Biochemical Pharmacology 77 (2009) 1364–1373

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

A new insight of anti-platelet effects of sirtinol in platelets aggregation via cyclic AMP phosphodiesterase

Fu-Chao Liu [a,b,c,d], Chang-Hui Liao [c,d,e], Yao-Wen Chang [b], Jiin-Tarng Liou [a,b,c,d], Yuan-Ji Day [a,b,c,d,*]

[a] Department of Anesthesiology, Chang Gung Memorial Hospital, Linkou Taiwan, ROC
[b] Transgenic & Molecular Immunogenetics Laboratory, Chang Gung Memorial Hospital, Linkou Taiwan, ROC
[c] Department of Medicine, Chang Gung University, Taiwan, ROC
[d] Graduate Institute of Clinical Medical Sciences, Chang Gung University, Taiwan, ROC
[e] Institution of Natural Products, Chang Gung University, Taiwan, ROC

ARTICLE INFO

Article history:
Received 14 October 2008
Accepted 20 January 2009

Keywords:
Sirtinol
Platelet aggregation
Prostaglandin E1
Calcium
cAMP
Phosphodiesterase

ABSTRACT

Sirtinol, a cell permeable six-membered lactone ring, is derived from naphthol and potent inhibitor of SIR2 and its naphtholic may have the inhibitory effects on platelets aggregation. In this study, platelet function was examined by collagen/epinephrine (CEPI) and collagen/ADP-induced closure times using the PFA-100 system reveal that CEPI-CT and CADP-CT were prolonged by sirtinol. The platelets aggregation regulated by physiological agonists such as: thrombin, collagen and AA and U46619 were significantly inhibited by sirtinol. Increases cAMP level was observed when sirtinol treated with Prostaglandin E1 in washed platelets. Moreover, sirtinol attenuated intracellular Ca^{2+} release and thromboxane B2 formation stimulated by thrombin, collagen, AA and U46619 in human washed platelets. This study indicated that sirtinol could inhibit the platelet aggregation induced by physiological agonists, AA and U46619. The mechanism of action may include an increase of cAMP level with enhanced VASP-Ser157 phosphorylation via inhibition of cAMP phosphodiesterase activity and subsequent inhibition of intracellular Ca^{2+} mobilization, thromboxane A2 formation, and ATP release during the platelet aggregation.

1. Introduction

A cohort of molecular signaling pathways participated in platelet activation, playing a crucial role in repertoire of the physiological and pathological functions, homeostasis and thrombosis in blood cells. Sirtinol (Sir two inhibitor naphthol), is a cell permeable six-membered lactone ring and derived from β-naphthol compound (2-hydroxy-1-naphthoic acid) Fig. 1, which mediates as a suppressors of sirtuin of member of histone deacetylases (HDAC) protein family [1–4]. We have recently promulgated the putative role of sirtinol on the hepatic injury and trauma-hemorrhage and it is associated with pro-inflammatory mediators such as: interleukin (IL)-6, intercellular adhesion molecule (ICAM)-1 and cytokine-induced neutrophil chemoattractant (CINC)-1, CINC-3 and also its crucial role against the hepatic injury [5]. Ota and colleague have divulged that sirtinol stimulates the senescence-like growth arrest via the activity of senescence associated β-galactosidase and the activation of Ras-MAPK signaling pathway, and it also initiate the expression plasminogen activator inhibitor 1 (PAI-1) in tumor cells [6]. Several studies have reported that the underlying mechanism of the platelets activation and aggregation are very important to prevent loss of blood at damaged site of blood vessels. Moreover, regulation of platelet inhibition is also one of the pivotal strategies for prevention of blood loss and treatment of disease. Platelets aggregation is correlated with the intracellular platelets granules contents which release physiological agonists, serotonin and nitric oxide (NO) to regulate platelets aggregation [7,8]. Most of the

* Corresponding author at: Department of Anesthesiology, Chang Gung Memorial Hospital, No. 5, Fushing Road, Gueishan Taoyuan, 333, Taiwan, ROC. Tel.: +886 3 3281200x3624; fax: +886 3 3281200x2787.
E-mail address: yd3j@adm.cgmh.org.tw (Y.-J. Day).

Abbreviations: SIR2, silent information regulator 2; TXB2, thromboxane B2; AA, arachidonic acid; HDAC, histone deacetylase; SIRT1, sirtuin1; FITC, fluorescein isothiocyanate; CEPI-CT, collagen/epinephrine closure time; CADP-CT, collagen/ADP closure time; PGE1, prostaglandin E1; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; SNP, sodium nitroprusside; IBMX, 3-isobutyl-1-methylxanthine; BSA, bovine serum albumin; EDTA, ethylenediamine tetraacetic acid; DMSO, dimethylsulfoxide; PRP, platelet-rich plasma; PPP, platelet-poor plasma; PKA, protein kinase A; PKG, protein kinase G; PDE, phosphodiesterase; EHNA, erythro-9-(2-hydroxy-3-nonyl)adenine; VASP, vasodilator-stimulated phosphoprotein.

0006-2952/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2009.01.011

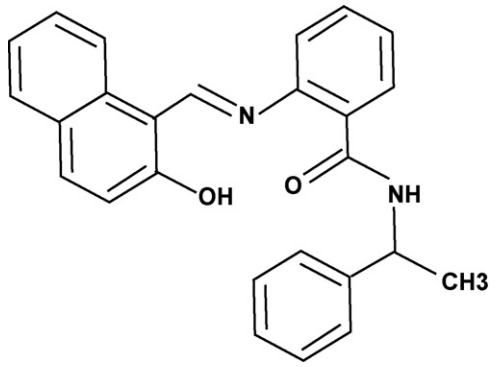

Fig. 1. Chemical structure of sirtinol.

physiological agonists such as ADP, ATP, thrombin and collagen play a broad range of physiological events in platelets. Adenine nucleotides regulate the cell membrane receptors such as P2X1 receptors and P2Y1 receptors coupled with phospholipase C (PLC) via G_q/G_i signaling pathways [9]. ATP and ADP stimulate the degradation of phosphotidylinositol bisphosphate (PIP) to inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG) through the PLC signaling pathways. The transient accumulation of IP3 promotes the calcium release from intracellular dense granules contents and the enhanced Ca^{2+} inturn stimulates the arachidonic acid through phospholipase A2 (PLA2) and DAG involved the phospho kinase C (PKC) activation.

In platelets, release of intracellular dense granules contents and the suppression of platelets aggregation are responsible for the effect of all aggregating agents including the enhanced cAMP levels. The ADP and ATP stimulate prostaglandin (PGE1) which is potent inhibitor of platelet aggregation. Adenylyl cyclase is directly activated by PGE1, and conversion of cAMP to AMP is restrained by the inhibitors of phosphodiesterase. Prostaglandin stimulates adenylate cyclase via the Gs protein, resulting in activation of cAMP-dependent protein kinase A (PKA). PKA phosphorylates could down-regulate Ca^{2+} mobilization and $\alpha IIb\beta 3$ integrin activation. The cAMP turnovered by cyclic nucleotide phosphodiesterases (PDEs) could regulate platelet activation. Platelets contain at least three types of PDEs. In human platelets, specific inhibition of PDE3 but not of PDE5 inhibited thrombin-induced Ca^{2+} responses. The effect of general PDE or PDE3 inhibition was accompanied by an increase in cAMP, and potentiated by Gs stimulation with prostaglandin E1 [8,10–13]. The enhanced activity of adenylate cyclase or inhibition of cAMP phosphodiesterase promotes the elevation of intracellular cAMP. In the presence of cAMP, the activation of phospholipase C, which results in the production of IP3 and diacylglycerol, is depressed by the agonist, leading to the inhibition of Ca^{2+} influx and PKC-dependent phosphorylations. Elevated cAMP is also shown to decrease the Ca^{2+} influx activated by thromboxane and the binding of thrombin to its receptor on human platelets [14]. It is well-known that cAMP is one of the most important mediators in platelet activation and an increase in intracellular cAMP leads to inhibition of platelet activation and release of granule contents, ATP release, synthesis of thromboxane and calcium efflux upon stimulation [15–17] with subsequently suppression of platelet function.

G-protein coupled receptors, agonists and thromboxane A2 mediates the mobilization of calcium in platelets. Ca^{2+} is a key regulator of several physiological incidents including the platelet shape change, aggregation, secretion and blood coagulation activities and its release activate several signaling pathways including the PLC mediated phosphoinositol signaling pathway [16]. Decreased intracellular calcium participates in the attenuation of platelets aggregation [18,19]. Inhibition of platelets function is a great hall mark therapeutic strategy for prevention of blood loss and for the treatment of variety of diseases. Conclusion: in this study, we examined the crucial role of sirtinol on normal human platelets and this knowledge can be utilized to characterize the possible mechanisms of sirtinol on platelets aggregation and for future studies.

2. Materials and methods

2.1. Blood sampling

Healthy volunteers without history of hematological diseases such as platelet or coagulation disorder, and without taking medication that might affect hematological function, were recruited for this study. Each volunteer was first signed the inform consent and then subjected to 30 ml blood drawn. All the experimental procedures and protocols were approved by the Institutional Review Board of Chang Gung Memorial Hospital (Linko, Taiwan, ROC)

2.2. Chemicals

All the chemicals used in these experiments were of the highest purity grade available from each supplier. Sirtinol, EHNA (erythro-9-(2-hydroxy-3-nonyl)adenine), cilostamide, dipyridamole and zaprinast were obtained from Calbiochem (Darmstadt, Germany). Thrombin, collagen, arachidonic acid, ATP standard and Chrono Lume were purchased from Chrono-Log Co. (PA, USA). ADP, U46619, epinephrine, indomethacin, apyrase, prostaglandin E1 (PGE1), sodium nitroprusside (SNP), 3-isobutyl-1-methylxanthine (IBMX), bovine serum albumin (BSA), ethylenediamine tetraacetic acid (EDTA) and dimethylsulfoxide (DMSO) were obtained from the Sigma Chemical Co. (St. Louis, MO, USA). Fluo-3 AM was bought from Molecular Probes (Eugene, Oregon, USA). Fluorescein isothiocyanate (FITC) and CD62P (P-selectin) antibody were obtained from BD Biosciences (San. Jose, CA, USA). Cyclic AMP, cyclic GMP and thromboxane B2 EIA kits were purchased from Cayman Chemical (Ann Arbor, USA). Ser157-phosphorylated vasodilator-stimulated phosphoprotein (VASP) antibody (#3111), Ser239-phosphorylated VASP antibody (#3114), and total VASP antibody (#3112) were purchased from Cell Signaling Technology (Danvers, MA, USA). Collagen/epinephrine (CEPI) and collagen/ADP (CADP) cartridges were purchased from Dade Behring, Inc. (Newark, DE, USA). Sirtinol was dissolved in dimethyl sulfoxide (0.5% DMSO) and stored at −20 °C until use.

2.3. Preparation of platelets

Healthy volunteers without taken any regimens within two weeks were subjected to 30 ml blood drawn. Blood samples were collected in 50 ml sample tubes containing anticoagulant with final concentration of 3.15% sodium citrate (1:9, v/v). Platelet-rich plasmas (PRP) were prepared by centrifuge the blood samples at 300 g for 10 min and collected the supernatants. The remaining samples were further subjected to another centrifugation at 850 g for 15 min and platelet-poor plasma (PPP) was harvested by collecting the upper layer of the samples. Washed platelets were prepared from prepared PRP which first centrifuged at 1050 g for 6 min at room temperature. The platelet pellets were washed with modified Tyrode-Hepes buffer (129 mmol/l NaCl, 2.8 mmol/l KCl, 8.9 mmol/l $NaHCO_3$, 0.8 mmol/l $MgCl_2$, 0.8 mmol/l KH_2PO_4, 2 mmol/l EGTA, 5.6 mmol/l glucose, 10 mmol/l Hepes, 0.35% BSA, pH 7.4) and then centrifuged at 1050 g for another 6 min. Finally, platelets were gently resuspended in Tyrode-Hepes buffer (129 mmol/l NaCl, 2.8 mmol/l KCl, 8.9 mmol/l $NaHCO_3$, 0.8 mmol/l $MgCl_2$, 0.8 mmol/l KH_2PO_4, 1 mmol/l $CaCl_2$, 5.6 mmol/l glucose,

10 mmol/l Hepes, 0.35% BSA, pH 7.4). The platelet concentrations were counted by Drew Hemavet 950FS® analyzer (Drew Scientific, Oxford, CT, USA) and adjusted to a concentration of 2×10^8 platelets/ml.

2.4. PFA-100 analyzer studies

To study the clot formation under the dynamic situation, we deployed the PFA-100 analyzer (Dade Behring, Marburg, Germany) for the study. Citrated blood samples were first sit at room temperature for 10 min and then incubated with sirtinol 50 and 100 μM 3 min or vehicle only before analysis. All blood samples were tested for closure times with collagen/epinephrine and collagen/ADP cartridges according to the manufacturer's instructions. The time required to obtain full occlusion of the aperture is defined as the collagen/ADP closure time (CADP-CT) or collagen/epinephrine closure time (CEPI-CT). All the samples were tested no later than 1 h after blood drawn and the upper limit closure time was set to 300 s by defaults.

2.5. Platelet aggregation and ATP release reaction

Both the platelets aggregation and release of ATP from platelets were simultaneously measured by Lumi-aggregometer Model 560 (Chrono-Log, Havertown, PA, USA). Washed platelets (or PRP) were incubated and stabilized at 37 °C in an aggregometry sample tube with stirring at 1000 rpm for 1 min before testing. The samples were further pretreated with sirtinol or vehicle for 3 min and then the platelets aggregation were induced by each of collagen (2 μg/ml), AA (5 mM), thrombin (0.1 U/ml), ADP (10 μM) and U46619 (2 μM). The resulting aggregations measured as the change of light transmission were recorded for 10 min. The extent of inhibition of platelet aggregation is expressed as %. The ATP released from platelets was detected by the Chrono Lume luciferase reagent according to the manufacturer's directions and gain control set at 0.005×. The final volume was made up to 0.5 ml. In order to eliminate the effect of the solvent on the aggregation and release reaction of platelets, the final concentration of DMSO was fixed at 0.5%. Platelets aggregation pretreated with PDE inhibitors IBMX (6 μM) (PDE general), EHNA (100 μM) (PDE II), cilostamide (50 μM) (PDE III), dipyridamole (20 μM) (PDE V), zaprinast (100 μM) (PDEV) and sirtinol of various concentration (25–100 μM) with or without PGE1 (4×10^{-3} μM) induced by thrombin (0.1 U/ml) were also recorded.

2.6. Measurement of Ca^{2+} influx

PRP was prepared as described above. After centrifugation of PRP at 1050 g for 6 min at room temperature, the platelet pellet was resuspended in Tyrode's buffer containing no calcium, at a density of 3×10^8 platelets/ml. Platelets were loaded with 5 μM/L fura-3/AM for 30 min at 37 °C in the dark, washed in Tyrode's buffer containing 0.35% human serum albumin, and finally resuspended at 22 °C, at a density of 3×10^8 platelets/ml, in Tyrode's buffer containing 0.1% bovine serum albumin. Aliquots of fura-3/AM-loaded platelets were transferred to a 10 mm × 10 mm quartz cuvette and prewarmed to 37 °C for 1 min, and fluorescence measurements were performed under continuous stirring. Measurement of the cytosolic Ca^{2+} concentration was performed using a Hitachi F4500 fluorescence spectrophotometer by a single wavelength program (excitation was measured at 505 nm, emission at 525 nm).

2.7. Platelet cAMP and cGMP level assay

Washed human platelets (5×10^8 platelets/ml) were warmed at 37 °C for 1 min in an aggregometer with stirring at 1000 rpm prior to incubation with prostaglandin E1 (1 μM), SNP (200 μM), IBMX (1 mM) or various concentrations of sirtinol (25, 50, 100 μM) and prostaglandin E1 (0.5 μM), SNP (100 μM) or IBMX (0.5 mM) plus sirtinol (25 μM) for 3 min. The reaction was stopped by addition of 10 mM EDTA and then boiled for 3 min. Upon cooling to 4 °C, precipitated protein was sediment by centrifugation in an eppendorf microcentrifuge. The supernatant was used to assay for cAMP and cGMP by enzyme immunoassay kit. In our experiment, IBMX was a known cyclic AMP, cyclic GMP phosphodiesterase inhibitor, prostaglandin E1 was an adenyl cyclase activator, and SNP was a guanylyl cyclase activator. The level of cAMP in sirtinol (25–100 μM) and PDE inhibitors IBMX (0.5 mM), EHNA (100 μM), cilostamide (50 μM), dipyridamole (20 μM) and zaprinast (100 μM) with or without PGE1 (0.5 μM) were measured.

2.8. Thromboxane B2 assay

Wash platelets were prepared as described above. PLT were pretreated with sirtinol 25, 50 or 100 μM for 3 min and addition of agonist thrombin (0.1 U), U46619 (2 μM), collagen (2 μg/ml), AA (5 mM) or ADP (10 μM) incubated at 37 °C for 7 min. Stop reaction at 10 min by 2 mM EDTA and 50 mM indomethacin. Sample was separated by centrifugation at 12000 g for 10 min at 4 °C. Remove pellet and supernatants stored and frozen at −80 °C. We measured the concentration of TXB2 using an enzyme immunoassay kit. The level of TXB2 in sirtinol (25–100 μM) and PDE inhibitors IBMX (0.5 mM), EHNA (100 μM), cilostamide (50 μM), dipyridamole (20 μM) and zaprinast (100 μM) with or without PGE1 (0.5 μM) was also recorded.

2.9. P-selectin expression

To study platelet surface expression of P-selectin (CD62P) on resting and activated platelets, FACScan instrument (Becton Dickinson, San Jose, CA) was used. Non-stirred washed platelets (3×10^8/ml) were incubated in the dark at 22 °C with CD62P conjugated fluorescein isothiocyanate monoclonal antibody for 10 min and then incubated at 37 °C with sirtinol (25, 50 or 100 μM) or without for 5 min. After treatment, the platelet were activated by agonist thrombin (0.1U) or U46619 (2 μM) 37 °C for 5 min and analysis was performed using flow cytometry.

2.10. Western blotting analysis

Washed human platelets (3×10^8 platelets/ml) were warmed at 37 °C for 1 min in an aggregometer and stirred at 1000 rpm, before incubated with various concentrations of sirtinol (12.5, 25, 50, 100 μM), prostaglandin E1 (0.2 μM) and IBMX (0.2 mM) for 3 min. The reaction was stopped by placing the platelets on ice and subjected them to immediate centrifugation. The pellet was re-suspended in 1× Laemmli sample buffer. After boiling for 10 min, the proteins were stored in −80 °C for immunoblotting assay. The sample was electrophoresed in 8–10% SDS-polyacrylamide gels and transferred electrophoretically to nitrocellulose. Blots were stained with Ponceau-S (0.2% Ponceau-S 3% TCA, and 3% sulfosalicylic acid) to visualize Mr markers and then destained with deionized water followed by TBST (10 mM Tris–HCl (pH 7.5), 100 mM NaCl, and 0.1% Tween-20). Blots were blocked for 1 h in 5% nonfat milk in TBST. Antibodies were diluted in PBS (pH 7.3) containing 3% bovine serum albumin and 0.02% sodium azide. Blots were incubated with the appropriate antibody (Phospho-VASP (Ser157), Phospho-VASP (Ser239) and VASP; 1/1000) for 2 h at 25 °C and then thoroughly washed (three times, 10 min each) with TBST. Next, blots were incubated for 1 h with an appropriate horseradish peroxidase-conjugated secondary antibody (1/5000)

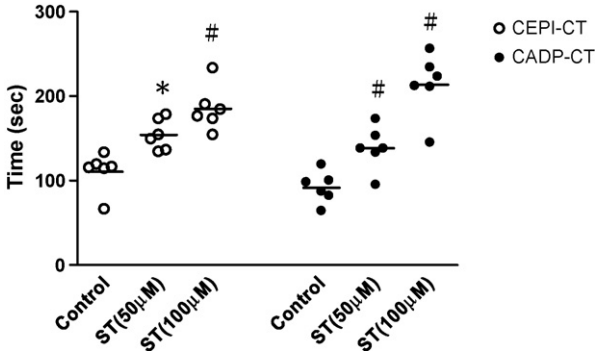

Fig. 2. Effect of sirtinol on human whole blood by platelet function analyzer. Closure time was measured with collagen/epinephrine (CEPI) or collagen/ADP (CADP) cartridge. Any closure time greater than 300 s was reported as 300 s.The mean control CEPI-CT was 110.5 s (range, 66–133 s) and CADP-CT was 91.7 s (range, 64–119 s). Sirtinol (50 and 100 μM) mean CEPI-CT was 154.0 s (range, 134–178 s) and 185 s (range, 154–233 s), CADP-CT was 138.3 s (range, 95–173 s) and 213.5 s (range, 145–256 s). Both CEPI-CT and CADP-CT closure time were significantly prolonged on sirtinol 50 and 100 μM. Data are shown as mean ± SD ($n = 6$). $^{\#}p < 0.05$ compared to control (DMSO 0.5%); $^{*}p < 0.05$ compared to sirtinol 50 μM.

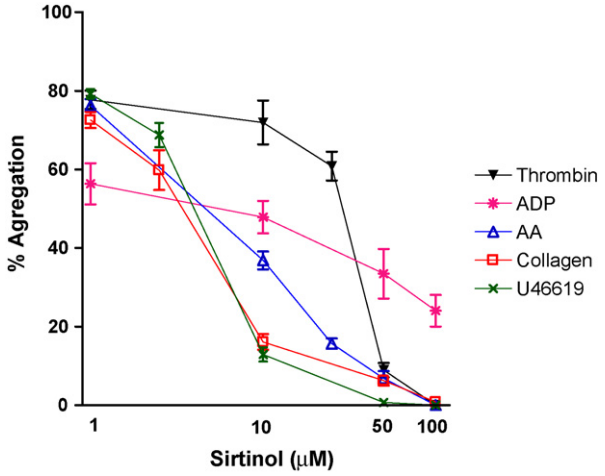

Fig. 3. Effect of sirtinol on washed human platelet aggregation. Dose-dependent inhibition by sirtinol of platelet aggregation induced by collagen (2 μg/ml), AA (0.5 mM), thrombin (0.1 U/ml), U46619 (2 μM) and ADP (10 μM) with an IC50 of 6.74 ± 0.58, 9.91 ± 1.13, 29.82 ± 4.02, 6.18 ± 0.70 and 66.93 ± 17.64 μM respectively. Values are shown as mean ± SD ($n = 6$).

in 5% nonfat milk in TBST, washed thoroughly and examined by enhanced chemiluminescence.

2.11. Statistical analysis

The experimental results are expressed as means ± SD. Statistical analysis was performed with analysis of variance ANOVA. Differences between the sample-treated group and control group were analyzed by Student's *t*-test. If the analysis showed significant differences among the group means, then each group was compared by the Newman–Keuls method. Results were considered significantly different at a value of $p < 0.05$.

3. Results

3.1. Sirtinol restrained clot formation with prolonged CEPI-CT and CADP-CT

We first examined whether sirtinol would have any effects on the normal coagulation and platelet function by PFA-100 following the rational that CEPI cartridges detect qualitative platelet defects while CADP cartridges detect only thrombocytopathies. Fig. 2 summarized the results of PFA-100 analysis for the effect of sirtinol on blood coagulation. As expected, both CEPI-CT and CADP-CT were significantly prolonged by sirtinol, whereas the CEPI-CT were 154.0 ± 18.4 SD, 185.0 ± 26.5 SD (control = 110.5 ± 22.9 SD) and CADP-CT were 138.3 ± 25.8 SD, 213.5 ± 37.5 SD (control = 91.7 ± 18.6 SD) with sirtinol 50, 100 μM separately. These findings raised a plausible conclusion that the sirtinol-induced prolongation of the blood coagulation might be mediated by direct suppression of normal platelet function

3.2. Sirtinol inhibited the aggregation and ATP release in washed platelet with dose-dependent manner

To study the possible suppressive effect on platelet function, we examined the influence of sirtinol on washed platelet aggregation and ATP release by Lumi-aggregometer. As shown in Fig. 3, our results demonstrated that sirtinol significantly inhibited the washed platelet aggregation triggered by each of collagen (2 μg/ml), AA (0.5 mM), thrombin (0.1 U/ml), U46619 (2 μM) and ADP (10 μM) respectively. Similar inhibitory results were also observed in ATP release induced by collagen and thrombin (Fig. 4) as well as induced by AA and U46619. Both sirtinol-induced suppression were in a dose-dependent manner

3.3. Sirtinol inhibited the intracellular calcium mobilization

In order to realize the possible mechanism of sirtinol-induced platelet suppression, we further studied the interaction between calcium mobilization and observed platelet suppression. Normally the increased intracellular calcium triggered by each of collagen (2 μg/ml), AA (0.5 mM), thrombin (0.1 U/ml) and U46619 (2 μM) respectively in the fluo-3 loaded platelets could be detected by fluorescence spectrophotometer (Fig. 5). However, these fluorescence signals were attenuated significantly in the sirtinol pretreated (50 μM, 3 min) fluo-3 loaded platelets with triggering agents described above. Furthermore, this attenuation of intracellular calcium release correlated well with observed suppression of platelet aggregation and ATP release. Therefore, sirtinol-induced platelet suppression is relevant to the mechanisms of calcium mobilization

3.4. Sirtinol and PGE1 increased intracellular cAMP level synergistically, but not cGMP

To realize the sirtinol-induced calcium mobilization suppression, we further measured the platelet cAMP level while interacted with different reagents. Our results were shown in Fig. 6A, in which sirtinol had mild effect on the basal cAMP level of resting platelets but significantly augmented the PGE1-induced elevation of intracellular cAMP in a synergistic manner. Though elevated intracellular cAMP was observed in IBMX-treated platelets, however, there was no further sirtinol-attributed cAMP increasing observed in sirtinol-IBMX-treated platelets. The cAMP level was significantly increased by sirtinol concentration in a dose-dependent manner and PDE inhibitors IBMX and cilostamide with or without PGE1, but not by PDE inhibitors EHNA, dipyridamole, and zaprinast (Fig. 6B). We further tested these results by platelet aggregation analysis as demonstrated in Fig. 7, whereas the synergistic suppression of thrombin-induced washed platelet aggregation was observed only in combination of sirtinol and PGE1 but not in other combinations. Similar experiments were conducted to study the influence of sirtinol on the alteration of intracellular cGMP. However, no significant influence or additive effect was noted in each combination between sirtinol, SNP and IBMX (Fig. 6A)

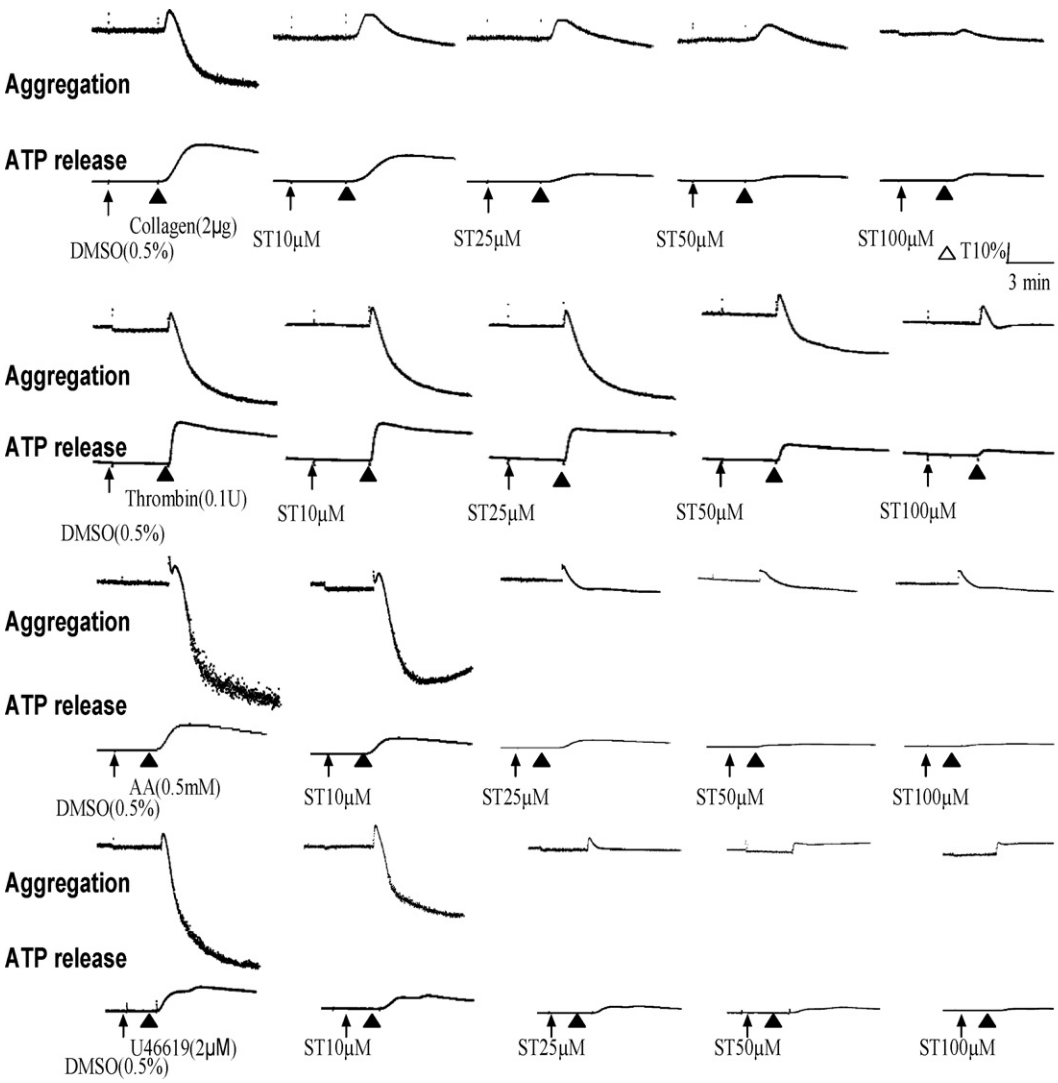

Fig. 4. Effect of various concentrations of sirtinol on the platelets aggregation and ATP release induced by collagen and thrombin. Washed human platelets were preincubated with sirtinol (10–100 μM), and then collagen (2 μg/ml), thrombin (0.1 U/ml), AA (0.5 mM) and U46619 (2 μM) was added to induced platelet aggregation and ATP release.

3.5. Sirtinol attenuated thromboxane B2 in platelets induced by collagen, AA, thrombin and U46619 but not ADP

We further studied whether sirtinol would affect intracellular thromboxane A2 level upon different stimulations. Due to the fast metabolism of thromboxane A2, therefore, we detected the thromboxane B2, a stable metabolite of thromboxane A2, instead. As the results shown in Table 1, thromboxane B2 were elicited by each of collagen (2 ug/ml), AA (0.5 mM), thrombin (0.1 U/ml) and U46619 (2 μM) but not by ADP. Sirtinol significantly attenuated the thromboxane B2 production described in above experiments, whereas the most prominent inhibition was observed in collagen-induced thromboxane B2 formation. No other considerable inhibition of thromboxane B2 production was observed in ADP triggered and resting washed platelets (Table 1). The thromboxane B2 level was significantly decreased by sirtinol concentration in a dose-dependent manner (25, 50, 100 μM) and PDE inhibitors IBMX and cilostamide with PGE1. (Supplementary Figure 1)

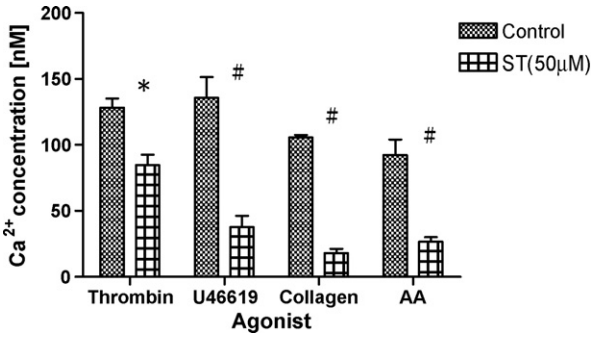

Fig. 5. Effect of sirtinol on washed human platelet intracellular calcium mobilization. Sirtinol (50 μM) inhibited the platelet intracellular calcium concentration, in fluo-3 loading platelets that induced by collagen (2 μg/ml), AA (0.5 mM), thrombin (0.1 U/ml), U46619 (2 μM). Data are shown as mean ± SD ($n = 6$). $^{*}p < 0.05$, $^{\#}p < 0.01$ compared to sirtinol 50 μM.

3.6. Sirtinol inhibited the washed platelet P-selectin expression by thrombin and U46619 in with dose-dependent manner

For further proof that the sirtinol could inhibit the intraplatelet granules substance release. We examinated the platelet surface P-selection expression was induced by thrombin (0.1 U/ml) and U46619 (2 μM) under various sirtinol

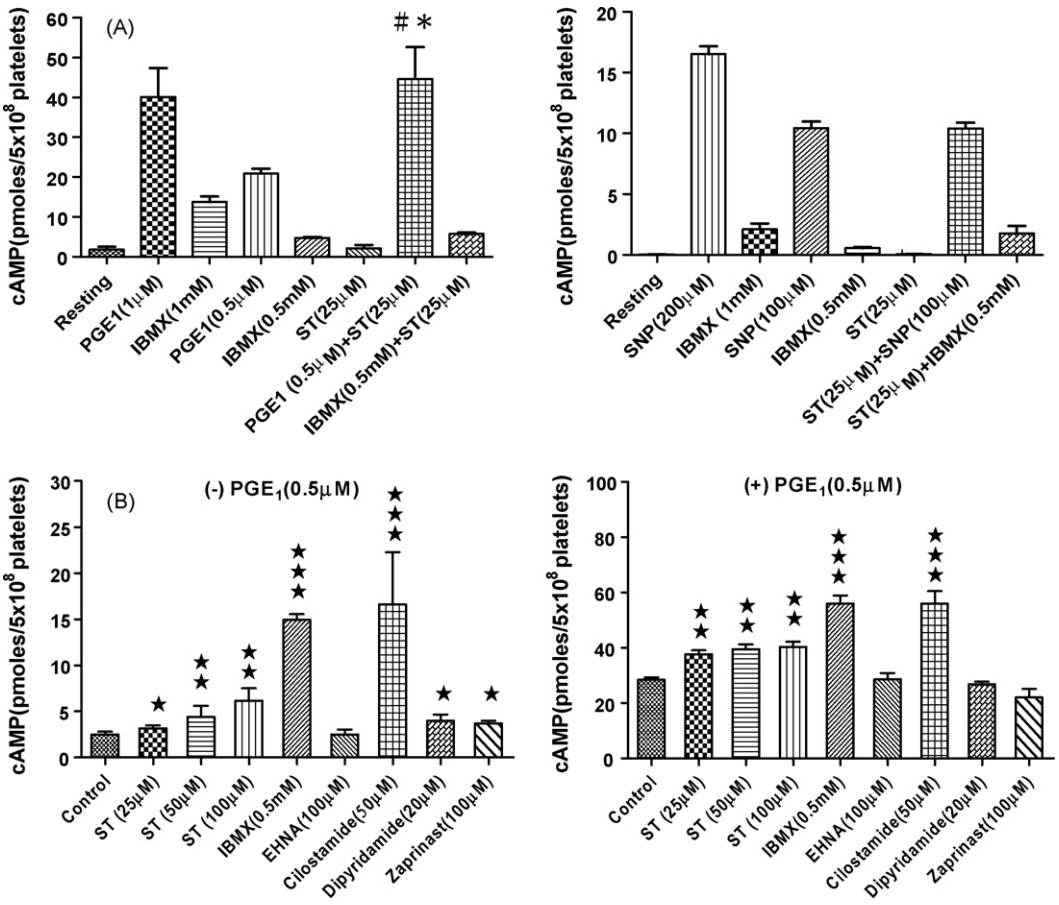

Fig. 6. Effect of sirtinol cAMP and cGMP Level in the resting washed Human Platelets. (A) The level of cyclic AMP in unstimulated platelets was 1.77 ± 1.72 pmol/5×10^8 platelets, there was significantly increased the cAMP level (44.62 ± 26.53 pmol/5×10^8 platelets) in sirtinol (25 μM) combined with PGE1 (0.5 μM), not with IBMX. $^*p < 0.01$ compared to sirtinol 25 μM (2.15 ± 2.05 pmol/5×10^8 platelets); $^\#p < 0.05$ compared to PGE1 0.5 μM (20.89 ± 2.91 pmol/5×10^8 platelets). No significant difference on the cGMP level, between the combination of sirtinol (25 μM) with PGE1 (0.5 μM) or IBMX (0.5 mM) compared to each single drug. Data are shown as mean ± SD. ($n = 6$); (B) The level of cAMP in sirtinol (25–100 μM) and PDE inhibitors IBMX (0.5 mM), EHNA (100 μM), cilostamide (50 μM), dipyridamole (20 μM) zaprinast (100 μM) with or without PGE1 (0.5 μM) were measured. Statistically significant at $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$ compared with control (DMSO 0.5%). Data are shown as mean ± SD. ($n = 4$).

concentration (25, 50 or 100 μM). Sirtinol significant reverse the thrombin-induced P-selectin expression by dose-dependence (Fig. 8)

3.7. Sirtinol increased the washed platelet VASP-Ser157 phosphorylation

For proof that the sirtinol could increase cAMP level with down-regulation VASP phosphorylation. We examinated the platelet phospho-VASP (Ser157), phospho-VASP (Ser239) and total VASP expression under various sirtinol concentration (12.5, 25, 50 or 100 μM). Sirtinol significantly enhanced the phosphorylation expression of total VASP and VASP-Ser157 in a dose-dependent manner (Fig. 9)

4. Discussion

Platelets play an important role in controlling blood loss through their adhesion to damaged blood vessels and the release of substances that act to further enhance aggregation [20,21]. In this report, we have primary examined the human whole blood coagulation affected by sirtinol using PFA-100 analyzer. Our results demonstrated that sirtinol delayed the coagulation by direct suppression of platelet function. In the subsequent aggregation

Table 1
Effects of sirtinol on the thromboxane B2 formation of washed human platelets.

	Collagen	AA	Thrombin	U46619	ADP
Thromboxane B_2 concentration (ng/ml)					
Control	17.46 ± 1.92	238.65 ± 15.84	6.07 ± 0.62	1.25 ± 0.11	1.76 ± 0.12
Sirtinol					
25 μM	6.61 ± 0.06$^\#$	96.40 ± 8.14$^\#$	5.57 ± 0.47	1.08 ± 0.12*	
50 μM	0.48 ± 0.03$^\#$	77.68 ± 7.20$^\#$	3.71 ± 0.45$^\#$	0.64 ± 0.12$^\#$	1.70 ± 0.13
100 μM		60.38 ± 5.26$^\#$	2.65 ± 0.27$^\#$		1.59 ± 0.12
Imidazole 1 mM	10.39 ± 0.83	53.78 ± 3.98	2.63 ± 0.33	1.14 ± 0.10	1.45 ± 0.16
Indomethacin 5 μM	0.48 ± 0.05	19.88 ± 2.60	3.01 ± 0.44	0.99 ± 0.13	0.94 ± 0.11

Sirtinol or control (0.5% DMSO) was preincubated with platelets, and then induced by collagen (2 μg/ml), AA (0.5 mM), thrombin (0.1 U/ml), U46619 (2 μM) and ADP (10 μM). The thromboxane B2.values are presented as mean ± SD ($n = 6$). Statistic significant at $^*p < 0.05$, $^\#p < 0.005$ compared with the control (DMSO 0.5%). The thromboxane B2 level of resting platelets was 0.88 ± 0.04.

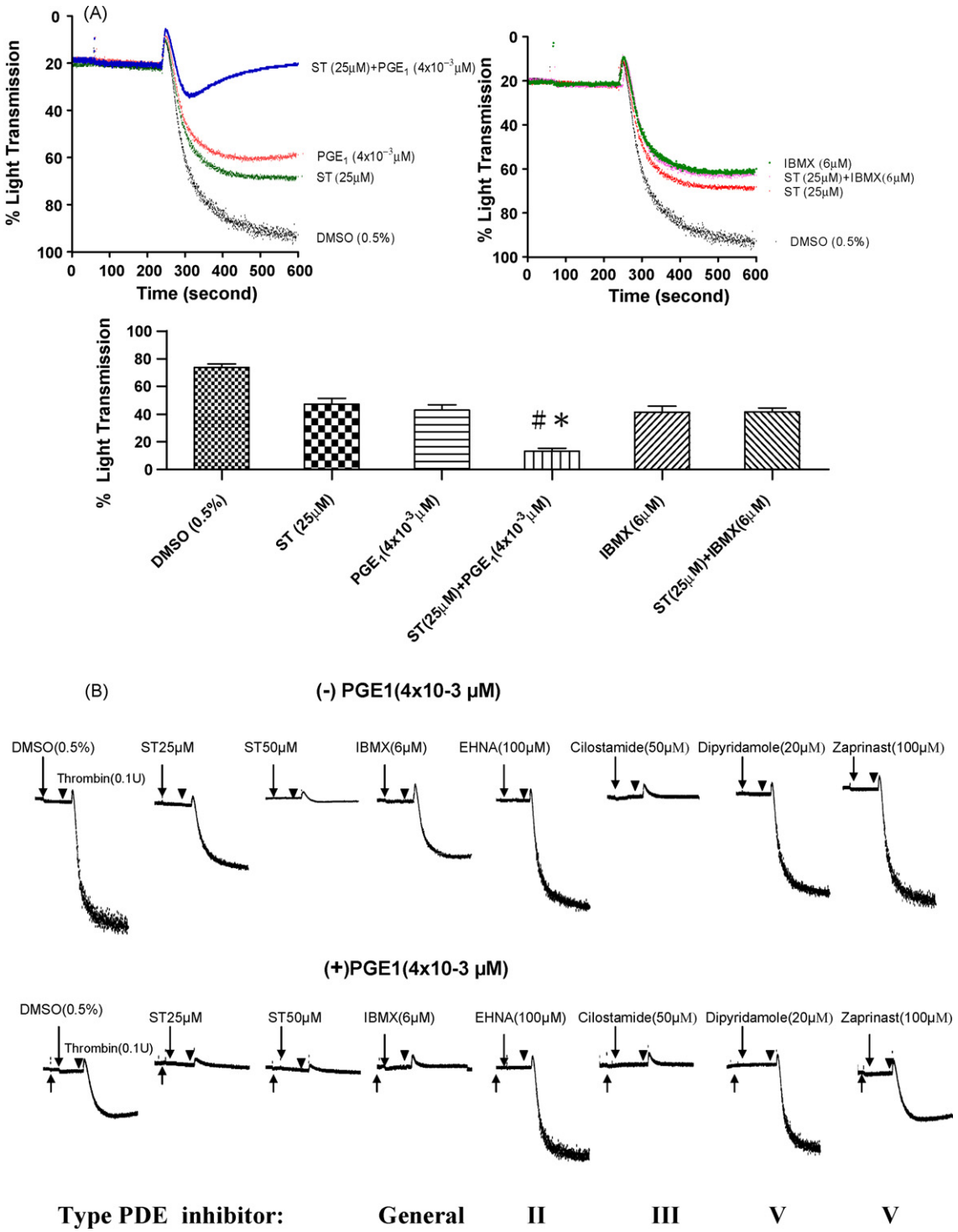

Fig. 7. (A) Effect of the combination of sirtinol (ST) with PGE1 or IBMX on washed human platelets aggregation is induced by thrombin 0.1 U with significant inhibited by the combination of sirtinol (25 μM) with PGE1 (4×10^{-3} μM), but not by the combination of sirtinol (25 μM) with IBMX (6 μM) $^{*}p < 0.005$ compared to sirtinol 25 μM; $^{\#}p < 0.005$ compared to PGE1 (4×10^{-3} μM). (B) Effect of sirtinol and PDE inhibitors on aggregation of washed human platelets are induced by thrombin 0.1 U with or without PGE1 (4×10^{-3} μM). Data are shown as mean ± SD. ($n = 6$).

and other functional analysis, we demonstrated that this platelet suppression effect of sirtinol is probably due to its naphthoic structure which direct inhibits the cyclic AMP phosphodiesterase activity.

Platelet activation is the result of a complex signal transduction cascade reaction mediated by various stimulants. Several potential mechanisms for inhibition of platelet aggregation have been suggested [12,22]. These, include increased cAMP [23,24], synthesis of prostacyclin analogues [25,26], inhibition of adenosine receptor [11] and thromboxane synthase [27,28]. One important mediator in regulating platelet activation is the level of cyclic AMP. It is well-known that an increased intracellular cyclic AMP level results in inhibition of platelet activation, adhesion, and release of granule contents [29–31]. The level of intracellular cyclic AMP is maintained by the homeostasis between the rate of synthesis mediated by adenylate cyclase and the rate of

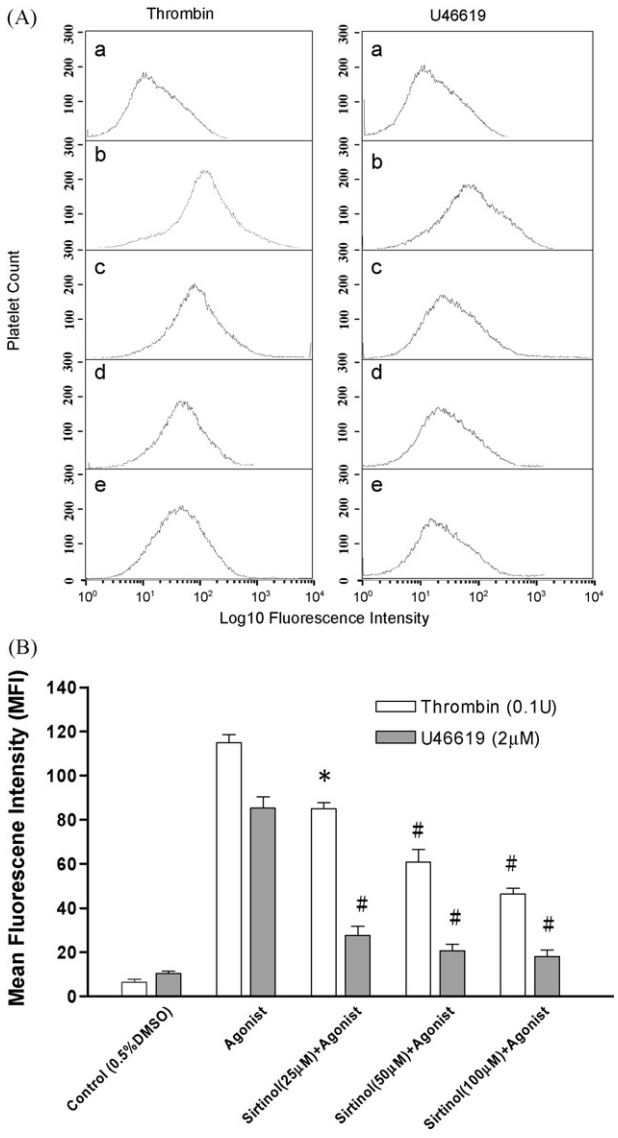

Fig. 8. Effect of sirtinol on washed human platelets surface P-selectin expression is induced by thrombin (0.1 U/ml) and U46619 (2 μM). Fluorescence intensity graph (A) and values (B) of P-selectin expression. (a) control (T: 6.37 ± 3.41; U: 10.35 ± 2.34); (b) agonist (T: 115.00 ± 8.01; U: 85.27 ± 11.41); (c) sirtinol (25 μM) with agonist (T: 85.01 ± 6.25; U: 27.69 ± 8.89); (d) sirtinol (50 μM) with agonist (T: 60.87 ± 12.90; U: 20.72 ± 6.57); (e) sirtinol (100 μM) with agonist (T: 46.40 ± 5.67; U: 18.16 ± 6.67). Data are shown as mean ± SD. (n = 5). $^{*}p < 0.01$, $^{\#}p < 0.005$ compared to agonist. T = thrombin (0.1 U/ml); U = U46619 (2 μM). Data are shown as mean ± SD. (n = 5).

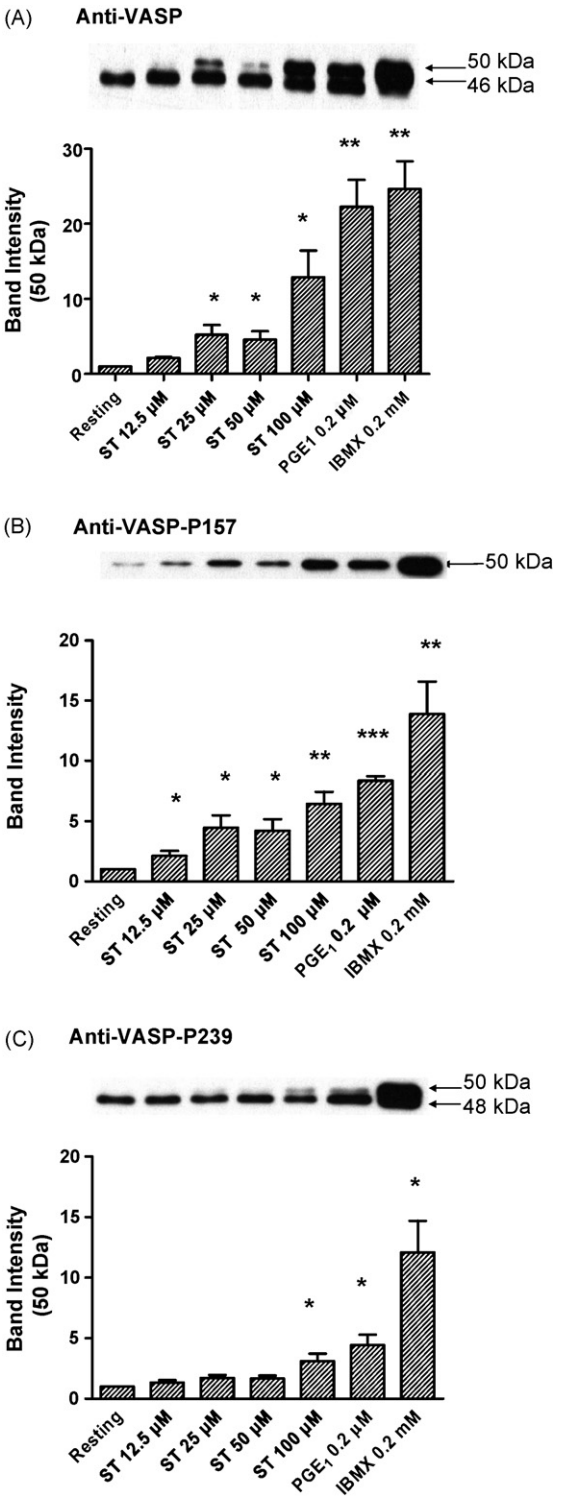

Fig. 9. Effect of sirtinol on VASP expression of washed human platelets. Sirtinol significantly enhanced the VASP phosphorylation level with increased percentage of 50-kDa VASP of total VASP (A) and VASP-Ser157 (B) in a dose-dependent manner. No significant increase of VASP phosphorylation level on VASP-Ser239 except for sirtinol (100 μM) (C) Statistic significant at $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$ compared with control. Data are shown as mean ± SD. (n = 4).

degradation regulated by cyclic AMP phosphodiesterase. Thus, either stimulation of adenylate cyclase activity or inhibition of cyclic AMP phosphodiesterase activity results in elevation of the intracellular cyclic AMP level and subsequently leads to suppression of platelet function. Therefore, to increase the intracellular cyclic AMP has been demonstrated with many inhibitory effects in normal physiological platelet response, which include platelet aggregation, ATP release, thromboxane formation and calcium efflux upon stimulation [15–17].

Our results demonstrated that sirtinol potentiated the PGE1-induced cyclic AMP increase, but not IBMX, in a synergistic manner; though sirtinol alone did not increase intracellular cyclic AMP markedly probably due to low level of cyclic nucleotide within the resting human platelets. These results imply that the sirtinol related cyclic AMP increasing is relevant to the inhibition of phosphodiesterase activity. Interestingly, sirtinol had no potentiating effect on cyclic GMP no matter administered alone or in combination with SNP, which indicates that sirtinol specifically targeted the cyclic AMP phosphodiesterase and modulated the human platelet function in our experiments.

In intact cells, cAMP-enhancing agents such as PGE1 can stimulate phosphorylation of VASP. VASP is an in vitro substrate for both cAMP-dependent protein kinase (PKA) and cGMP-dependent protein kinase (PKG). Three phosphorylation sites have been identified in VASP (Ser157, Ser239, Thr278); PKA has been reported to prefer the Ser157 site, and PKG the Ser239 site [32]. Our result showed that sirtinol could enhance VASP-Ser157 phosphorylation in a dose-dependent manner. Previous study demonstrates a cross-talk of cGMP- and cAMP-signaling pathways in human platelets. However, the cGMP analog–induced VASP phosphorylation is a delayed event requiring more than 10 min. This showed that the enhancement of VASP phosphorylation by sirtinol on Ser157 is mediated by activation of PKA which increased platelet cAMPs.

As the other cell lineages, many of the platelet physiological function and activation are calcium dependent. Thus the mobilization of the cytosolic Ca^{2+} seems to be one of the critical events in platelet activation and aggregation. Therefore, factors that decrease the intracellular calcium often lead to the inhibition of platelet aggregation and activation. Furthermore, the increasing intracellular calcium either by Ca^{2+} influx or releasing from intracellular reservoir is the primitive response of platelet to the various stimuli [33]. Accordingly, agents that inhibit calcium mobilization may suppress the platelet aggregation and subsequent activation [18,19]. Interestingly, cyclic AMP, observed increasing by sirtinol, is one of the many factors that may affect intracellular calcium concentration. Increased cyclic AMP would stimulate protein kinase mediated phosphorylation of ATP-dependent calcium pump and subsequently remove calcium from the cytosol. Besides, cyclic AMP also exerts both phospholipase C inhibition [34] and calcium release from the dense tubular system [35]. Furthermore, our data fully supported that sirtinol would have potentiating effect on activation of adenyl cyclase induced increasing of cAMP. Coincidently, these effects were paralleled to observed anti-aggregation of sirtinol. Taken together, our results indicated that sirtinol suppressed platelet aggregation first by increasing cAMP and subsequently inhibited the increase of intracellular calcium concentration upon activation.

While platelet activated by ADP or collagen, the phosphorylation of $PLC_{\beta\gamma}$ catalyzes the formation of IP3 and DAG with subsequent calcium mobilization. In the mean time, calcium dependent phospholipase A2 is activated which promotes the release of arachidonic acid from the membrane and later the thromboxane A2 production [36,37]. However, the thromboxane A2 per se is also an important mediator amplifying platelet activation, secretion and aggregation. It binds to a G_q and G_i coupled thromboxane receptor $\alpha\beta$ to further potentiate the platelet response to triggering factors and causes platelet to change shape, extend pseudopods, and adhere to other platelets on the damaged surface. Therefore, thromboxane A2 become an important intermediate metabolite to evaluate how the sirtinol exerts the suppressive effect upon activated platelet. However, it is difficult to measure the thromboxane A2 directly due to its fast metabolism. Instead, we measured thromboxane B2, a stable metabolite of thromboxane A2 catalyzed by PGE2, to evaluate the possible signaling pathway interfered by sirtinol. Although it has been demonstrated that cyclic AMP inhibited phospholipase A - mediated arachidonic acid release and subsequent thromboxane B2 formation [38], but did not affect the activity of cyclooxygenase [39] and phosphatidic acid formation [40,41], which mostly explained what we observed the suppression effect of sirtinol on collagen-induced thromboxane B2; however, our data also supported that sirtinol might have direct inhibitory effect on cyclooxygenase or thromboxane synthase activity due to observed arachidonic acid induced thromboxane B2 suppression by sirtinol. The mechanism and the influence of cyclooxygenase or thromboxane synthase suppression by sirtinol remains obscured and will be further investigated in our future studies.

Sirtinol, a synthetic compound, is potent selectively inhibitor of Sir2. Sir2 protein is nicotinamide adenine dinucleotide (NAD^+) dependent protein belongs to the histone deacetylase (HDAC) class III protein family and be found in prokaryotes and all eukaryotes. There are seven human sirtuin type homologs (SIRT1–7) [42]. They are an important member of a group of enzymes that modify chromatin conformation. Sirtinol is a specific inhibitor to SIRT1 and SIRT2. Due to platelet is differential from megakarocyte and not a nuclear cell. The sirtuins almost link metabolic activity and gene expression by means of the histone/protein acetylation activity, including genomic stability, DNA repair, transcriptional silencing, p53-mediated apoptosis, and adipogenesis [4,43,44]. It is implicated that sirtinol to platelet effect through of sirtuins is excluded. So we thought that sirtinol naphthol chemical structure is important factor to affect platelet function.

5. Conclusion

This study evaluated the anti-platelets effect of sirtinol on human platelets. Sirtinol has a potential inhibitor to SIRT1 and SIRT2 and its naphthol structure is a great hallmark to affect platelets function. The critical events of the mobilization of Ca^{2+} and elevation of cAMP regulated by sirtinol suppressed the platelet activation and aggregation. Sirtinol is directly inhibited the intracellular calcium release through ATP which is mediated by agonist, and reducing Ca^+ release leads to inhibition of platelets aggregation and activation. An increased intracellular cAMP mediates several platelet responses including ATP release, thromboxane formation and calcium efflux upon stimulation in the platelets. Sirtinol and PG1 synergistically increased cAMP regulate the PLA signaling pathway. The mechanism action of sirtinol may be to increase the cAMP and suppression of intracellular Ca^{2+} mobilization, TXB2 formation and ATP release which contribute in the platelets aggregation.

Acknowledgements

This work was supported by a research grant from Chang Gung Memorial Hospital of Taiwan (CMRPG350301) and approved by the Institutional Review Board of Chang Gung Memorial Hospital (IRB:95-0904B). The work is attributed to Department of Anesthesiology and Transgenic & Molecular Immunogenetics Laboratory of Chang Gung Memorial Hospital.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2009.01.011.

References

[1] Denu JM. The Sir 2 family of protein deacetylases. Curr Opin Chem Biol 2005;9:431–40.
[2] North BJ, Verdin E. Sirtuins: Sir2-related NAD-dependent protein deacetylases. Genome Biol 2004;5:224.
[3] Frye RA. Characterization of five human cDNAs with homology to the yeast SIR2 gene: Sir2-like proteins (sirtuins) metabolize NAD and may have protein ADP-ribosyltransferase activity. Biochem Biophys Res Commun 1999;260:273–9.
[4] Kruszewski M, Szumiel I. Sirtuins (histone deacetylases III) in the cellular response to DNA damage–facts and hypotheses. DNA Repair (Amst) 2005;4:1306–13.
[5] Liu FC, Day YJ, Liou JT, Lau YT, Yu HP. Sirtinol attenuates hepatic injury: pro-inflammatory cytokine production following trauma-hemorrhage in male Sprague-Dawley rats. Acta Anaesthesiol Scand 2008;52:635–40.

[6] Ota H, Tokunaga E, Chang K, Hikasa M, Iijima K, Eto M, et al. Sirt1 inhibitor, Sirtinol, induces senescence-like growth arrest with attenuated Ras-MAPK signaling in human cancer cells. Oncogene 2006;25:176–85.
[7] Lo B, Honemann CW, Kohrs R, Hollmann MW, Polanowska-Grabowska RK, Gear AR, et al. Local anesthetic actions on thromboxane-induced platelet aggregation. Anesth Analg 2001;93:1240–5.
[8] Packham MA, Kinlough-Rathbone RL, Mustard JF. Thromboxane A2 causes feedback amplification involving extensive thromboxane A2 formation on close contact of human platelets in media with a low concentration of ionized calcium. Blood 1987;70:647–51.
[9] Tolhurst G, Vial C, Leon C, Gachet C, Evans RJ, Mahaut-Smith MP. Interplay between P2Y(1), P2Y(12), and P2X(1) receptors in the activation of megakaryocyte cation influx currents by ADP: evidence that the primary megakaryocyte represents a fully functional model of platelet P2 receptor signaling. Blood 2005;106:644–51.
[10] Hankey GJ, Eikelboom JW. Antiplatelet drugs. Med J Aust 2003;178:568–74.
[11] Boullin DJ, Green AR, Price KS. The mechanism of adenosine diphosphate induced platelet aggregation: binding to platelet receptors and inhibition of binding and aggregation by prostaglandin E 1. J Physiol 1972;221:415–26.
[12] Booyse FM, Rafelson Jr ME. Regulation and mechanism of platelet aggregation. Ann NY Acad Sci 1972;201:37–60.
[13] Feijge MA, Ansink K, Vanschoonbeek K, Heemskerk JW. Control of platelet activation by cyclic AMP turnover and cyclic nucleotide phosphodiesterase type-3. Biochem Pharmacol 2004;67:1559–67.
[14] Cavallini L, Coassin M, Borean A, Alexandre A. Prostacyclin and sodium nitroprusside inhibit the activity of the platelet inositol 1,4,5-trisphosphate receptor and promote its phosphorylation. J Biol Chem 1996;271:5545–51.
[15] Rhee SG, Lee CW, Jhon DY. Phospholipase C isozymes and modulation by cAMP-dependent protein kinase. Adv Second Messenger Phosphoprotein Res 1993;28:57–64.
[16] Siess W. Molecular mechanisms of platelet activation. Physiol Rev 1989;69:58–178.
[17] Geiger J, Nolte C, Butt E, Sage SO, Walter U. Role of cGMP and cGMP-dependent protein kinase in nitrovasodilator inhibition of agonist-evoked calcium elevation in human platelets. Proc Natl Acad Sci USA 1992;89:1031–5.
[18] Kang WS, Chung KH, Chung JH, Lee JY, Park JB, Zhang YH, et al. Antiplatelet activity of green tea catechins is mediated by inhibition of cytoplasmic calcium increase. J Cardiovasc Pharmacol 2001;38:875–84.
[19] Shah BH, Nawaz Z, Pertani SA, Roomi A, Mahmood H, Saeed SA, et al. Inhibitory effect of curcumin, a food spice from turmeric, on platelet-activating factor- and arachidonic acid-mediated platelet aggregation through inhibition of thromboxane formation and Ca^{2+} signaling. Biochem Pharmacol 1999;58:1167–72.
[20] Gerrard JM. Platelet aggregation: cellular regulation and physiologic role. Hosp Pract (Off Ed) 1988;23:89–94. 107.
[21] Mustard JF, Packham MA, Kinlough-Rathbone RL. Platelets, blood flow, and the vessel wall. Circulation 1990;81:I24–7.
[22] Tobelem G. Mechanism of platelet aggregation and mode of action of platelet antiaggregants. Rev Prat 1989;39:2219–22.
[23] Hettasch JM, Le Breton GC. Modulation of Ca^{2+} fluxes in isolated platelet vesicles: effects of cAMP-dependent protein kinase and protein kinase inhibitor on Ca^{2+} sequestration and release. Biochim Biophys Acta 1987;931:49–58.
[24] Wang TY, Hussey CV, Sasse EA, Fobian JE. Human platelet aggregation and cAMP system—cAMP level, adenyl cyclase, phosphodiesterase. Ann Clin Lab Sci 1978;8:403–12.
[25] Demolis JL, Robert A, Mouren M, Funck-Brentano C, Jaillon P. Pharmacokinetics and platelet antiaggregating effects of beraprost, an oral stable prostacyclin analogue, in healthy volunteers. J Cardiovasc Pharmacol 1993;22:711–6.
[26] Katsube N, Sakaguchi K, Fujitani B, Aishita H. Anti-platelet and anti-thrombotic effects of OP-41483.alpha-CD, a prostacyclin analogue, in experimental animals. Prostaglandins Leukot Essent Fatty Acids 1993;49:795–804.
[27] Rajtar G, Cerletti C, Castagnoli MN, Bertele V, de GG. Prostaglandins and human platelet aggregation. Implications for the anti-aggregating activity of thromboxane-synthase inhibitors. Biochem Pharmacol 1985;34:307–10.
[28] Wallis RB, Zelaschi D. Differential effects of cyclo-oxygenase inhibitors and thromboxane synthase inhibitors on rabbit platelet aggregation: evidence for the importance of prostaglandin endoperoxides as mediators in arachidonate-induced aggregation but not in collagen-induced aggregation. Biochem Soc Trans 1980;8:726–7.
[29] Kimura Y, Yasunaga K. Interaction of prostaglandin and cyclic AMP involved in regulation of platelet function. Nippon Ketsueki Gakkai Zasshi 1980;43:1130–8.
[30] Salzman EW. Cyclic AMP and platelet function. N Engl J Med 1972;286:358–63.
[31] Cole B, Robison GA, Hartmann RC. The role of cyclic AMP in platelet function. Ann NY Acad Sci 1971;185:477–547.
[32] Li Z, Ajdic J, Eigenthaler M, Du X. A predominant role for cAMP-dependent protein kinase in the cGMP-induced phosphorylation of vasodilator-stimulated phosphoprotein and platelet inhibition in humans. Blood 2003;101:423–9.
[33] Jackson SP, Nesbitt WS, Kulkarni S. Signaling events underlying thrombus formation. J Thromb Haemost 2003;1:1602–12.
[34] Lazarowski ER, Lapetina EG. Activation of platelet phospholipase C by fluoride is inhibited by elevation of cyclic AMP. Biochem Biophys Res Commun 1989;158:440–4.
[35] Feoktistov IA, Paul S, Hollister AS, Robertson D, Biaggioni I. Role of cyclic AMP in adenosine inhibition of intracellular calcium rise in human platelets. Comparison of adenosine effects on thrombin- and epinephrine-induced platelet stimulation. Am J Hypertens 1992;5:147S–53S.
[36] Bell RL, Kennerly DA, Stanford N, Majerus PW. Diglyceride lipase: a pathway for arachidonate release from human platelets. Proc Natl Acad Sci USA 1979;76:3238–41.
[37] Billah MM, Lapetina EG, Cuatrecasas P. Phospholipase A2 activity specific for phosphatidic acid. A possible mechanism for the production of arachidonic acid in platelets. J Biol Chem 1981;256:5399–403.
[38] Xing M, Post S, Ostrom RS, Samardzija M, Insel PA. Inhibition of phospholipase A2-mediated arachidonic acid release by cyclic AMP defines a negative feedback loop for P2Y receptor activation in Madin-Darby canine kidney D1 cells. J Biol Chem 1999;274:10035–8.
[39] Minkes M, Stanford N, Chi MM, Roth GJ, Raz A, Needleman P, et al. Cyclic adenosine 3′,5′-monophosphate inhibits the availability of arachidonate to prostaglandin synthetase in human platelet suspensions. J Clin Invest 1977;59:449–54.
[40] Lapetina EG. Incorporation of synthetic 1,2-diacylglycerol into platelet phosphatidylinositol is increased by cyclic AMP. FEBS Lett 1986;195:111–4.
[41] Lerea KM, Glomset JA, Krebs EG. Agents that elevate cAMP levels in platelets decrease thrombin binding. J Biol Chem 1987;262:282–8.
[42] Michan S, Sinclair D. Sirtuins in mammals: insights into their biological function. Biochem J 2007;404:1–13.
[43] Longo VD, Kennedy BK. Sirtuins in aging and age-related disease. Cell 2006;126:257–68.
[44] Smith JS, Avalos J, Celic I, Muhammad S, Wolberger C, Boeke JD. SIR2 family of NAD(+)-dependent protein deacetylases. Methods Enzymol 2002;353:282–300.