Cyclic-AMP Agonists Inhibit Antiphospholipid/ß$_2$-Glycoprotein I Induced Synthesis of Human Platelet Thromboxane A$_2$ *in Vitro*

REGINALD OPARA, DICK L. ROBBINS, and VINCENT A. ZIBOH

ABSTRACT. ***Objective.*** To investigate mechanisms responsible for increased thrombotic activity in systemic lupus erythematosus (SLE) associated with the antiphospholipid syndrome (APS). We had reported that anticardiolipin/ß$_2$-glycoprotein I (aCL/ß$_2$-GPI) complexes induce platelet overactivity resulting in excessive production of thromboxane A$_2$ (TXA$_2$). Presumably this occurs by decreased platelet cyclic AMP (cAMP) activity and results in increased platelet aggregation.

Methods. We stimulated platelet intracellular cAMP generation with known cAMP agonists (dibutyryl cAMP, theophylline, and prostaglandin E$_1$) and measured aCL/ß$_2$-GPI induced platelet TXB$_2$ production *in vitro*. Isolated human platelets were prelabeled with ^{14}C-arachidonic acid and then challenged with aCL/ß$_2$-GPI in the presence or absence of cAMP-activating substances. The resulting ^{14}C labeled TXB$_2$ was quantified by thin layer chromatography and radioactive scanning.

Results. We found a marked decrease in aCL/ß$_2$-GPI induced platelet TXB$_2$ production by the cAMP agonists in a dose dependent manner.

Conclusion. Our findings suggest the usefulness of cAMP agonists in the control of thrombosis in some patients with SLE and APS. (J Rheumatol 2003;30:55–9)

Key Indexing Terms:
ANTIPHOSPHOLIPID ANTIBODIES
ANTIPHOSPHOLIPID SYNDROME
THROMBOXANE A$_2$
PLATELETS

Systemic lupus erythematosus (SLE) is an autoimmune disease in which some patients are predisposed to vascular thrombosis and/or fetal loss. Antiphospholipid syndrome (APS) can be defined as an observable correlation between the presence of antiphospholipid (aPL) antibodies, e.g., anticardiolipin (aCL) and/or lupus anticoagulant and vascular thrombosis and/or repeated fetal loss[1]. aPL are autoantibodies that can recognize endogenous phospholipids and coagulation cofactors as antigens[2]. Thus, it is believed that aCL is responsible for the APS in some patients with SLE. However, it is unclear how aCL produces the effects of fetal loss and thrombosis.

Thromboxane A$_2$ (TXA$_2$) is the major cyclooxygenase product in platelets and it is likely that APS involves increased platelet production of TXA$_2$ and aggregation[3]. We previously hypothesized that ß$_2$-glycoprotein I (ß$_2$-GPI) might mediate aCL binding to the activated platelet cell surface by binding with phosphatidylserine, thereby promoting increased platelet activation by the aCL/ß$_2$-GPI complexes. We have shown that aCL/ß$_2$-GPI complexes induce an increase in platelet TXB$_2$ (a stable metabolite of TXA$_2$) production and aggregation[4,5]. It is also likely that fetal loss associated with APS occurs secondary to thrombosis of placental vessels and subsequent placental insufficiency[6].

While the mechanism of thrombosis in APS is vague, the negative effect of prostacyclin (prostaglandin I$_2$, PGI$_2$) on platelet TXA$_2$ production and platelet aggregation is better understood. PGI$_2$ is an endothelially derived cyclooxygenase product that leads to decreased platelet aggregation and vasodilatation. Specifically, PGI$_2$ binds to prostacyclin receptor[7], leading to activation of a signaling system that controls vascular tone and platelet aggregation[8], resulting in elevations of cyclic AMP (cAMP) and cyclic guanosine monophosphate. The increase in cAMP results in a broad alteration of platelet function, one of which is inhibition of platelet aggregation via cAMP phosphorylation of specific protein kinases and suppression of intracellular Ca^{++}.

Since a mechanism of PGI$_2$ inhibition of platelet aggregation is via the stimulation of adenyl cyclase and accumulation of platelet cAMP, we hypothesized that stimulation of platelet intracellular cAMP generation with compounds known to enhance intracellular cAMP should inhibit aCL/ß$_2$-GPI induced platelet TXA$_2$ production *in vitro*. These effects were measured by the metabolic conversion of

From the Department of Dermatology and Department of Internal Medicine, School of Medicine, University of California at Davis, Davis, California, USA.

R. Opara, BS, Medical Student; V.A. Ziboh, PhD, Professor of Dermatology, Department of Dermatology; D.L. Robbins, MD, Professor of Medicine, Department of Internal Medicine.

Address reprint requests to Dr. D.L. Robbins, Department of Internal Medicine, TB 192, School of Medicine, University of California at Davis, Davis, CA 95616. E-mail: dlrobbins@ucdavis.edu

Submitted May 18, 2001; revision accepted July 10, 2002.

^{14}C-arachidonic acid (^{14}C-AA) labeled platelets into platelet ^{14}C-TXB$_2$. Thrombin, a naturally occurring protein with high affinity binding sites for platelets, was used as an agonist to stimulate basal platelet TXA$_2$ production. In a further attempt to mimic *in vivo* conditions found in SLE, aCL/ß$_2$-GPI complexes previously isolated from plasma of patients with SLE were preincubated with normal platelets prior to each incubation and the effect on the metabolism of ^{14}C-AA labeled platelets into platelet ^{14}C-TXB$_2$ was determined. Interestingly, we found that compounds that stimulated cAMP generation produced significant inhibition of aCL/ß$_2$-GPI induced platelet TXB$_2$ biosynthesis *in vitro*.

MATERIALS AND METHODS

Chemicals and reagents. ^{14}C-AA was purchased from DuPont (Boston, MA, USA). Thrombin, theophylline, and indomethacin all were purchased from Sigma Chemical Company (St. Louis, MO, USA). Dibutyryl cAMP (db-cAMP) and PGE$_1$ were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). The aCL/ß$_2$-GPI complex was isolated in our laboratory from the plasma of a patient with SLE and APS[4].

Preparation of ^{14}C-AA labeled platelets. Platelets were prepared as described[4,5]. Briefly, donor blood was mixed with citrate, an anticoagulant, and centrifuged at room temperature to obtain the platelet-rich plasma. ^{14}C-AA prepared for preincubation with platelet-rich plasma consisted of drying ^{14}C-AA in a tube under nitrogen gas, then it was dissolved with 1 ml of ethyl ether; 1 ml of Hanks' balanced salt solution (HBSS) was added to each 1 µCi of ^{14}C-AA in the tube and the ethyl ether in the sample was evaporated off under nitrogen gas. The remaining solution was sonicated. The platelet-rich plasma was incubated 4 h with the ^{14}C labeled AA to incorporate the ^{14}C-AA into platelet membrane phospholipids. After centrifugation, the ^{14}C labeled platelet pellets were washed with Tris-HCl, pH 7.5, and centrifuged. The ^{14}C labeled platelets were then resuspended in HBSS. From this, sample cells were counted and 1×10^8 platelets were used for each incubation. Aliquots were also taken because platelets containing 10,000–20,000 dpm were necessary for each incubation.

Isolation of aCL/ß$_2$-GPI complex. Isolation of the aCL/ß$_2$-GPI complex was performed as described[4,5]. Although we use the term complex, this methodology may instead isolate a mixture of aCL and ß$_2$-GPI rather than an actual complex. Briefly, donor plasma from a patient with SLE-APS was subjected to ion exchange chromatography with DEAE-Sephadex (Pharmacia, Uppsala, Sweden). The isolated IgG was then subjected to cardiolipin affinity chromatography, from which the aCL/ß$_2$-GPI complex was eluted by linear gradient from 0 to 100% with 0.01 M phosphate buffer (pH 7.5) containing 1 M NaCl. The eluate was assayed for aCL by ELISA and for aCL and ß$_2$-GPI by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10%).

Incubation of platelets with cAMP agonists or a cyclooxygenase inhibitor (indomethacin). Because cAMP is known to inhibit platelet TXA$_2$ biosynthesis, we tested the effects of cAMP agonists on aCL/ß$_2$-GPI induced platelet TXA$_2$ biosynthesis. Specifically, ^{14}C-AA labeled platelets were first preincubated for 10 min with varying concentrations of the cAMP activating substances or cyclooxygenase inhibitors to determine the dose dependent effects of these compounds on TXB$_2$ production. Second, 1 µg of the aCL/ß$_2$-GPI complex was added to the preincubated platelets and allowed to preincubate an additional 50 min. Third, the platelets were challenged with 5 units of thrombin and incubated for 20 min. Finally, after centrifugation, the radioactive supernatant was extracted with Folch mixture (chloroform/methanol, 2:1) to isolate the desired radioactive metabolites.

Separation of reaction products by thin layer chromatography (TLC). The samples containing ^{14}C-AA metabolites were dried under nitrogen gas, then redissolved with 100 µl of chloroform/methanol (2:1) mixture. Samples were then spotted onto activated TLC plates. The plates were developed in the TLC solvent mixture consisting of ethyl acetate:iso-octane:acetic acid:water, 165:75:30:150 (v/v/v/v). After the separation, the plates were allowed to dry at room temperature and then scanned using a Berthold Linear TLC Analyzer to determine the peaks of radioactive metabolites (particularly TXB$_2$) from ^{14}C-AA.

Separation of radioactive metabolites by TLC. After incubation of ^{14}C-AA platelets with thrombin and aCL/ß$_2$-GPI, the incubation compounds were extracted by Folch mixture, dried under nitrogen gas, and spotted onto TLC plates. The ^{14}C radioactive band that comigrated with authentic TXB$_2$ was identified by scanning plates on the Berthold Linear TLC Analyzer.

RESULTS

Inhibition of thrombin/aCL/ß$_2$-GPI induced platelet TXB$_2$ production by indomethacin. Figure 1 shows the dose dependent effects of thrombin, aCL/ß$_2$-GPI complexes, and indomethacin on platelet conversion of AA (substrate) to TXB$_2$ *in vitro*. Data illustrated in Figure 1 show that thrombin produced about a 6-fold increase in AA conversion to TXB$_2$. Moreover, the aCL/ß$_2$-GPI complexes produced roughly 4-fold enhancement of platelet TXB$_2$ biosynthesis compared with platelets incubated with thrombin alone.

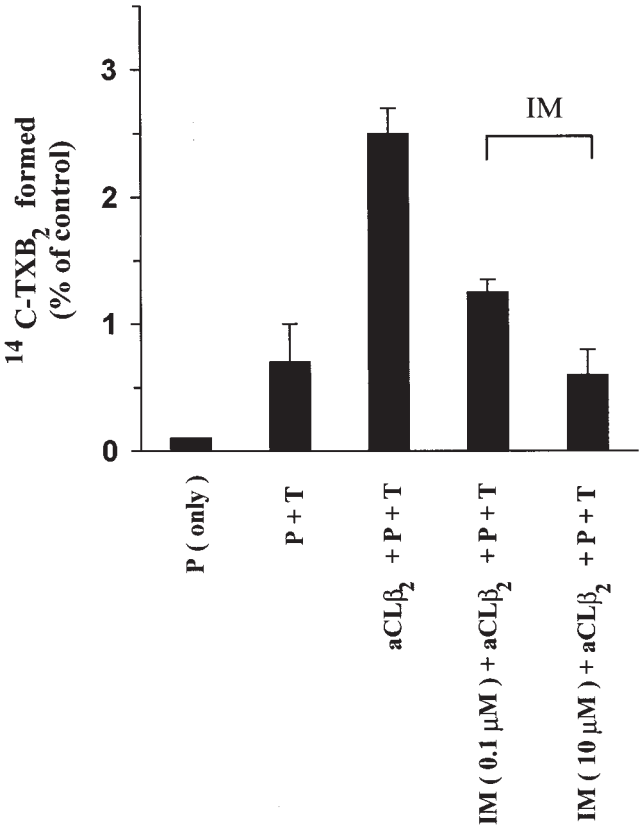

Figure 1. Dose dependent inhibitory effects of thrombin/aCL/ß$_2$-GPI activated platelet conversion of AA to TXB$_2$ by a NSAID, indomethacin. aCL/ß$_2$-GPI complexes produced roughly a 4-fold increase in TXB$_2$ biosynthesis compared to thrombin alone, which was inhibited by indomethacin. IM: indomethacin, P: platelets, T: thrombin. Each experiment was done in triplicate in 3 separate experiments; results are expressed as mean ± 1 SD.

Further, addition of indomethacin in concentrations of 0.1 and 10 µM to the incubations containing thrombin/aCL/ß$_2$-GPI complex decreased platelet TXB_2 production roughly 50% and 75%, respectively.

Inhibition of platelet TXB_2 production by cAMP agonists. As illustrated in Figure 2, we tested 2 concentrations of PGE_1, reported to directly stimulate cAMP by activating adenyl cyclase[12]. As observed, about 60% inhibition occurred at a concentration of 0.3 µM. No further significant suppression occurred at concentrations as high as 3.0 µM.

Similarly (Figure 3), we tested 3 low concentrations of theophylline on thrombin/aCL/ß$_2$-GPI induced platelet AA conversion to TXB_2. In this case, 0.225 mM and 0.5 mM theophylline produced 16% and 35% inhibition, respectively, and complete inhibition occurred at a concentration of 1.0 mM.

Inhibition of platelet TXB_2 production by synthetic cAMP. Since stimulation of intracellular cAMP by 2 compounds (PGE_1 and theophylline) via different mechanisms of action produced significant inhibition of platelet AA conversion to TXB_2, we examined the direct effect of synthetic dibutyryl cAMP (db-cAMP) on thrombin/aCL/ß$_2$-GPI activated platelets. Using 3 concentrations known to enhance cAMP dependent effects in other systems[9], we observed dose dependent inhibition of platelet AA conversion to TXB_2 by db-cAMP (Figure 4). Maximal inhibition (45%) occurred at a concentration of 0.3 mM, the lowest db-cAMP concentration examined.

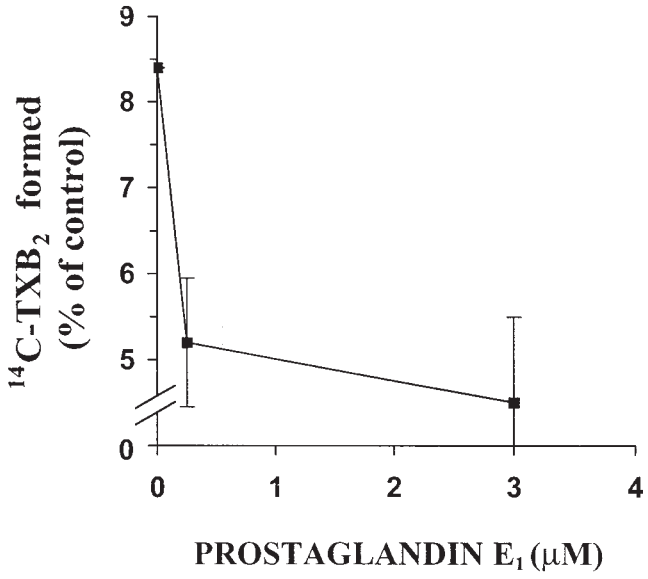

Figure 2. Dose dependent inhibitory effects of PGE_1 on thrombin/aCL/ß$_2$-GPI activated platelet TXB_2 production. Roughly 60% inhibition occurred at the lowest PGE_1 concentration (0.3 µM). Each experiment was done in triplicate in 3 separate experiments; results are expressed as mean ± 1 SD.

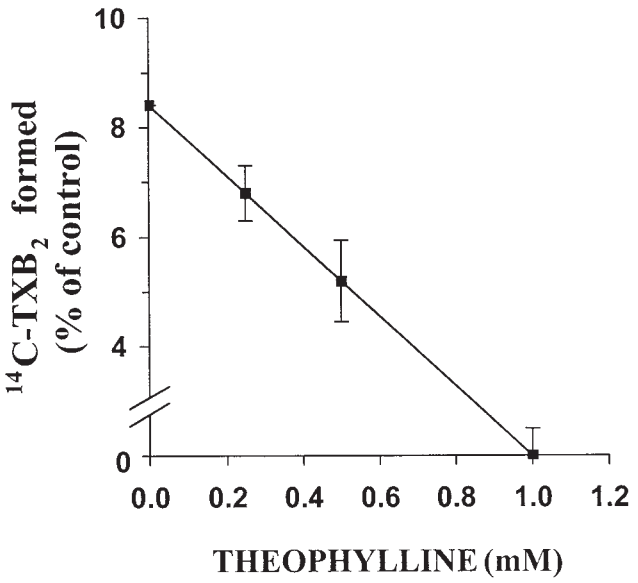

Figure 3. Dose dependent inhibitory effects of theophylline on thrombin/aCL/ß$_2$-GPI activated platelet AA conversion to TXB_2. Theophylline concentrations of 0.5 mM and 1.0 mM resulted in 35% and complete inhibition, respectively. Each experiment was done in triplicate in 3 separate experiments; results are expressed as mean ± 1 SD.

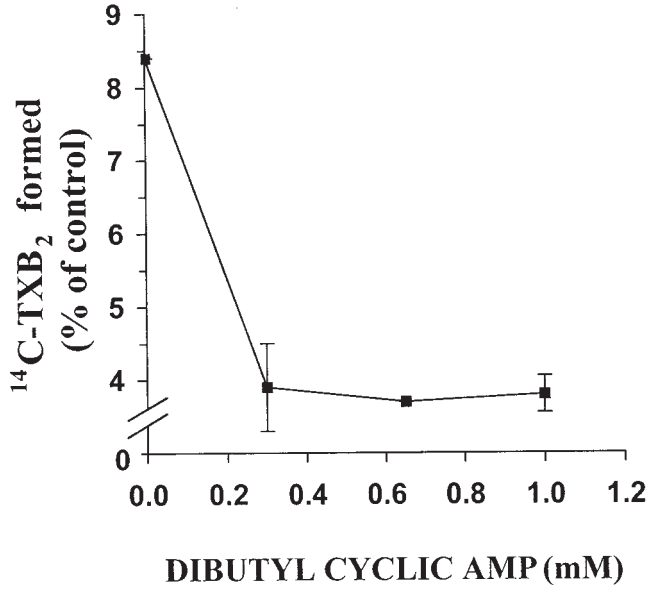

Figure 4. Dose dependent inhibitory effects of db-cAMP on thrombin/aCL/ß$_2$-GPI activated platelet AA conversion to TXB_2. A 45% inhibition occurred at db-cAMP concentration of 0.3 mM, the lowest concentration examined. Each experiment was done in triplicate in 3 separate experiments; results are expressed as mean ± 1 SD.

DISCUSSION

Thrombin stimulates platelet TXA_2 biosynthesis, and APS derived aCL/ß$_2$-GPI complexes stimulate even greater platelet TXA_2 production[4,5]. Thus, we investigated the effect of cAMP agonists on aCL/ß$_2$-GPI complex induced platelet TXB_2 biosynthesis. Clearly, there was a significant increase

in platelet TXB_2 production by thrombin/aCL/$ß_2$-GPI activated platelets compared to platelets incubated with thrombin alone[4,5] (Figure 2). Previous studies in our laboratory showed stimulation of platelet TXB_2 production by heat aggregated IgG (nonspecific Fc/FcR effect), and even greater stimulation by aCL $(Fab')_2$ fragments (specific antibody effect)[5]. Since we used intact aCL in these experiments, TXB_2 stimulation could be due to one or both effects[5].

As a control, thrombin stimulated platelets incubated with aCL/$ß_2$-GPI complexes were incubated with a nonsteroidal antiinflammatory drug (NSAID), indomethacin. This drug inhibits platelet TXA_2 production by inhibiting the cyclooxygenase pathway (COX-1). Our data showed that while the thrombin/aCL/$ß_2$-GPI complex stimulated platelet TXB_2 production, the addition of a NSAID, indomethacin, caused a decrease in thrombin/aCL/$ß_2$-GPI activated platelet TXB_2 production. Specifically, our data show a dose dependent decrease in platelet TXB_2 production in the presence of a NSAID. These results demonstrate that a NSAID inhibits platelet metabolism of AA via the cyclooxygenase pathway.

A simplified scenario suggests that aCL/$ß_2$-GPI induced enhancement of platelet TXA_2 generation from platelet AA is followed by associated platelet aggregation. Associated with this cascade is the vascular endothelial cell generation of PGI_2 from endothelial AA, which is known to stimulate platelet adenyl cyclase activity and thus increase platelet cAMP, an inhibitor of platelet TXA_2 generation and associated platelet aggregation. An increase in platelet cAMP is central in the inhibition of TXA_2. Thus, the stimulation of platelet adenyl cyclase by PGE_1 [9], a cyclooxygenase metabolite derived from dietary gamma-linolenic acid, should have an effect similar to PGI_2 [10] (Figure 5). Receptors for both PGE_1 and PGI_2 in human platelets have been described[11], supporting the role of these autocoids in the modulation of platelet/vascular homeostasis.

In another experiment, we preincubated thrombin/aCL/$ß_2$-GPI activated platelets with theophylline. Theophylline is an antiphosphodiesterase that inhibits the intracellular breakdown of cAMP by phosphodiesterase enzyme to 5'-AMP[12]. We observed a dose dependent inhibition of platelet TXB_2 production by theophylline, presumably from its agonistic effects on cAMP activity (Figure 5).

Although we observed the dose dependent inhibitory effect of PGE_1 and theophylline on platelet TXB_2 biosynthesis, we attempted to confirm the mechanism for the effect was due to intracellular cAMP. To examine this, thrombin/aCL/$ß_2$-GPI activated platelets were preincubated with db-cAMP, a synthetic cAMP. Indeed, db-cAMP produced a dose dependent inhibition of platelet TXB_2 production. Taken together, these data strongly support the notion that cAMP stimulation is involved in the inhibition of TXB_2 production.

The major current but potentially dangerous therapeutic intervention for patients with APS is high level anticoagula-

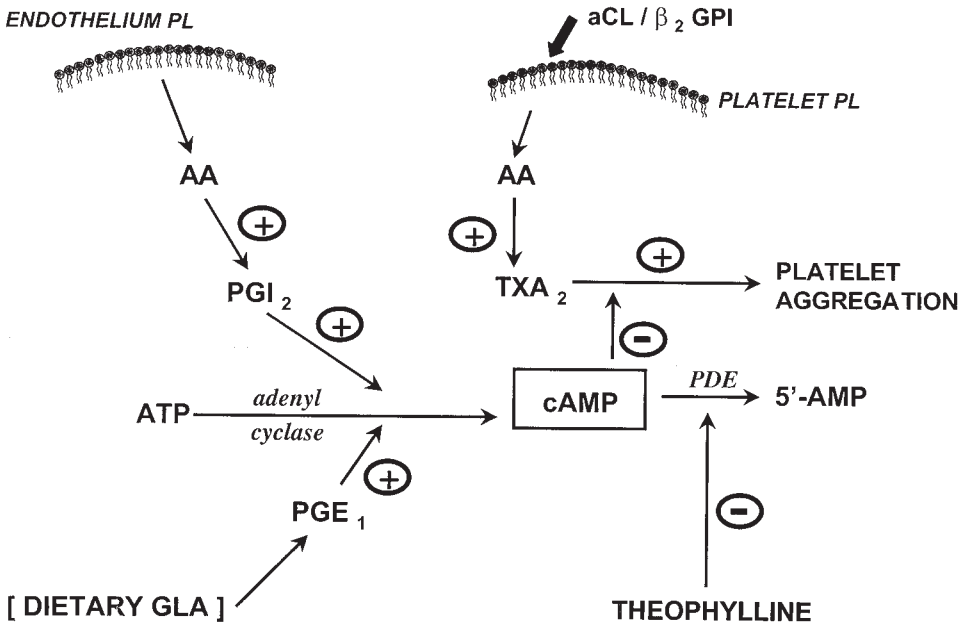

Figure 5. Metabolism of arachidonic acid (AA) in endothelial cells and platelets. The figure indicates sites of inhibitory activity produced by cAMP agonists examined in this study. PGI_2 from endothelial cells and PGE_1 from dietary sources stimulate conversion of ATP to cAMP (via adenyl cyclase) in platelets. Theophylline inhibits conversion of cAMP to 5'-AMP by inhibiting platelet phosphodiesterase. db-cAMP is a stable analog of cAMP. Increased cAMP decreases platelet TXA_2 generation and thus platelet aggregation. +: stimulation, –: inhibition, PL: phospholipid, PDE: phosphodiesterase, GLA: gamma-linolenic acid.

tion. The use of aspirin, a COX-1 inhibitor, is only mildly beneficial (if at all), and can in some cases result in gastric irritation and the more dangerous induction of anaphylactic asthma. Since data from our study indicate *in vitro* inhibition of thrombin/aCL/ß$_2$-GPI induced platelet TXB$_2$ production by cAMP agonists, other therapeutic strategies in management of APS might involve the use of intracellular cAMP-stimulating agents, which might inhibit *in vivo* TXA$_2$ production. The possibilities for such therapeutic intervention deserve to be explored in light of our findings, perhaps mitigating the negative side effects associated with high level anticoagulation.

ACKNOWLEDGMENT

The authors thank Stanley Leung for his help in conducting this study.

REFERENCES

1. Harris EN. Antiphospholipid syndrome. In: Klippel JH, Weyand CM, Wortmann RL, editors. Primer on the rheumatic diseases. 11th ed. Atlanta: Arthritis Foundation; 1997:313-5.
2. Roubey RAS. Immunology of the antiphospholipid syndrome. Arthritis Rheum 1996;39:1444-54.
3. Martinuzzo ME, Maclouf J, Carreras LO, Levy-Toledano S. Antiphospholipid antibodies enhance thrombin-induced platelet activation and thromboxane formation. Thromb Haemost 1993;70:667-71.
4. Leung S, Ziboh VA, Miller-Blair DJ, Robbins DL. Isolation and purification of anticardiolipin antibody from plasma of a patient with antiphospholipid syndrome: induced generation of platelet thromboxane-A$_2$ synthesis. Prostaglandins Leukot Essent Fatty Acids 1996;55:385-93.
5. Robbins DL, Leung S, Miller-Blair DJ, Ziboh V. Effect of anticardiolipin ß$_2$-glycoprotein I complexes on production of thromboxane A$_2$ by platelets from patients with the antiphospholipid syndrome. J Rheumatol 1998;25:51-6.
6. MacLachlan NA, Elder MG. Antiphospholipid antibodies and fetal loss — proposed mechanisms. In: Harris EN, Exner T, Hughes GRV, Asherson RA, editors. Phospholipid binding antibodies. Boston: CRC Press; 1991:355-69.
7. Walsh M-T, Foley JF, Kinsella BT. The α, but not the ß, isoform of the human thromboxane A$_2$ receptor is a target for prostacyclin-mediated desensitization. J Biol Chem 2000;275:20412-23.
8. Schwarz UR, Welter U, Ergenthaler M. Taming platelets with cyclic nucleotides. Biochem Pharmacologica 2001;62:1153-61.
9. Gorman RR, Bunting S, Miller OV. Modulation of human platelet adenylate cyclase by prostacyclin (PGX). Prostaglandins 1977;13:377-88.
10. Asby B. Comparison of iloprost, cicaprost and prostacyclin effects on cyclic AMP metabolism in intact platelets. Prostaglandins 1992;43:255-61.
11. Dutta-Roy AK, Sinha AK. Purification and properties of prostaglandin E$_1$/prostacyclin receptor of human blood platelets. J Biol Chem 1987;262:12685-91.
12. Brown W, Shiozawa C. Cyclic AMP and phosphodiesterase levels in tumor cells. In: Kahn RH, Lands WEM, editors. Prostaglandins and cyclic AMP — biological actions and clinical applications. New York: Academic Press; 1973:21-4.