Thromboxane A₂ and prostaglandin endoperoxide receptors in platelets and vascular smooth muscle

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ABSTRACT 9,11-Dimethylmethano-11,12-methano-16-(3-iodo-4-hydroxyphenyl)-13,14-dihydro-13-aza-15αβ-ω-tetranor-TXA₂, (I-PTA-OH), a recently synthesized thromboxane (TX) A₂/prostaglandin (PG) H₂ receptor antagonist, was shown to be a competitive antagonist of human platelet aggregation induced by the stable endoperoxide analog U46619. This antagonism was due to competitive blockade of the platelet TXA₂/PGH₂ receptor since I-PTA-OH did not antagonize the first phase of ADP-induced aggregation which is TXA₂/PGH₂ independent, nor did it inhibit TXA₂ synthesis. In addition, analysis of dose-response curves to U46619 (0.1 to 40 μM) in the presence of increasing concentrations of I-PTA-OH (0.5 to 10 μM) showed that I-PTA-OH produced a parallel rightward shift of the dose-response curve. Further analysis of the data in the form of a Schild plot yielded a straight line with a slope (m = 1.03) not significantly different from −1. These results are consistent with the notion that I-PTA-OH acts as a competitive antagonist of the TXA₂/PGH₂ receptor.

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SINCE THE DISCOVERY of the prostaglandin (PG) endoperoxides PGG₂ and PGH₂ and thromboxane (TX) A₂,¹⁴ their actions on platelets and vascular smooth muscle have been the subject of numerous investigations. Initial studies were descriptive in nature, characterizing PGG₂, PGH₂, and TXA₂ as potent vasoconstrictors²⁻⁷ and stimulators of platelet aggregation.¹⁻⁶,⁸,⁹ Since the development of TX synthetase inhibitors, the potential roles of the PG endoperoxides and TXA₂ in platelet aggregation¹⁰ and in cardiovascular diseases¹¹ have been studied. It was anticipated that this group of compounds could provide new therapeutic approaches for the treatment of a variety of cardiovascular diseases.¹¹

More recently, the development of stable analogs of the PG endoperoxides and TXA₂, which act as either mimics or antagonists of the actions of the naturally occurring eicosanoids, has provided tools for fundamental investigations into the mechanisms of action of these compounds and the nature of their receptors. Studies with the receptor antagonists have provided some preliminary evidence for more than one class of receptors.¹² Like the TX synthetase inhibitors, the receptor antagonists also provide the promise of potential new therapeutic approaches for the treatment of a variety of cardiovascular diseases.¹¹

This report provides information of the effects of 9,11-dimethylmethano-11,12-methano-16-(3-iodo-4-hydroxyphenyl) -13,14- dihydro -13-aza -15αβ-ω-tetranor-TXA₂, (I-PTA-OH), a recently synthesized TXA₂/PGH₂ receptor antagonist, on human platelet aggregation. This compound was originally synthesized for its potential to be radiolabeled with ¹²⁴I for radioligand binding studies. Current concepts concerning platelet and vascular TXA₂/PGH₂ receptors are also reviewed.

Materials and methods

**Platelet aggregation.** Blood obtained by venipuncture from four healthy male subjects who had not received any medication for 2 weeks before the study was collected into plastic syringes containing 3.8% trisodium citrate (9:1). Platelet-rich plasma (PRP) was prepared by centrifugation of whole blood at 100 g for 30 min at room temperature. The PRP (425 μl) was added to individual silanized glass cuvettes. Platelet aggregation was monitored, by the method of Born,¹⁴ in a Chronolog model 300 aggregometer (Havertown, PA) maintained at 37°C. Platelets were preincubated with 25 μl of indomethacin (10 μM) and 25 μl of the antagonist, I-PTA-OH, 0.5 to 10 μM (figure 1) or vehicle for 1 min before the addition of 25 μl of the aggregating agent, the stable endoperoxide analog U46619 (0.1 to 40 μM). The aggregatory response was followed for 2 min. The percent aggregation was determined 1 min after the addition of U46619. The pA₂ value for I-PTA-OH was determined by constructing

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dose-response curves to U46619 in the presence and absence of varied concentrations of I-PTA-OH, as previously described.15, 16

I-PTA-OH was synthesized by a previously described method.13 A 10 mM stock solution of I-PTA-OH was prepared in ethanol and was diluted appropriately in barbital-buffered saline (final concentration of ethanol in the cuvette was 0.1%). U46619 was a generous gift of Dr. John Pike, Upjohn Company, Kalamazoo, MI.

This study was approved by the Institutional Review Board of the Medical University of South Carolina. Written informed consent was obtained from all subjects.

TX synthetase assay. The effect of I-PTA-OH on platelet TX synthetase was measured in human platelet homogenates. PRP was prepared as described above. Platelet pellets were prepared from PRP by centrifugation at 650 g for 20 min and resuspended in 25 mM Tris HCl, pH 7.4. Platelet homogenates were prepared from the resuspended platelet pellets by 20 passes of a tight-fitting Dounce glass homogenizer. Platelet homogenates (1 ml) were preincubated with either vehicle or 10 µM I-PTA-OH for 1 min at 37°C before the addition of 1 mM arachidonic acid (Nucheck Prep, Elysian, MN). Samples (100 µl) were removed at 15, 30, and 60 sec intervals, immediately added to 900 µl of 500 µM imidazole to stop synthesis of TXA₂ and quick-frozen in a dry ice-acetone bath. TXA₂ was measured as its stable metabolite, iTXB₂ (immunoreactive TXB₂), by a previously described radioimmunoassay.17 Protein was determined by the method of Lowry et al.18

Statistics. iTXB₂ levels in control (vehicle) and treated (10 µM I-PTA-OH) platelet homogenates were compared with Student’s t test for paired observations.19

Results

To determine if I-PTA-OH was an antagonist of the platelet TXA₂/PGH₂ receptor, platelets were aggregated with U46619. The latter is considered to be a TXA₂/PGH₂ receptor agonist. I-PTA-OH produced a dose-dependent inhibition of U46619-induced platelet aggregation (figure 2). As shown in figure 3, increasing concentrations of I-PTA-OH produced successive parallel shifts in the dose-response curves to U46619. The Schild plot15, 16 of the data (figure 4) revealed a straight line with a slope not significantly different from −1. Thus, I-PTA-OH appears to be a competitive inhibitor of U46619-induced platelet aggregation. The pA₂ value (6.68) yields a Kᵦ of 208 nM for I-PTA-OH. I-PTA-OH had no effect on the first phase of ADP-induced aggregation.12

I-PTA-OH (10 µM) did not have any significant effect on platelet TX synthetase, as assayed in human platelet homogenates. Sixty seconds after the addition of 1 mM arachidonic acid, iTXB₂ was 6.5 ± 1.4 ng/mg protein in control samples and 6.4 ± 1.9 ng/mg protein in 10 µM I-PTA-OH–treated samples (n = 3, p > .05).

Discussion

Until recently characterization of TXA₂/PGH₂ receptors has been predominately by pharmacologic approaches. The use of radiolabeled ligands for characterization of the receptors has lagged behind the pharmacologic approach. The major reason for this has been the unavailability of a suitable radiolabeled ligand. In particular, these efforts have been hampered by the lack of molecules with high affinity for the putative receptors and that may be easily radiolabeled to high specific activity and retain biological activity. Figure 1 shows the ligands that have been synthesized and used for radioligand binding studies to date.

The first molecule to be synthesized and used for radioligand binding assays was [³H]-13-azaprostanoic

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FIGURE 1. Structures of radiolabeled PGH₂/TXA₂ receptor agonists or antagonists used in radioligand binding studies. 13-APA and U44069 are labeled with ³H, and 1-cis-13-APO and I-PTA-OH are labeled with ¹²⁵I.

FIGURE 2. Dose-dependent inhibition of U46619-induced human platelet aggregation by I-PTA-OH. U46619 (1 µM) was added to the cuvette containing PRP, 10 µM indomethacin and I-PTA-OH, or vehicle, and the aggregation response was followed for 1 min. Numbers to the right of the trace indicate the concentration (µM) of I-PTA-OH present. Experiments were performed as described in methods.
acid (13-APA) (figure 1), a TXA₂/PGH₂ antagonist.²⁰,²¹ Experiments with [³H]-13-APA and human platelet membranes demonstrated that the binding of the drug was saturable, stereospecific, and displaceable by a series of compounds thought to interact with TXA₂/PGH₂ receptors in human platelets.²² However, this compound will probably prove not to be an ideal ligand since its potency is too low to make it suitable for extensive radioligand binding studies. There was also a 100-fold discrepancy between the reported Kₐ and the concentration at which it inhibited 50% (IC₅₀) of U46619-induced platelet aggregation. In addition, [³H]-13-APA was displaced from its binding site by PGF₂α, a compound that reportedly does not act at the TXA₂/PGH₂ receptor,²³,²⁴ although this is somewhat controversial.²⁵

Radiolabeled ligand binding studies with [³H]-U44069 (figure 1), a stable PGH₂ analog, have also been conducted with the use of intact washed human platelets.²⁴ With this ligand, it was shown that binding was sensitive to the stereochemistry of the C-15 OH group. In addition, a series of full and partial agonists and antagonists of TXA₂/PGH₂ displaced the bound ligand with potency ratios similar to those found for their pharmacologic activities in platelets. More recently, with the use of the same ligand,²⁶ it was found that there was a correlation between receptor occupancy by the ligand, induction of phosphatidate formation, and elevation of cytosolic free calcium levels in human platelets, thus establishing pharmacologic relevance to the binding site.

Work in our laboratory has focused on the synthesis and pharmacologic characterization of a series of TXA₂/PGH₂ antagonists, two of which have been radiolabeled with ¹²⁵I and used in radioligand binding studies. The first such compound synthesized, cis-7-(2-p-hydroxyphenylethanolaminocyclopentyl) heptanoic acid (cis-13-APO) (figure 1), a structural analog of 13-APA, was iodinated by a modification of the chloramine T method. It was successfully used in radioligand binding studies, thus demonstrating the utility of ¹²⁵I-labeled ligands in characterizing platelet TXA₂/PGH₂ receptors.²⁷ The iodinated molecule retained the biological activity of the parent compound, although there was some loss of activity. The amount of radiodinated material that bound to membranes from human platelets was saturable, displaceable, dependent on protein concentration, and was reduced by treatment with trypsin or inactivation of the protein by boiling. In addition, the Kₐ determined from binding studies agreed well with the IC₅₀ obtained from platelet aggregation experiments.
However, I-cis-13-APO, like 13-APA, proved to be of insufficient potency to be useful for extensive investigations of platelet TXA$_2$/PGH$_2$ receptors. Therefore, a series of compounds were synthesized that are derivatives of 13-azapine-TXA$_2$, a series of TXA$_2$/PGH$_2$ receptor antagonists that are more potent than 13-APA. One of these compounds was I-PTA-OH (figure 1). Experiments described herein have demonstrated that I-PTA-OH is a competitive antagonist of the human platelet TXA$_2$/PGH$_2$ receptor. In addition, it is considerably more potent than both I-cis-13-APO and 13-APA. Thus, it has the characteristics necessary for it to be used as a radioligand in binding assays. Preliminary studies using [125I]-PTA-OH in radioligand binding assays with human platelet membranes have demonstrated that binding is saturable, displaceable, and dependent on protein concentration. The $K_d$ has been estimated to be approximately 30 nM from equilibrium binding studies. We have recently found that the $K_d$ determined from the $pA_2$ value for I-PTA-OH in washed human platelets is 7.9 nM. Thus, these two $K_d$ values agree well with each other. The higher $K_d$ obtained in PRP in this study probably reflects binding of I-PTA-OH to plasma proteins. However, this binding to plasma proteins apparently does not affect the rank orders of potency for agonists or antagonists. We have found that the rank orders for agonists as stimulators of aggregation in PRP and for antagonists as inhibitors of U46619-induced aggregation in PRP were the same as the rank orders for displacement of [125I]-PTA-OH from its binding sites in washed human platelets. In addition, binding is displaceable by a number of TXA$_2$/PGH$_2$ agonists and antagonists. Thus, [125I]-PTA-OH may prove to be a suitable ligand for use in binding studies of human platelet TXA$_2$/PGH$_2$ receptors.

That both the PG endoperoxides and TXA$_2$ can cause platelet aggregation and contraction of vascular smooth muscle is well documented. In the platelets and blood vessels, TXA$_2$ seems to be more potent than either of the endoperoxides. It is generally assumed that their effects are mediated at discrete receptors, most commonly termed TXA$_2$ and/or TXA$_2$/PGH$_2$ receptors. However, efforts in which the naturally occurring eicosanoids were used to pharmacologically characterize these receptors were hindered due to their lack of stability under physiologic conditions. In 1975, the first potent stable analogs of PGH$_2$ were reported, while the corresponding stable TXA$_2$ analogs were not synthesized until 1979 and 1980. Since that time, a wide variety of synthetic PGH$_2$ and TXA$_2$ analogs that act as agonists or antagonists have been described. These compounds provided useful tools in the elucidation of the physiologic and pathophysiologic roles of PGH$_2$ and TXA$_2$ in the cardiovascular system and platelet function.

As these studies progressed, structure-activity relationships among the various analogs examined became apparent. For example, the most potent derivatives, agonist or antagonist, possess bicyclic ring systems of a variety of types that not only confer biological activity but also lend stability to the molecules. Some of these molecules include the pinane, carbocyclic, and sulfur-substituted isosteres among the TXA$_2$ analogs and the azo, ether, and ethano analogs among the PGH$_2$ derivatives. In general, the retention of electron-negative atoms (O,S,N) in the bicyclic nucleus leads to agonist activity, while substitution to an all-carbon system results in both agonist (CTA$_2$) and antagonist (PTA$_2$) properties, depending upon the degree of substitution of the bicyclic ring system. However, a notable exception is 11a-carba-TXA$_2$, which is an antagonist of human platelet aggregation but devoid of any activity in rat aorta.

Certain structural groupings among the side chains of the analogs examined also appear to be important. Among the agonists, both the presence and the orientation of the C-15 hydroxy group in the natural (S) configuration is crucial for activity as a mimic. Reversal of the configuration at C-15 often results in lower potency or a loss of mimetic activity, indeed many (R)-epimers of agonists are antagonists. In contrast, the influence of the orientation of the C-15 hydroxy group upon the activity of antagonists may be unpredictable, and may depend on the tissue that is studied. We have found in platelets, for example, that the 15(R)- and 15(S)-epimers of PTA-OH, I-PTA-OH, and ONO11120 were equipotent as antagonists of U46619-induced platelet aggregation; in canine saphenous veins the epimers were not equipotent. Other examples exist in which the most potent antagonist is of either the (R) or (S) configuration, which suggests that other structural features play a more important role in these molecules. In addition, the presence of the $\Delta^3$ and $\Delta^9$ double bonds are important determinants of potency for compounds with either agonist or antagonist activity. However, the $\Delta^9$ double bond appears to be the most important determinant of activity. For example, pinane TXA$_2$, an antagonist in platelets and vascular smooth muscle, loses its activity when the $\Delta^9$ double bond is saturated, while carbocyclic TXA$_2$ loses its agonist effect in smooth muscle.

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loss in activity has also been observed with other molecules both in platelet and vascular smooth muscle.37

Previous studies with the “classic” PGs have shown that the lower (ω) side chain could be modified distal to the C-15 position without a loss of agonist activity.51 Furthermore, 16-p-halophenoxy and 16,16-dimethyl derivatives of PGF2α, 11-deoxy-PGF2α, and PGD2 and 11-deoxy-16-phenoxy derivatives of PGE exhibit TXA2/PGH2-like activity.24, 39, 52 In addition, 16-p-halophenoxy derivatives of stable TXA2/PGH2 analogs are more potent agonists than their parents. Indeed, some 16-p-halophenoxy derivatives of TXA2/PGH2 analogs are agonists, while the parent compound acts as an antagonist.46 Likewise, many TXA2/PGH2 antagonists have undergone significant modifications distal to the C-15 position and retained antagonist activity.51, 53 Our work with a series of ω-substituted congeners that are analogs of I-PTA-OH and TXA2/PGH2 receptor antagonists indicate that the receptors in platelets and vascular smooth muscle are different. It was determined that the potency ratios of the compounds as antagonists of U46619-induced platelet aggregation or contraction of saphenous vein were significantly different in tissues from canine and human sources.12 Furthermore, while the 15(R)- and 15(S)-epimers of PTA-OH, 1-PTA-OH, and ONO11120 were equipotent as antagonists of U46619-induced platelet aggregation, there were differences in their potencies as inhibitors of U46619-induced canine saphenous vein contraction.50 This confirms the suggestion put forward by several other investigators5, 37, 42, 47, 54-56 that the receptors are different in platelets than in blood vessels.

In summary, pharmacologic approaches have begun to characterize the TXA2/PGH2 receptor(s) in platelets and vascular smooth muscle. Structure-activity relationships among the variety of analogs synthesized have implicated the existence of different TXA2/PGH2 receptors among platelets and vascular smooth muscle. The strongest evidence for this is the presence of agonist activity in vascular smooth muscle and antagonistic activity in platelets6, 37, 42, 47, 55 within individual molecules and changes in the rank order of potency among a series of 13-azapinane-TXA2 antagonists.12, 50 While pharmacologic studies remain a powerful tool for characterizing the TXA2/PGH2 receptor(s), studies using radioligand binding assays should provide greater understanding of the nature of the TXA2/PGH2 receptor in many tissues, particularly quantitative information on receptor number and regulation.

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