Biochemical Pharmacology, Vol. 36, No. 12, pp. 1913–1917, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00

DECREASE IN AGONIST AFFINITY FOR HUMAN PLATELET THROMBOXANE A_2/PROSTAGLANDIN H_2 RECEPTORS INDUCED BY A PLATELET-DERIVED SUPERNATANT

GERALD W. DORN II, RONALD M. BURCH, PAMELA J. KOCHEL, DALE E. MAIS and PERRY V. HALUSHKA*

Departments of Pharmacology, Cardiology and Medicine and the Drug Science Foundation, Medical University of South Carolina, Charleston, SC 29425, U.S.A.

(*Received* 15 *August* 1986; *accepted* 3 *Novmber* 1986)

Abstract—Platelets possess membrane receptors which mediate the aggregatory response to thromboxane A_2 (TXA_2) and prostaglandin H_2 (PGH_2). It has been observed recently that the affinities for a series of TXA_2/PGH_2 mimetics are decreased in crude human platelet membranes and solubilized membranes compared to intact washed platelets. The present study investigated the notion that platelets contain a substance that is released during platelet lysis that reduces the affinity of the TXA_2/PGH_2 receptor for agonists. The displacement of 9,11-dimethylmethano-11,12-methano-16-(3-iodo-4-hydroxyphenyl)-13,14-dihydro-13-aza-15$\alpha\beta$-ω-tetranor-TXA_2 ([^{125}I]PTA-OH), a TXA_2/PGH_2 receptor antagonist, from its binding site in intact washed platelets by TXA_2/PGH_2 mimetics and antagonists was characterized in the presence or absence of the supernatant (50,000 *g*) obtained from sonicated platelets. In the presence of the supernatant, there was a significant ($P < 0.025$) increase in the IC_{50} values for the TXA_2/PGH_2 mimetics U46619, SQ26655, and ONO11113. The increase in the IC_{50} for U46619 induced by the supernatant was abolished by either boiling or treating the supernatant with trypsin. The supernatant did not affect the K_d or B_{max} of [^{125}I]PTA-OH or the IC_{50} of the TXA_2/PGH_2 antagonist, SQ29548. Pretreatment of the platelets with the supernatant resulted in a significant ($P < 0.02$) reduction in the aggregation response induced by U46619. Gel filtration (Sephacryl S200) of the supernatant revealed a fraction (molecular weight ~100,000 daltons) which significantly increased the IC_{50} for U46619 to displace [^{125}I]PTA-OH from its binding site. Thus, human platelets appear to possess a protein(s) that is released into the supernatant upon sonication and inhibits the binding of TXA_2/PGH_2 agonists but not antagonists to their receptor. This protein may play a role in the regulation of platelet responses to the aggregatory stimuli TXA_2/PGH_2.

Human platelets aggregate in response to thromboxane A_2 (TXA_2†) and prostaglandin H_2 (PGH_2) through interaction with a specific class of membrane receptors [1, 2]. The recent synthesis of the TXA_2/PGH_2 antagonist [^{125}I]PTA-OH has provided a tool to help in the characterization of human platelet TXA_2/PGH_2 receptors [3]. Binding of [^{125}I]PTA-OH to intact washed human platelets [4], platelet membranes [5], and detergent solubilized platelet membrane proteins [6] has demonstrated that the affinity of TXA_2/PGH_2 mimetics for their receptor is decreased in the membrane preparations compared to intact platelets. This suggested the possibility that human platelets possess a substance liberated during their lysis which decreases the affinity of the TXA_2/PGH_2 receptor for its mimetics. If such a substance existed, it could act to modulate the aggregation of platelets *in vivo*. The present studies examined the effects of a crude and gel-filtered superantant of sonicated human platelets on the binding of and aggregation response to TXA_2/PGH_2 mimetics in washed intact platelets.

* Send all correspondence to : Perry V. Halushka, M.D., Ph.D., Department of Pharmacology, Medical University of South Carolina, 171 Ashley Ave., Charleston, SC 29425.

† Abbreviations: TXA_2, thromboxane A_2; PGH_2, prostaglandin H_2; I-PTA-OH, 9,11-dimethylmethano-11,12-methano-16-(3-iodo-4-hydroxyphenyl)-13,14-dihydro-13-aza-15$\alpha\beta$-ω-tetranor-TXA_2; ONO11113, 9-11-epithio-11,12-methano-TXA_2; U46619, 15*S*-hydroxy 11α,9α-(epoxymethano)prosta-5Z,13E-dienoic acid; SQ26655, [1*S*-(1α-2β(5Z)3α(1E,3*S*)),4α]-7-[3-(3-hydroxy-1-octenyl)-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid; SQ29548, [1*S*]1α,2β(5Z),3β,4α]]-7-[3-[[2-[(phenylamino)carbonyl]-hydrazino]methyl]-7-oxabicyclo[2.2.1]-hept-2-yl]-5-heptenoic acid; PMSF, phenylmethylsulfonyl fluoride; and SBTI, soybean trypsin inhibitor.

MATERIALS AND METHODS

[^{125}I]PTA-OH was prepared and stored as previously described [3]. U46619 was purchased from the Upjohn Co. (Kalamazoo, MI). ONO11113 was a gift from the ONO Pharmaceutical Co. (Osaka, Japan). SQ26655 and SQ29548 were gifts from E. R. Squibb & Sons Inc. (Princeton, NJ). All chemicals and marker proteins were purchased from Sigma (St. Louis, MO). Sephacryl S-200 was purchased from Pharmacia (Piscataway, NJ). Platelets were incubated in 50 mM Tris (pH 7.4 at 37°), 100 mM NaCl, 5 mM glucose (incubation medium) unless otherwise noted. All TXA_2/PGH_2 agonists and antagonists

were stored as concentrated stock solutions in ethanol at −20°. Aliquots of the stock solutions were evaporated under nitrogen and dissolved in vehicle (pH 8.5) to a final concentration of 1–5000 nM, and added to the binding reaction in a volume of 20 μl.

Washed platelets were prepared as previously described [4] and diluted in either incubation medium, crude supernatant, or column eluate to 5×10^8 platelets/ml. Indomethacin (10 μM) was present in the incubation medium at all times.

For preparation of platelet supernatant, the platelet pellet obtained from 60 ml of whole blood was suspended in 2 ml of buffer containing 10 μM indomethacin and sonicated with a Heat Systems-Ultrasonics, Inc. (Plainview, NY) model 185F sonicator with the micro tip at setting 6 for 15 sec at 4°. The sonicate was centrifuged at 50,000 *g* for 20 min at 4° and the supernatant was used the same day. The activity of the supernatant declined significantly after storage for 24 hr at −70° (data not shown).

Platelet aggregation in response to U46619 was studied as previously described [4, 7]. Briefly, washed platelets (5×10^8 platelets/ml) were prepared and diluted in incubation medium to which was added $CaCl_2$ (250 μM) and indomethacin (10 μM). The washed platelets (1 ml) were incubated with supernatant (1.5 ml) or incubation medium containing bovine serum albumin (20 mg/ml) as a control for 30 min at 37° and centrifuged at 350 *g* for 8 min. The resultant platelet pellet was then resuspended in incubation medium to a final platelet count of 2.5 to 5×10^8 platelets/ml. Platelet aggregation was carried out in a Chronolog model 300 aggregometer (Haverton, PA). Cuvettes containing platelets (475 μl) were preincubated and stirred for 2 min at 37° prior to the addition of the TXA_2/PGH_2 mimetic U46619 (25 μl) (concentrations from 0.08 to 40 μm). Higher concentrations of U46619 than those which are routinely used were required to aggregate the platelets because of the additional washing procedure. The percent aggregation 1 min after the addition of U46619 was determined. Incubation medium alone was used to represent 100% aggregation.

[^{125}I]PTA-OH binding to washed platelets was determined using a previously described procedure [4]. Briefly, washed platelets at a final concentration of 2.5×10^8 platelets/ml were incubated in the presence of competing ligand (0.5 nM to 1 μM final concentration) in a total volume of 200 μl of which 50 μl was either the supernatant, column eluate (50 μl), or buffer for 30 min at 37° in a shaking water bath with [^{125}I]PTA-OH (~0.05 to 0.15 nM) (30,000–80,000 cpm). [^{127}I]PTA-OH (5 μM) or competing ligand (5 μM) was added to determine displaceable binding. The assays were terminated by addition of ice-cold buffer (4 ml) (50 mM Tris, 100 mM NaCl, pH 7.4 at 4°) and vacuum filtered through Whatman GF/C glass fiber filters followed by three 4-ml washes with ice-cold buffer. Specific binding was defined as total bound cpm minus cpm bound in the presence of excess displacing ligand. All measurements were means of duplicate or triplicate determinations. Specific binding of [^{125}I]PTA-OH was unchanged by the presence of platelet supernatant. To determine if [^{125}I]PTA-OH bound to the platelet supernatant, 90 μg of supernatant protein was incubated for 30 min at 37° with [^{125}I]PTA-OH and then filtered on Whatman GF/C filters presoaked with 0.3% polyethyleneimine [8]. There was no significant binding of [^{125}I]PTA-OH to the supernatant alone (data not shown).

The effects of the protease inhibitors PMSF and SBTI on supernatant activity were determined in the following manner. PMSF freshly dissolved in Me_2SO (75 mg/ml) and SBTI (50 mg/ml in incubation medium) were added to the washed platlets to achieve final concentrations of 75 and 100 μg/ml, respectively, immediately prior to incubation with or without supernatant. After the usual incubations for 30 min, the radioligand binding was terminated by filtration as described above.

Gel filtration experiments were carried out at 4° using a Sephacryl S-200 column (3.2 cm × 24 cm) pre-equilibrated with incubation medium. Platelet supernatant (25–30 mg/2 ml) was placed on the column and eluted at a flow rate of 2 ml/min. Fractions were collected every 30 sec. Void volume (V_0) was determined using blue dextran and total volume (V_t) using $CuSO_4$. Molecular weight markers were used to calibrate the column. γ-Globulin and carbonic anhydrase were assayed by the method of Lowry *et al.* [9]. Elution volumes (V_e) of hemoglobin, myoglobin, and cytochrome *c* were determined by visual inspection of the individual collected fractions. Protein content of each fraction was analyzed by the method of Lowry *et al.* [9].

For analysis of the radioligand binding data, a log-logit transformation of the competition curves was constructed. The IC_{50}, the concentration required to displace 50% of the specifically bound ligand, for each of the mimetics was determined from this transformation. Differences in IC_{50} values and aggregation responses were analyzed using a group Student's *t*-test. Differences between the K_d and B_{max} values for I-PTA-OH in the presence or absence of the supernatant were analyzed using a paired Student's *t*-test. All values are expressed as mean ± SEM.

This study was approved by the Medical University of South Carolina Office for Protection from Research Risk. Informed consent was obtained from all subjects.

RESULTS

The IC_{50} of U46619 to displace [^{125}I]PTA-OH in washed platelets was shifted significantly ($P < 0.005$) by the platelet supernatant almost 3-fold (Table 1).

Table 1. Effects of platelet supernatant on the IC_{50} values of TXA_2/PGH_2 agonists in washed human platelets

Ligand (agonists)	IC_{50} (nM) − Supernatant	+ Supernatant
U46619	148 ± 15 (12)	413 ± 68* (6)
SQ26655	41 ± 4 (7)	153 ± 28† (7)
ONO11113	55 ± 5 (5)	149 ± 26† (5)

Values are the mean ± SEM (N).
* $P < 0.005$ compared to minus (−) supernatant.
† $P < 0.025$ compared to minus (−) supernatant.

The slopes of the displacement curves were not affected significantly by addition of supernatant (-1.06 ± 0.07 for control versus -1.18 ± 0.12 with supernatant). Similar shifts in the IC_{50} values for the TXA_2/PGH_2 mimetics SQ26655 and ONO11113 were also observed in the presence of platelet supernatant (Table 1). Boiling the platelet supernatant for 5 min or incubation of the supernatant with 1% trypsin for 30 min followed by the addition of soy bean trypsin inhibitor (SBTI) abolished the supernatant-induced decrease in receptor affinity for U46619. The IC_{50} for displacement of U46619 was 188 ± 3 nM for the trypsin-treated supernatant, 182 ± 13 nM for boiled supernatant, 190 ± 0 nM in the absence of supernatant, and 310 ± 30 nM in the presence of supernatant (means of two experiments).

To determine if the supernatant also affected the binding of antagonists, two series of experiments were conducted. Equilibrium binding experiments conducted with [^{125}I]PTA-OH and subjected to Scatchard analysis revealed that the K_d of [^{125}I]PTA-OH was 9.5 ± 1.2 nM ($N = 6$) in the absence of the supernatant and 15.1 ± 3.3 nM ($N = 6$) in the presence of the supernatant (difference NS). The B_{max} was 16.8 ± 1.4 fmol/10^7 platelets in the absence of the supernatant and 22.6 ± 3.8 fmol/10^7 platelets in the presence of the supernatant (difference NS).

In a separate series of experiments, the effect of the supernatant on the IC_{50} for the TXA_2/PGH_2 antagonist SQ29548 [10] to displace [^{125}I]PTA-OH was determined. The IC_{50} value for SQ29548 was 4.0 ± 0.5 nM ($N = 6$) in the absence of supernatant and 4.3 ± 0.6 nM ($N = 6$) (NS) in the presence of supernatant.

SBTI (100 μg/ml) and PMSF (75 μg/ml) were added to the incubation mixture to determine if protease inhibitors attenuated the activity of the supernatant. The protease inhibitors did not alter significantly the IC_{50} values of U46619: 150 ± 40 nM without supernatant and 293 ± 50 nM ($N = 4$, $P < 0.025$) with supernatant.

To determine if the supernatant itself bound U46619, which would then lower the effective concentration of U46619 and increase the apparent IC_{50} value, the following experiments were performed. Crude supernatant (16 mg/ml), bovine serum albumin (19 mg/ml), and buffer were coincubated for 30 min with 32 nM [^{3}H]U46619 (130,000 cpm) and filtered on Whatman GF/C filters presoaked with 0.3% polyethyleneimine. The supernatant bound slightly more [^{3}H]U46619 (240 ± 6 cpm) than buffer control (202 ± 2 cpm) and significantly less than the albumin control (553 ± 65 cpm) ($N = 4$). To further confirm this in aggregation experiments, U46619 in various concentrations was used to aggregate washed platelets either after incubation with supernatant for 30 min at 37° or mixed with the supernatant and used immediately. The EC_{50} for U46619 determined for the above groups was 58 ± 2 and 59 ± 2 nM ($N = 2$) respectively. Thus, the supernatant did not appear to reduce the effective concentration of U46619 to any appreciable extent.

The crude supernatant was subjected to gel filtration using Sephacryl S-200. A representative elution curve is shown in Fig. 1.. The peak of activity which maximally increased the IC_{50} of the column

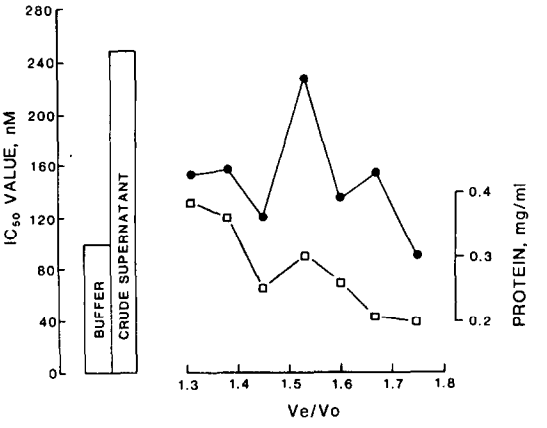

Fig. 1. Representative elution curve for gel filtration of the crude supernatant. A peak of activity was found which increased the IC_{50} of U46619 in the binding studies to approximately the level seen with addition of the crude, unfractionated supernatant. The bars represent the IC_{50} values for U46619 in the control (buffer) or unfractionated supernatant-treated platelets. Key: (□) protein and (●) IC_{50} values.

fractions in the binding assay eluted at an apparent molecular weight of approximately 100,000 daltons. Incubation of the platelets with those fractions increased the IC_{50} value from 112 ± 20 to 212 ± 32 nM ($N = 5$) ($P < 0.05$).

The effect of the crude supernatant on U46619-induced aggregation was determined. A representative set of aggregation curves is shown in Fig. 2. Preincubation of the platelets with supernatant resulted in an average 41% inhibition ($P < 0.02$) of U46619 (0.08 to 40 μM) induced aggregation measured 1 min after the initiation of aggregation.

DISCUSSION

These studies demonstrate the presence of protein(s) with a molecular weight of approximately 100,000 daltons in the supernatant of sonicated platelets that decreases the affinity of TXA_2/PGH_2 agonists but not antagonists for their receptor in washed human platelets. The total number of antagonist binding sites was not altered as determined from the equilibrium binding studies, making it unlikely that the effect of the supernatant was due to a change in the number of receptors. However, since binding experiments were not carried out with a radiolabeled agonist, it is uncertain if the number of binding sites for the agonist changed. The recent availability of [^{3}H]U46619, a TXA_2/PGH_2 agonist, may help to resolve this issue. Since all studies were carried out in the presence of indomethacin, preventing the synthesis and release of endogenous PGH_2/TXA_2, the effect of the supernatant was probably not due to homologous receptor desensitization.

Inhibition of U46619-induced platelet aggregation by the supernatant demonstrates that the inhibitory effect on binding of TXA_2/PGH_2 mimetics to their receptor was associated with a modulating effect on platelet function. The specificity of the effect of the

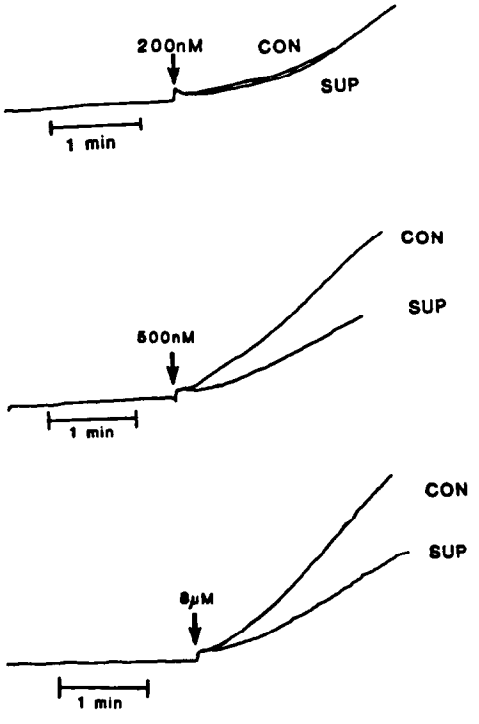

Fig. 2. Representative aggregation responses to U46619 in washed platelets. After incubation with either bovine serum albumin (20 mg/ml) or crude supernatant for 30 min, the platelets were washed and exposed to various concentrations of U46619. The arrow indicates the time of addition of the various concentrations of U46619. Con = control aggregation response. Sup = aggregation response of platelets preincubated with the crude supernatant.

supernatant on other aggregatory stimuli is unknown at this time.

The present study establishes the existence but not the mechanism of the effect of the supernatant on TXA_2/PGH_2 agonist binding. Treatment of platelets with the protease chymotrypsin increases the B_{max} for fibrinogen [11] and increases the IC_{50} of the α_2-adrenergic agonist epinephrine without changing the B_{max} for the α_2-adrenergic antagonist yohimbine [12]. This latter combination of findings is analogous to the findings of the present study and raised the possibility that proteolytic activity may have been responsible for the effects of the supernatant. However, we found that the protease inhibitors PMSF and SBTI did not block the effect of the supernatant, making proteolysis an unlikely mechanism for the effect of the supernatant.

Evidence exists for a cytosolic factor which is involved in the desensitization of membrane receptors. A novel protein kinase, β-adrenergic receptor kinase (β-ARK), has been isolated and partially purified. β-ARK desensitizes the β-adrenergic receptor by phosphorylation but only in the presence of β-adrenergic agonists [13]. The supernatant effect on the TXA_2/PGH_2 receptor differs from that reported for β-ARK because the presence of a TXA_2 receptor agonist was not necessary for desensitization to occur. However, it is still possible that the TXA_2/PGH_2 receptor may also be a substrate for a kinase which is responsible for desensitization.

The increase in K_d for TXA_2/PGH_2 agonists which we observed after exposure of platelets to platelet supernatants, while significant, did not approach the K_d of these agonists seen in platelet membranes [5] and detergent-solubilized membranes [6]. The reasons for this are unknown at this time. It has been demonstrated recently that β-ARK must be translocated from the cytosol to the plasma membrane to desensitize the β-adrenergic receptor. This occurs when cells are exposed to β-adrenergic agonists or PGE_1 [14]. Perhaps the active factor in platelet cytosol must be translocated to the platelet membrane to modify the TXA_2/PGH_2 receptor. It is possible that incubation of platelets with supernatants is less effective in bringing together the enzyme(s) and receptor than disruption of platelets during membrane preparation.

In summary, the effects of a supernatant of sonicated human platelets to inhibit TXA_2/PGH_2 agonist binding to washed platelets have been described. The supernatant did not alter the number of binding sites and, therefore, may be presumed to have decreased the affinity of existing TXA_2/PGH_2 receptors for agonists. Inhibition by the supernatant of platelet aggregation induced by a TXA_2/PGH_2 mimetic provided a functional parallel to the binding studies. The supernatant protein(s) may be released during platelet aggregation and interact with surrounding platelets to desensitize the TXA_2/PGH_2 receptor as a form of feed-back inhibition of platelet aggregation. Clearly, further studies of this protein(s) are warranted.

Acknowledgements—Supported in part by HL 29566, RR1070, HL07260 and a Medical University of South Carolina institutional research fellowship. P. V. H. is a Burroughs-Wellcome Scholar in Clinical Pharmacology. We gratefully acknowledge the secretarial assistance of Nita Pike, Ginny Minchoff, Marie Meadowcroft and Connie Hill.

REFERENCES

1. M. Hamberg, J. Svensson, T. Wakabayashi and B. Samuelsson, *Proc. natn. Acad. Sci. U.S.A.* **71**, 345 (1974).
2. M. Hamberg, J. Svensson and B. Samuelsson, *Proc. natn. Acad. Sci. U.S.A.* **72**, 2994 (1975).
3. D. E. Mais, D. R. Knapp, K. Ballard, N. Hamanaka and P. V. Halushka, *Tetrahedron Lett.* **25**, 4207 (1984).
4. D. E. Mais, R. M. Burch, D. L. Saussy, Jr., P. J. Kochel and P. V. Halushka, *J. Pharmac. exp. Ther.* **235**, 729 (1985).
5. D. L. Saussy, Jr., D. E. Mais, R. M. Burch and P. V. Halushka, *J. biol. Chem.* **261**, 3025 (1986).
6. R. M. Burch, D. E. Mais, D. L. Saussy, Jr. and P. V. Halushka, *Proc. natn. Acad. Sci. U.S.A.* **82**, 7434 (1985).
7. D. E. Mais, D. L. Saussy, Jr., A. Chaikhouni, P. J. Kochel, D. R. Knapp, N. Hamanaka and P. V. Halushka, *J. Pharmac. exp. Ther.* **233**, 418 (1985).
8. R. F. Bruns, K. Lawson-Wendling and T. A. Pugsley, *Analyt. Biochem.* **132**, 74 (1983).
9. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
10. H. Darius, J. B. Smith and A. M. Lefer, *J. Pharmac. exp. Ther.* **235**, 274 (1985).

11. S. Niewiarowski, A. Z. Budzynski, T. A. Morinelli, T. M. Brudzynski and G. J. Stewart, *J. biol. Chem.* **256**, 917 (1981).
12. N. Ferry, S. Adnot, A. Borsodi, L. Marie-Lise, G. Guellaën and J. Hanoune, *Biochem. biophys. Res. Commun.* **108**, 708 (1982).
13. J. L. Benovic, R. H. Strasser, M. G. Caron and R. J. Lefkowitz, *Proc. natn. Acad. Sci. U.S.A.* **83**, 2797 (1986).
14. R. H. Strasser, J. L. Benovic, M. G. Caron and R. J. Lefkowitz, *Proc. natn. Acad. Sci. U.S.A.* **88**, 6362 (1986).