cAMP regulates ADP-induced HSP27 phosphorylation in human platelets

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Abstract. Elevation of cAMP in platelets is recognized to play a suppressive role in platelet functions. We have previously shown that adenosine diphosphate (ADP)-induced phosphorylation of heat shock protein 27 (HSP27) via p38 mitogen-activated protein (MAP) kinase is correlated with platelet-derived growth factor (PDGF)-AB secretion and soluble CD40 ligand (sCD40L) release. In the present study, we investigated the relationship between cAMP and HSP27 phosphorylation in platelet function. 8-Bromoadenosine-3',5'-cyclic monophosphate (8-bromo-cAMP), a plasma membrane-permeable cAMP analogue, or cilostazol, an inhibitor of cAMP phosphodiesterase, markedly attenuated the ADP-induced phosphorylation levels of p38 MAP kinase. In addition, the ADP-induced HSP27 phosphorylation was suppressed by 8-bromo-cAMP or cilostazol. 8-Bromo-cAMP, forskolin and cilostazol remarkably reduced the ADP-stimulated PDGF-AB secretion and sCD40L release. These results strongly suggest that cAMP regulates ADP-stimulated platelet activation due to inhibition of HSP27 phosphorylation via p38 MAP kinase.

Introduction

Platelet adhesion and aggregation represents the first step in thrombogenesis. Platelets are activated by a variety of stimuli, resulting in shape change, adhesion, aggregation, and subsequently thrombus formation. Platelet aggregation plays a pivotal role in thrombus formation. Thrombus formation provokes platelet granule secretion, such as platelet-derived growth factor (PDGF)-AB and serotonin (5-HT), and release of inflammatory substances, such as soluble CD40 ligand (sCD40L) (1).

Adenosine diphosphate (ADP) activates human platelets through P2-receptors such as P2Y1, P2Y12 and P2X receptors (2,3). Although ADP is considered to be a weak agonist compared to thrombin or collagen, ADP is an essential cofactor for the activation of platelets by other agonists (4,5). Low concentrations of ADP amplify the agonist-induced platelet activation (6). It is well known that the activation of P2-receptors leads to both platelet aggregation through P2Y1 and P2Y12 receptors and to shape changes through P2X receptors (7). P2Y1 or P2Y12 receptor activation by ADP reportedly results in the activation of either p44/p42 mitogen-activated protein (MAP) kinase or p38 MAP kinase in human platelets (8).

In response to environmental stress such as heat and chemicals, heat shock proteins (HSPs) are expressed in a variety of cells (9). HSPs are classified into high-molecular-weight HSPs and low-molecular-weight HSPs based on their apparent molecular weights. Low-molecular-weight HSPs with molecular masses from 10-30 kDa, such as HSP27, have high homology in their amino acid sequences (10). It is generally recognized that low-molecular-weight HSPs may have chaperoning functions similarly to the high-molecular-weight HSPs (10). It is recognized that HSP27 activity is regulated by post-translational modifications such as phosphorylation (11). HSP27 becomes rapidly phosphorylated in response to various stresses, as well as exposure to cytokines and mitogens (12,13). Human HSP27 is phosphorylated at three serine residues (Ser-15, Ser-78 and Ser-82). While HSP27 exists in an aggregated form under unstimulated conditions, it rapidly dissociates as a result of stimulation-induced phosphorylation. It has been shown that HSP27 phosphorylation is catalyzed by members of the MAP kinase superfamily, such as the...
 Addition of an ice-cold EDTA (10 mM) solution. The mixture was centrifuged at 10,000 x g at 4°C for 2 min. To measure PDGF-AB and sCD40L as described below, the supernatant was isolated and stored at -20°C for subsequent enzyme-linked immune-sorbent assay (ELISA). For Western blot analysis, the pellet was washed twice with phosphate-buffered saline and then lysed and immediately boiled in a lysis buffer containing 62.5 mM Tris/HCl, pH 6.8; 2% sodium dodecyl sulfate (SDS); 50 mM dithiothreitol; and 10% glycerol.

**Western blot analysis.** Western blot analysis was performed as previously described (21). Briefly, SDS-PAGE was performed by the method of Laemmli (22) in a 12 or 10% polyacrylamide gel. Proteins were fractionated and transferred onto Immobillon-P membranes (PVDF). Membranes were blocked with 5% fat-free dry milk in Tris-buffered saline with 0.1% Tween-20 (TBS-T; 20 mM Tris, pH 7.6; 137 mM NaCl; 0.1% Tween) for 2 h before incubation with primary antibodies against GAPDH, HSP27, phospho-HSP27 (Ser-15), phospho-HSP27 (Ser-78), phospho-HSP27 (Ser-82), p38 MAP kinase, and phospho-p38 MAP kinase. Peroxidase-labeled antimouse IgG was used as a secondary antibody. The antibodies were diluted for optimum concentration with 5% fat-free dry milk in TBS-T. Peroxidase activity on PVDF membranes was visualized on an X-ray film by means of an ECL Western blotting detection system.

**Measurement of PDGF-AB and sCD40L levels.** The PDGF-AB and sCD40L levels in samples were determined using PDGF-AB Quantikine and sCD40Ligand Quantikine ELISA purchased from R&D Systems (Minneapolis, MN), respectively, according to the manufacturer's protocol.

**Statistical analysis.** The data were analyzed by ANOVA followed by the Bonferroni method for multiple comparisons between the indicated pairs or the paired t-test, and a p<0.05 was considered significant. All data are presented as the mean ± SEM.

**Results**

**Effect of 8-bromo-cAMP and cilostazol on platelet aggregation stimulated by ADP.** It is well known that cAMP elevation induces the suppression of platelet aggregation (19). We confirmed that 8-bromo-cAMP suppressed the ADP-induced platelet aggregation in a dose-dependent manner at concentrations from 0.3 and 3 µM. In addition, we showed that cilostazol, an inhibitor of cAMP phosphodiesterase (23), slightly reduced the ADP-stimulated platelet aggregation in a dose-dependent manner at concentrations ranging between 3 and 20 µM (Fig. 1). According to the analysis of the size of the platelet aggregates, large aggregates (50-70 µm) were dose-dependently decreased by cilostazol (Fig. 1). On the other hand, cilostazol increased small aggregates (9-25 µm) in a dose-dependent manner (Fig. 1).

**Effects of 8-bromo-cAMP or cilostazol on the ADP-induced phosphorylation of p38 MAP kinase or HSP27 in human platelets.** We have previously demonstrated that ADP induces the phosphorylation of HSP27 via activation of p38 MAP kinase.
kinase in human platelets, resulting in secreting PDGF-AB and releasing sCD40L (17,18). We have previously demonstrated that 4 min-stimulation by ADP is sufficient for the activation of p38 MAP kinase (17). Therefore, we next examined the effects of 8-bromo-cAMP or cilostazole on the ADP-induced phosphorylation of p38 MAP kinase dose-dependently in the concentration range of 3-20 μM (Fig. 3).

In our previous report (17), we showed that ADP stimulates HSP27 phosphorylation at three serine residues (Ser-15, Ser-78 and Ser-82) in human platelets. We further examined the effect of 8-bromo-cAMP on ADP-induced phosphorylation of HSP27 (Ser-15, Ser-78 and Ser-82). As shown in Fig. 4, the phosphorylation levels of HSP27 (Ser-15, Ser-78 and Ser-82) induced by ADP were almost completely suppressed by 8-bromo-cAMP. In addition, cilostazole attenuated the phosphorylated levels of HSP27 (Ser-15, Ser-78 and Ser-82) induced by ADP (Fig. 5) in a dose-dependent manner between 3 and 20 μM.

Effects of 8-bromo-cAMP or forskolin on the ADP-induced PDGF-AB secretion and the sCD40L release from human platelets. In our previous studies (17,18), we have shown that
ADP stimulates PDGF-AB granule secretion and sCD40L release at least in part via HSP27 phosphorylation through p38 MAP kinase activation from human platelets. We next examined the effect of 8-bromo-cAMP on the ADP-induced secretion of PDGF-AB and release of sCD40L from human platelets. 8-Bromo-cAMP significantly suppressed the ADP-induced PDGF-AB secretion and sCD40L release at concentrations ranging from 3-20 µM (Fig. 8).

**Discussion**

In the present study, we focused on the relationship between cAMP and HSP27 phosphorylation in ADP-stimulated human platelets. First, we confirmed that 8-bromo-cAMP and cilostazol significantly suppressed ADP-induced platelet aggregation which is consistent with the previous study (19). We showed that cell membrane-permeable 8-bromo-cAMP suppressed ADP-induced HSP27 phosphorylation (Ser-15, Ser-78 and Ser-82). In addition, we also demonstrated that the HSP27 phosphorylation at three serine residues by ADP was reduced by cilostazol, an inhibitor of cyclic nucleotide phosphodiesterase III that induces cAMP hydrolysis (23). Therefore, these findings suggest that cAMP elevation attenuates the ADP-induced HSP27 phosphorylation in human platelets.
We have previously reported that ADP stimulates the HSP27 phosphorylation via p38 MAP kinase activation in human platelets (17). Thus, we next investigated the effect of cAMP on the ADP-induced activation of p38 MAP kinase. We showed that 8-bromo-cAMP and cilostazol remarkably reduced the p38 MAP kinase phosphorylation by ADP. MAP kinases are recognized to be activated by phosphorylation of threonine and tyrosine residues by the dual specificity MAP kinase kinase (25). Thus, it is likely that cytosolic cAMP elevation attenuates p38 MAP kinase activity under the stimulation of ADP. Taking these findings into account, it is most likely that the effect of cAMP is exerted at a point upstream from p38 MAP kinase in human platelets, resulting in the inhibition of HSP27 phosphorylation.

Among the P2 receptors, it is generally recognized that the P2Y12 receptors play a central role in platelet activation when stimulated by ADP (2,3,26). Among the heterotrimeric GTP-binding protein family, the P2Y12 receptors couple primarily to the Gi protein which functions as an intermediary resulting in the inhibition of adenylyl cyclase. It has been reported that Akt, Rap and potassium channels are crucial functional effectors downstream of the P2Y12 receptor stimulation (26). On the other hand, the reduced levels of cAMP are not directly responsible for the downstream effects of the receptors (26). Interestingly, we herein showed that cilostazol, an inhibitor of cAMP phosphodiesterase, attenuated the phosphorylated levels of HSP27 in the ADP-stimulated platelets. Based on these findings, it is possible that the elevated cytosolic cAMP by cilostazol suppressed the secretion of PDGF-AB and the release of sCD40L due to the reduction of HSP27 phosphorylation in the P2Y12 receptor activation. Further investigation is necessary to clarify the exact mechanism of cAMP action in ADP-stimulated platelet functions.

It is well known that activated platelets secrete the materials stored in the specific granules such as dense-granules and α-granules. Dense-granules contain small non-protein molecules including ADP. On the other hand, α-granules contain large adhesive and healing proteins such as PDGF-AB (1). PDGF-AB released from platelet α-granules is a potent mitogenic growth factor, which mainly acts on connective tissue such as vascular smooth muscle cells and promotes arteriosclerosis (27). In addition, activated platelets release inflammatory mediators of atherosclerosis, such as CD40 ligand (CD40L). CD40L is stored in the cytoplasm of unstimulated platelets and rapidly translocates on the surface after platelet activation by agonists, such as ADP (28,29). The CD40L expressed on the activated platelet surface undergoes cleavage that generates a functional soluble fragment termed sCD40L. It is known that sCD40L release from platelets induces inflammatory responses via CD40, which is expressed on vascular endothelial cells and neutrophils (30). We found that ADP-induced secretion of PDGF-AB from α-granules was significantly inhibited by the pretreatment with 8-bromo-cAMP or cilostazol. In addition, the ADP-stimulated sCD40L release was also attenuated by 8-bromo-cAMP or cilostazol. Taking these findings and our results into account, it is most likely that elevation of cAMP suppresses the PDGF-AB secretion and the sCD40L release due to the inhibition of HSP27 phosphorylation via p38 MAP kinase in ADP-stimulated platelets. Cilostazol is recognized as an effective alternative to aspirin for prevention of stroke recurrence (31,32). These findings could provide a novel mechanism of cilostazol as an anti-platelet medicine. In conclusion, our results strongly suggest that cAMP regulates ADP-stimulated platelet activation due to inhibiting HSP27 phosphorylation via p38 MAP kinase.

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References


