been widely implicated as a receptor for ox-LDL, it plays an important role in atherosclerosis and atherothrombosis formation. LOX-1 was exposed on the surface of platelets in an activation-dependent manner. LOX-1 is up-regulated after ADP treatment determined by flow cytometry. To study whether CRP and pravastatin have an effect on platelet LOX-1 expression induced by ADP, Platelets were treated with CRP (50 $\mu\text{g}/\text{mL}$) or pravastatin (1 μM , 10 μM) incubated for 10 min at room temperature. This was followed by the addition of 5 μM ADP for 15 min. CRP (50 $\mu\text{g}/\text{mL}$) had no effect on LOX-1 expression induced by ADP. In

contrast, upon treatment with 1 to 10 μ MP pravastatin, LOX-1 expression was found to be reduced in a concentration-dependent manner ($p < 0.05$, Fig.6).

Discussion

In the present study, we have presented evidences that PAR-1 is critical for ADP-induced platelet aggregation as well as platelet activation. In resting platelets, PAR-1 were detectable in the surface of the platelet by flow cytometry. The exposure of PAR-1 on platelet surface can increase about 35% during platelet activation by ADP. The experiment revealed that this phenomenon occurs as early as 5 min into incubation of platelets with ADP, and the period is not long enough to translate and synthesis new PAR-1 protein. Flow cytometry is commonly applied to determine platelet PAR-1 levels, but this measurement only allow for the enumeration of platelets that bear the specified antigen on the surface exposure. Western blot analysis of whole cell lysates comprises all specified receptor molecules in the sample, independently of its original position within the cell (12). We also used western blot to determine platelets total PAR-1 expression. Our result showed that the platelets total PAR-1 doesn't change 5 min after ADP stimulation, which means that in resting platelets approximately one-third of the PAR-1 is restored and shed in the platelet. ADP stimulation exposed these thrombin receptor on the platelet plasma membrane. Actually, the lower dose of ADP (1 μ M) provokes the release of intracellular calcium, which represents the "primary wave" of aggregation. This initial aggregation response can be followed by a "secondary wave" if the higher dose of ADP is added, which could amplify the primary aggregation response. Thrombin and protease-activated receptor-1 (PAR-1) mediates dense granule secretion and second wave of platelet aggregation in ADP-stimulated citrated PRP. Ample data from both clinical and experimental studies suggests that CRP is not only a well-known marker of cardiovascular disease, but is also probably a mediator of atherothrombosis disease (13).

In the CRPtg mice, Danenberg *et al.* (14) showed that there was more complete thrombotic occlusion in the femoral artery than in the WT mice. The experiment also implied that CRP promotes atherothrombosis. It is becoming apparent that CRP may be important in modulating a spectrum of host responses during inflammation. CRP acts as an early protective recognition mechanism in states that follow infection. CRP has been shown to inhibit certain activities of the T lymphocyte as well as of the platelet (15,16), while promoting activation of the complement system and coagulation (17, 18). In the present study, we have presented evidences that CRP reduce PAR-1 expression on platelet induced by ADP as well as platelet aggregation. It was interesting that the total PAR-1 protein in platelet is also reduced by western blot analysis. Our study implied PAR1 expression may be down-regulated on platelets induced by inflammation, which is accordance with another study (19). CRP may promote atherothrombosis in coagulation other than activating platelet. CRP has been linked to thrombosis via direct effects on plasminogen activator inhibitor-1 (PAI-1), tissue plasminogen activator, and tissue factor activity in vascular cells (20-

22). The mechanism modulation of PAR1 on platelet by CRP is not clear. We confirmed the TXB2 expression, which reflects TXA2 production, is down-regulated on platelet by CRP incubation. TXA2 synthesis has long been suggested to underlie the ADP induced platelet second wave of aggregation in citrated PRP. Other studies showed that CRP inhibits platelet reactivity by interfering with an aspect of prostaglandin metabolism, and which occurs subsequent to the hydrolytic accumulation of arachidonic acid. CRP prevents further tissue destruction and stabilizes cellular membranes by binding to membrane phospholipids (10), which may lead to inhibit TXA2 formation. On the other hand, we also confirmed that CRP stimulation increases prothrombin fragment F1+2 level after ADP stimulation. We can concluded that CRP promotes thrombin generation in this study. Thrombin will bind and cleave PAR1 NH2-terminal ectodomain to unmask a new receptor NH2-terminus even at low concentrations. As soon as to be activated, the PAR1 will fail to recover their ability to respond to thrombin as well as to be detected by PAR-1 antibody.

The 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors (statins) have been shown to exhibit several vascular protective effects, including anti-thrombotic properties, which are not related to changes in lipid profile. By flow cytometry, we confirmed that pravastatin decreases PAR1 expression on platelet stimulated by ADP. A clinic investigation also showed that statins can inhibit the activity and antigen level of the platelet PAR-1 thrombin receptor (11). Our results indicate that pravastatin down-regulates ADP-dependent thromboxane generation. Other pieces of evidence for the effects of statin on platelet thromboxane generation originated from clinical research in patients receiving these drugs (23).

Luzak *et al.* (24) reported that simvastatin or pravastatin may directly interact with blood platelets and down-regulate the activation of GPIIb-IIIa, as well as thromboxane generation. We may suppose that the effect of pravastatin on ADP-induced TXA2 generation in PRP is downstream of PAR1 activation.

Statins reduce oxidative stress by blocking the generation of ROS and reducing the NAD⁺/NADH ratio (25). It is possible that the antioxidant properties of statins contribute to their protective cardiovascular effects. LOX-1 has been widely implicated as a receptor for ox-LDL, which is up-regulated in the presence of oxidative stress. Muhammad *et al* (26) found LOX-1 plays an important role in ADP-induced platelet aggregation. Our studies determined that pravastatin reduces platelet LOX-1 expression induced by ADP by either flow cytometry or western blot. It imply LOX-1, like P-selectin, may be restored in granules. Flow cytometric analyses revealed that LOX-1 was exposed on the surface of platelets in an activation-dependent manner. Anti-LOX-1 antibody can block ADP mediated platelet aggregation, which suggests that pravastatin may mediate PAR-1 reduction and may be associated with inhibition of LOX-1. When we combine CRP and pravastatin incubation into PRP before ADP stimulation, it showed a synergistic effect in reducing PAR-1 expression. We believe that the different mechanism between CRP and pravastatin may contribute to this effect.

Taken together, the present study suggested that PAR-1 serve as a critical mechanism to relay the platelet activation process induced by ADP. The novelty of this study is that the synergistic effect of CRP combine pravastatin could reducing the PAR-1 expression in platelet induced by ADP, which was reported for the first time. Statins exerting antithrombotic effects in the inflammatory process may have important clinical implications. The clinical implications of inhibition of PAR-1 provide a plausible mechanism for the pleiotropy of statins in reducing ischemic vascular events.

Acknowledgments

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