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ENHANCEMENT BY BERAPROST SODIUM, A STABLE ANALOGUE OF PROSTACYCLIN, IN THROMBOMODULIN EXPRESSION ON MEMBRANE SURFACE OF CULTURED VASCULAR ENDOTHELIAL CELLS VIA INCREASE IN CYCLIC AMP LEVEL

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Abstract—Prostacyclin and beraprost sodium (beraprost), a stable analogue of prostacyclin, increased cyclic AMP (cAMP) levels of cultured human umbilical vein endothelial cells (HUVEC) in a concentration-dependent manner. The elevation of cAMP by beraprost was sustained longer than that by prostacyclin. The expression of thrombomodulin (TM) on membrane surface of HUVEC was enhanced by beraprost and prostacyclin, and the persistence of the increase in TM expression by beraprost was greater than prostacyclin. Dibutyryl cAMP (db-cAMP) mimicked the effects of beraprost and 3-isobutyl-1-methylxanthine enhanced the effects. Beraprost, prostacyclin and db-cAMP also effectively blocked the interleukin-1- and tumor necrosis factor-induced depression of TM expression substantially. These results suggest that TM expression is positively regulated by cAMP in HUVEC, and that beraprost may be potentially effective for reducing thrombotic events through the mechanism which initiates the stimulation of cAMP/TM system in vascular endothelial cells.

Prostacyclin (PGI_2§), released from vascular endothelial cells, modulates functions of blood cells and vascular cells. Beraprost (sodium (±)-(IR*,2*R**,3*aS**,8*bS**)-2,3,3*a*,8*b*-tetrahydro-2-hydroxyl-1-[(*E*)-(3*S**)-3-hydroxy-4-methyl-1-octen-6-ynyl] 1*H*-cyclopenta[*b*]benzofuran-5-butyrate), a stable and orally active agent with a PGI_2-like structure [1] (Fig. 1), has not only anti-thrombotic action in animal models [1] but also potent inhibitory effects on platelet aggregation [2], vasoconstriction [3] and leukocyte chemotaxis [4] regulated by cAMP levels in the platelets, vascular smooth muscle cells and leukocytes like PGI_2. However, at present, the effect of beraprost on cyclic AMP levels in vascular endothelial cells has not been examined. TM is an endothelial cell surface glycoprotein, and either inactivates procoagulant reactions of thrombin or activates protein C-promoting anticoagulant mechanisms [5, 6]. Although second messengers concerning the regulation of TM expression are not been fully elucidated [5], it has been reported that the expression of fetomodulin in embryos, which have a cDNA clone identical to that of TM [7], is increased by cAMP [8]. Recently, preliminary experiments by one of the authors showed a possible role of cAMP in the control of TM using mouse hemangioma cells and HUVEC [9]. Moreover, our preliminary study also indicates that beraprost actually increased in cAMP of HUVEC as well as PGI_2. These findings urged us to examine precisely the effects of beraprost and PGI_2 on cAMP level and TM expression in HUVEC. The present study shows that TM expression in HUVEC is regulated positively by cAMP, and that beraprost may reduce thrombotic events through the stimulation of cAMP/TM system.

MATERIALS AND METHODS

Materials. db-cAMP, BSA (fatty acid and globulin free) and IBMX were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). IL-1β and TNF-α were from Boehringer Mannheim Yamanouchi (Tokyo, Japan). M199, fetal calf serum and ECGS were from Flow Laboratories (McLean, VA, U.S.A.), Hyclone Laboratories (Logan, UT, U.S.A.) and Collaborative Research Inc. (Bedford,

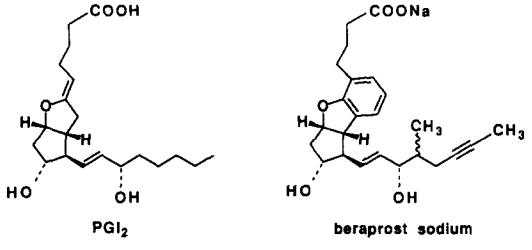

Fig. 1. Chemical structures of PGI_2 and beraprost sodium.

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§ Abbreviations: cAMP, cyclic AMP; HUVEC, human umbilical vein endothelial cells; beraprost, beraprost sodium; TM, thrombomodulin; db-cAMP, dibutyryl cAMP; IBMX, 3-isobutyl-1-methylxanthine; PGI_2, prostacyclin; IL-1, interleukin-1; TNF, tumor necrosis factor; BSA, bovine serum albumin; ECGS, endothelial cell growth supplement; PBS, phosphate-buffered saline.

MA, U.S.A.), respectively. [^{125}I]cAMP RIA kit and [^{125}I]anti-mouse IgF(ab')$_2$ were purchased from New England Nuclear (Boston, MA, U.S.A.). Biotinated anti-mouse IgG and fluorescein isothiocyanate-labeled avidin were from Zymed Laboratories (San Francisco, CA, U.S.A.). Beraprost and PGI_2 were synthesized in our chemical laboratory. Anti-human TM IgG (mouse) was prepared as described previously by one of the authors [10].

Preparation and culture of HUVEC. HUVEC were prepared by the method of Jaffe *et al.* [11] and cultured in M199 supplemented with 10% fetal calf serum and 30 mg/L ECGS at 37° in an atmosphere of 95% air/5% CO_2. After confluence the cells were subcultured, and the cells just before confluence in the second subculture were used for the experiments.

cAMP assay. The cells on 12-well plates were washed three times with 15 mM Hepes/Hanks buffer containing 1% BSA and preincubated for 10 min at 37° in the same buffer. The cells were treated with beraprost or PGI_2 and further incubated at 37°. After the indicated period, the reaction was terminated by an addition of cold 5% trichloroacetic acid solution. The cells were collected, sonicated and centrifuged at 100 *g* for 15 min at 4°. Trichloroacetic acid in the supernatant was removed by an extraction using water-saturated ether, and the resulting aqueous layer was dried by a centrifugal vaporizer (EYELE). The dried samples were resolved in sodium acetate buffer and cAMP was determined using a RIA kit.

Binding assay of TM using anti-TM antibody and ^{125}I-labeled second antibody. The cells on 24-well plates were treated with beraprost, PGI_2, db-cAMP, IL-1 or TNF for the time indicated at 37°. The cells were washed three times with PBS containing 0.1% sodium azide and 1% BSA, and incubated at 4° for 1 hr in the presence of anti-human TM IgG of mouse. Normal mouse IgG was used for control treatments. Moreover, the cells were washed three times with the above-mentioned buffer and further incubated at 4° for 1 hr in the presence of [^{125}I]anti-mouse IgF(ab')$_2$ (0.26 mCi/well). The cells were washed three times and solubilized with the buffer containing 1% Triton X-100. Radioactivity of each well was determined in a γ-counter. In the experiments for combined treatments, the cells were preincubated with IL-1 or TNF for 10 min before the addition of beraprost, PGI_2 or db-cAMP.

Analysis of TM expression using flow cytometry. The cells in 60-mm dishes were treated with beraprost and/or IL-1 for 16 hr at 37° in a manner similar to that described above. After the incubation the cells were washed, removed from plates and collected by collagenase treatment and centrifugation. The cells were resuspended in PBS containing 1% bovine serum albumin and 0.02% sodium azide, and further incubated at 4° for 1 hr in the presence of anti-human TM IgG of mouse or normal mouse IgG. Moreover, the cells were washed three times with the same buffer, and incubated at 4° for 30 min in the presence of biotinated anti-mouse IgG. The cells were again washed three times and incubated at 4° for 30 min in the presence of fluorescein isothiocyanate-labeled avidin. After washing, the cells were resuspended in PBS. The cells passed through nylon mesh were analysed using a flowcytometer (FACScan model, Beckton Dickinson Co.).

Statistical analyses. Statistical analyses were done by Anova-test and Dunnett-test.

RESULTS

Treatment of HUVEC with beraprost resulted in a transient increase in cAMP level in a concentration-dependent fashion (10–1000 nM) and a peak activity was observed within 10 min after the treatment (Fig. 2A). The duration of cAMP elevation was increased concentration-dependently (Fig. 2A). PGI_2 (1 mM) also increased the cAMP level in HUVEC 10 min after treatment, but the cAMP level declined to the basal level immediately (Fig. 2B). When beraprost (1 mM) was removed from the medium 10 min after treatment, the increased cAMP level rapidly declined to the basal level (Fig. 2B). The time course of cAMP level caused by PGI_2 was not affected by removal of the drug (Fig. 2B). The effect of beraprost on cAMP was much enhanced by the pretreatment with 0.5 mM IBMX (2.82 ± 0.11 pmol/10^5 cells for beraprost; 5.96 ± 0.33 pmol/10^5 cells for beraprost plus IBMX).

The effects of beraprost and PGI_2 on TM expression on the membrane surface of HUVEC were evaluated 3 hr after treatment by binding assay of TM. Beraprost (1, 10 and 100 nM) and PGI_2 (10 and 100 nM) significantly increased TM expression (Fig. 3 and Table 1). The effect of beraprost was mimicked by db-cAMP (2 and 3 mM) and further enhanced by 0.5 mM IBMX treatment (Fig. 3).

Time course effects of beraprost (100 nM) and PGI_2 (100 nM) on TM expression showed that persistence of the increase in TM expression by beraprost was longer than that by PGI_2. The PGI_2-induced increase in TM expression was maximal 3 hr after treatment and decreased subsequently (Fig. 4). Meanwhile, the beraprost-induced increase in TM expression was greater at 6 hr than at 3 hr after the treatment (Fig. 4). The removal of beraprost from the medium 30 min after treatment diminished the sustained effect of beraprost on TM expression, and the pattern of time course for beraprost became almost identical to that for PGI_2 (Fig. 4). The removal of PGI_2 from the medium 30 min after treatment did not affect the effect of PGI_2 on TM expression (Fig. 4). db-cAMP (2 mM) mimicked the effect of beraprost for TM expression (Fig. 4).

It is known that cytokines such as IL-1 [12] and TNF [13, 14] reduce TM expression. In the present experiments, IL-1 (3 units/mL) and TNF (3 units/mL) substantially decreased TM expression of HUVEC to 70 and 55% of the control value 3 and 9 hr after the treatment, respectively (Fig. 5 and Table 1). Pretreatment with beraprost (1, 10 and 100 nM), PGI_2 (10 and 100 nM) and db-cAMP (2 mM) effectively suppressed the IL-1- and TNF-induced a decrease of TM expression (Fig. 5 and Table 1). After treatment of the cells with IL-1 (3 units/mL) for 16 hr, TM expression was considerably decreased to 35% of the control value (Fig. 6B). Even in that case, pretreatment with beraprost (100 nM) partially but significantly counteracted the

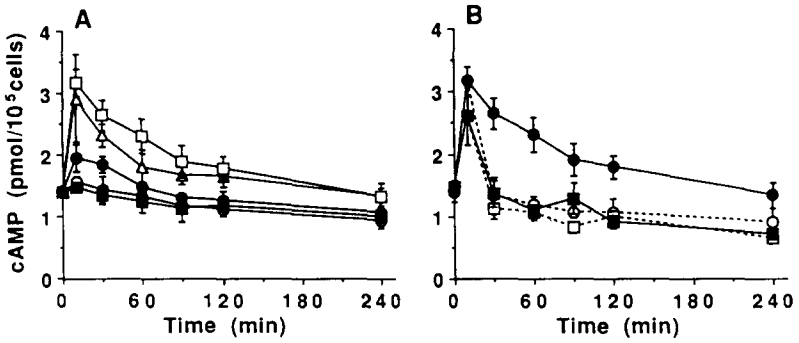

Fig. 2. Effects of beraprost and PGI_2 on cAMP level in HUVEC. In panel A, the cells were treated with 1–1000 nM beraprost (○, 1; ●, 10; △, 100; □, 1000 nM) or vehicle (■) at time 0. In panel B, the cells were treated with 1 mM beraprost (●, ○) or PGI_2 (■, □) at time 0. The cells of some plates were washed to remove beraprost (○) and PGI_2 (□) at 10 min after treatment. After the incubation for the indicated periods, cAMP levels in the cells were determined. Each point and vertical bar represent mean ± SE of four repeated experiments in which triplicate assay was performed.

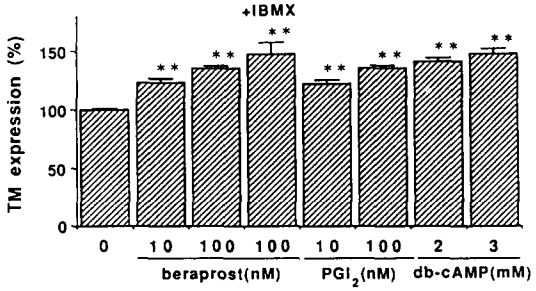

Fig. 3. Effects of beraprost, PGI_2, db-cAMP and IBMX on TM expression in HUVEC. The cells were treated with beraprost (10, 100 nM), PGI_2 (10, 100 nM), db-cAMP (2, 3 mM) and IBMX (0.5 mM). After 3 hr incubation, TM expression on the membrane surface was determined by binding assay as described in Materials and Methods. Data are expressed as percentage against [^{125}I]anti-mouse $IgF(ab')_2$ binding to the non-treated cells (control). The radioactivity of non-treated cells (control) i.e. 100% was approximately 6950 ± 85.9 cpm/well. Each column and vertical bar represent mean ± SE of four repeated experiments in which triplicate assay was performed. ** $P < 0.01$ versus non-treated (control).

IL-1-induced decrease of TM expression (Fig. 6B). Analysis of TM expression using fluorescence flow cytometry also supported the results of the binding assay. In fact, distribution of cells treated with IL-1 (3 units/mL) for 16 hr was shifted much to that of the cells treated with normal mouse IgG instead of anti-human TM IgG (Fig. 6A). The pretreatment with beraprost (100 nM) substantially blocked the IL-1-caused shift of the cell distribution (Fig. 6A).

DISCUSSION

PGI_2 has a number of important actions, physiologically, such as potent anti-platelet and vasodilator activities, but its clinical and experimental use have been limited by chemical instability. Beraprost was synthesized to overcome such defect and was shown to be stable and orally active [1]. It has been reported that a half-life of PGI_2 at 25° in phosphate buffer, pH 7 ~ 8, was estimated to be 3.5 min, and that PGI_2 is about 100-fold more stable in carbonate buffer pH 9 ~ 10, than in phosphate buffer, pH 7.4 [15]. Therefore, addition of freshly-dissolved PGI_2 in carbonate buffer stored at 4° to culture medium is essential to evaluate its effect correctly. Decomposition of PGI_2 is accelerated in the culture medium, pH 7.4, after the treatment of HUVEC with PGI_2. On the other hand, it was reported that beraprost in various buffer solutions, pH 4 ~ 10, was stable at 40° at least for 10 days [1]. Moreover, decomposition of beraprost in aqueous solution, pH 7.0, at 50° was not observed for at least 4 weeks (unpublished data). Beraprost is surely supposed to be stable in the HUVEC culture system. Beraprost is much superior to PGI_2 in stability.

The present results clearly showed that beraprost, like PGI_2 [16], caused an effective increase in the cAMP level of HUVEC as platelets [2] and leukocytes [4]. The persistence of the increase in cAMP by beraprost was superior to PGI_2. Such difference between the two agents might be explained by a difference in the stability of the compounds in HUVEC and/or medium. This possibility was clarified by the experiments on removal of beraprost and PGI_2 from the cells and medium. Namely, washout of beraprost extinguished the sustained effect of beraprost. Of course we cannot completely exclude any other possibility such as a difference in the effect on phosphodiesterase activity. But both compounds are known to lack anti-phosphodiesterase activity. Therefore beraprost may exert its effect on vascular endothelial cells at least more effectively than PGI_2 because of its stability, with a more efficient increase in cAMP.

TM, which is located on the membrane surface of endothelial cells, inactivates procoagulant actions of thrombin and activates protein C by the formation of a thrombin–TM complex [6]. Elevation of TM activity on the membrane surface promotes the anti-coagulation pathway of the blood coagulation

Table 1. Effect of beraprost at low concentrations on TM expression and IL-1-caused decrease in TM expression in HUVEC

Beraprost (nM)	IL-1(units/mL)	TM expression (%)*
[Experiment I]		
0	—	100.0 ± 0.6†
0.01	—	98.0 ± 1.0
0.1	—	104.1 ± 1.0
1	—	113.6 ± 0.7‡
10	—	125.0 ± 1.6‡
[Experiment II]		
0	0	100.0 ± 0.6†
0	3	68.9 ± 3.3
0.01	3	66.5 ± 2.3
0.1	3	71.1 ± 3.8
1	3	80.5 ± 3.4‡
10	3	90.9 ± 0.1‡

The cells were treated with beraprost. In Experiment II, IL-1 was further added to the medium 10 min after beraprost treatment. The cells were incubated for 3 hr after the addition of beraprost (Experiment I) or IL-1 (Experiment II). After the incubation, TM expression on the membrane surface was determined by binding assay as described in Materials and Methods.

* Data are expressed as percentage against [^{125}I]anti-mouse IgF(ab')$_2$ binding to the vehicle-treated cells.

† The radioactivity of vehicle-treated cells i.e. 100% was approximately 7094 ± 40.0 cpm/well in both experiments.

‡ $P < 0.01$ versus vehicle-treated (Experiment I) and versus treated with IL-1 alone (Experiment II).

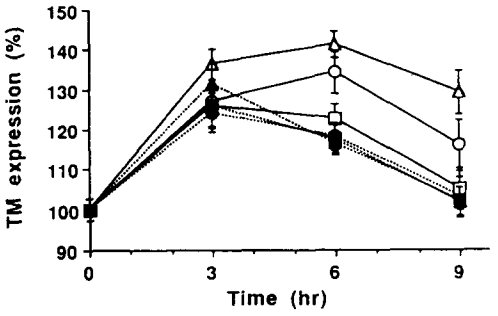

Fig. 4. Effects of removal of beraprost, PGI_2 and db-cAMP at 30 min after treatment on TM expression caused by these agents in HUVEC. The cells were treated with 100 nM beraprost (○, ●), 100 nM PGI_2 (□, ■) and 2 mM db-cAMP (△, ▲) at time 0. The cells of some plates were washed to remove drugs (●, ■, ▲) at 30 min after treatment. After the incubation for the indicated periods, TM expression on the membrane surface was determined by binding assay as described in Materials and Methods. Data are expressed as percentage against [^{125}I]anti-mouse IgF(ab')$_2$ binding to the non-treated cells (control). The radioactivity of non-treated cells (control) i.e. 100% was approximately 7394 ± 203.1 cpm/well. Each point and vertical bar represent mean ± SE of four repeated experiments in which triplicate assay was performed.

cascade, and depression of such activity predominates the procoagulation pathway. Thus, blood coagulation on the cell surface is controlled by changes of TM expression [5, 6]. The present study demonstrated first and foremost a possibility that TM expression is enhanced by PGI_2 increasing cAMP in vascular endothelial cells. Moreover, beraprost seems to be superior to PGI_2 in that increased TM expression was persistent, i.e. the beraprost-induced sustained increase in cAMP was persistent as compared to PGI_2. During preparation of the manuscript, Ito *et al.* [17] reported the enhanced expression of TM in human megakaryoblastic leukemia cell lines by cAMP-increasing agents.

It has been reported that cytokines such as IL-1, TNF and endotoxin, which are putative mediators of the inflammatory process, cause a decreased TM expression [5, 12–14]. The precise mechanism of reduced TM expression in response to cytokines remains unclear, but increased endocytosis and lysosomal degradation of TM might be involved [5, 14]. The present results confirmed the IL-1- and TNF-induced reduction of TM expression on the membrane surface of the endothelial cells. Moreover, the present results also showed that beraprost as well as PGI_2 and db-cAMP counteracted the decreased TM expression caused by these cytokines. After the incubation with IL-1 for a relatively long time (16 hr), TM expression was markedly suppressed down to 35% of the control. Even in that case, flow cytometry and binding assay analyses clearly showed that the TM expression suppressed by IL-1 was partially but significantly restored by beraprost. It has been suggested that platelets, leukocytes and endothelial cells interact with each other in the amplification of vascular injury and inflammation as seen in ischemic diseases [18]. Products of lymphocytes or macrophages also regulate various aspects of vascular cell functions, including pro-

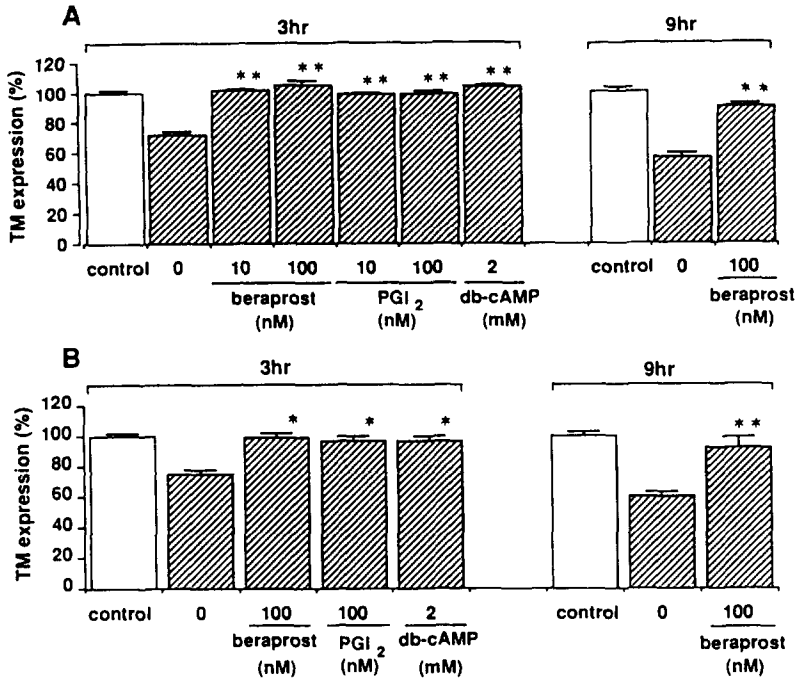

Fig. 5. Effects of beraprost, PGI_2 and db-cAMP on the IL-1- and TNF-caused decrease in TM expression in HUVEC. The cells were treated with beraprost (10, 100 nM), PGI_2 (10, 100 nM) and db-cAMP (2 mM), and (A) IL-1 (3 units/mL) and (B) TNF (3 units/mL) were added to the medium after 10 min. The cells were further incubated for 3 or 9 hr. After the incubation, TM expression on the membrane surface was determined by binding assay as described in Materials and Methods. Data are expressed as percentage against [^{125}I]anti-mouse IgF(ab')$_2$ binding to the non-treated cells (control). The radioactivity of non-treated cells (control) i.e. 100% was approximately 7095 ± 191.5 cpm/well. Open and hatched columns show either non-treated or IL-1/TNF-treated cells. Each point and vertical bar represent mean ± SE of four repeated experiments in which triplicate assays were performed. $^{**}P < 0.01$, $^{*}P < 0.05$ versus the cells treated with IL-1 or TNF alone.

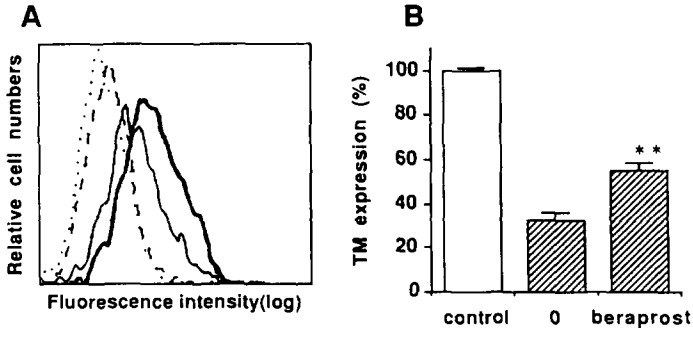

Fig. 6. Effect of beraprost on the decreased TM expression caused by IL-1 exposure for 16 hr—analysis of flow cytometry. The cells were treated with beraprost (100 nM) and IL-1 (3 units/mL) at 10 min interval. After further incubation for 16 hr, TM expression was determined by (A) flow cytometry and (B) binding assay as described in Materials and Methods. In panel A, the abscissa shows the intensity of fluorescence and ordinate shows cell number. Each line indicates non-treated (——), IL-1-treated (– – –), IL-1 plus beraprost-treated (— — —) and negative control without anti-human TM antibody (· · · ·). In panel B, data are expressed similar to Fig. 5. $^{**}P < 0.01$ versus IL-1-treated.

coagulant activity. Thus, cytokines such as IL-1 and TNF released from activated macrophages play a role in the vascular pathogenesis of thrombosis. This means that beraprost possibly exerts an anti-thrombotic effect by acting not only on inflammatory cells [4] but also on vascular endothelial cells.

The present results suggest that beraprost promotes and preserves vascular endothelial cell functions

such as TM expression. Therefore, beraprost may be effective in reducing thrombotic disorders via a mechanism that includes activation of cAMP/TM system.

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