Epithio-11,12-Methano-Thromboxane A₂ Stimulates Inositol Phosphates Accumulation in Isolated Canine Mesenteric Artery Strips

Haruaki Ninomiya¹,# and Motohatsu Fujiwara²,∗

¹Department of Pharmacology, Faculty of Medicine, Kyoto University, Kyoto 606, Japan
²Department of Pharmacology, Faculty of Pharmaceutical Sciences, Mukogawa Women's University, Nishinomiya 663, Japan

Received April 1, 1991 Accepted July 24, 1991

ABSTRACT—Epithio-11,12-methano-thromboxane A₂ (STA₂), a stable analog of thromboxane A₂ (TXA₂), stimulated inositol phosphates (IPs) accumulation in canine mesenteric artery strips, but not in cerebral (basilar) artery strips. When canine mesenteric artery strips were incubated with 0.1 μM [³H]myo-inositol for 15 min and then stimulated with 10 μM STA₂ for 30 min, there was a significant increase in ³H-IPs accumulation as measured by anion exchange chromatography (2,028 ± 204 and 3,526 ± 210* dpm/mg protein for basal and stimulated accumulations, respectively; means ± S.E.M., n = 3, *P < 0.01, significantly different from the basal value). This effect of STA₂ was dose-dependent with an EC⁵₀ value of 1.6 ± 0.2 μM. The presence of equimolar concentrations of TXA₂ receptor antagonists, either ONO-3708 (9,11-dimethylmethano-11,12-methano-13,14-dihydro-13-aza-14-oxo-15-cyclopental-16,17,18,19,20-pentanor-15-epi-thromboxane A₂) or S-1452 (5Z-7-(3-endo-phenylsulfonamino(2.2.1.)-bicyclohept-2-exo-yl)heptenoic acid), completely blocked the effect of STA₂. These results suggest the presence of TXA₂ receptors coupled with IPs accumulation in canine mesenteric artery strips. The exact location of the TXA₂ receptor-IPs system, however, remains unknown.

Thromboxane A₂ (TXA₂) is a potent stimulator of platelet aggregation and a constrictor of vascular smooth muscles. As for the intracellular signal transduction mechanism(s) for TXA₂, it is generally assumed that the TXA₂ receptors on platelets are coupled with phosphoinositide (PI) turnover through a GTP-binding protein(s) and phospholipase C (1). The "rhodopsin-type" molecular structure of the receptor that was deduced from the cloned cDNA strongly supports this notion (2). In contrast, the intracellular signal transduction mechanism(s) for the putative TXA₂ receptors on smooth muscles is largely unknown. Radioligand binding studies using radioactive derivatives of TXA₂ demonstrated the presence of putative TXA₂ receptors on vascular smooth muscles from various species and tissues (3–5). It is, however, not yet determined whether the platelet and vascular receptors are the same or not (6–11). Furthermore, even if the molecular structures of the platelet and vascular receptors are the same, it
is still possible that vascular receptors have an intracellular signal transduction mechanism(s) distinct from platelet receptors. Thus, the present study was undertaken to determine if the vascular smooth muscle TXA₂ receptors are coupled with PI turnover. Canine cerebral (basilar) and mesenteric arteries were chosen because of their well-characterized responsiveness to exogenous TXA₂ analogs (12).

MATERIALS AND METHODS

Myo-[2-³H]inositol (4.01 GBq/mmol) was purchased from Amersham (Buckinghamshire, U.K.). Dowex-1 anion exchange resin (100–200 mesh, X 8 in the formate form) was obtained from BioRad (NY, U.S.A.). STA₂ (epithio-11,12-methano-thromboxane A₂) and ONO-3708 (9,11-dimethylmethano-11,12-methano-13,14-dihydro-13-aza-14-oxo-15-cyclo-pental-16,17,18,19,20-pentanor-15-epi-thromboxane A₂) were kindly provided by Ono Pharmaceutical Co., Ltd. (Osaka, Japan). S-1452 (5Z-7-(3-endo-phenylsulfonylamino-(2.2.1.)-bicyclohept-2-exo-yl)heptenoic acid) was kindly provided by Shionogi & Co., Ltd. (Osaka, Japan). All other chemicals were of reagent grade and were obtained commercially.

Preparation of artery strips

Cerebral (basilar) and mesenteric arteries were isolated from mongrel dogs of either sex, weighing from 8 to 14 kg, anesthetized with sodium pentobarbital (30 mg/kg, i.v.) and given calcium heparin (500 U/kg, i.v.). The arteries were cut into helical strips of 2 mm X 5 mm and dispersed in Krebs buffer (120 mM NaCl, 4.7 mM KCl, 1.3 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 25 mM NaHCO₃, 11.7 mM glucose) equilibrated to pH 7.4 with 95% O₂/5% CO₂ at 37°C.

Assay of inositol phosphates (IPs) accumulation

Assay of IPs accumulation was done according to the method of Ninomiya et al. (13) with the modifications detailed below: Each artery strip was incubated at 37°C with 270 µl Krebs buffer containing 0.1 µM [³H]inositol and 8 mM LiCl. At 15 min, 30 µl of STA₂ solution was added, and the reaction was allowed to continue, usually for 30 min. When an antagonist was used, the initial incubation was started in 240 µl Krebs buffer and 30 µl of antagonist solution was added 5 min before the addition of STA₂. The samples were exposed to a continuous gas flow of 95% O₂/5% CO₂ throughout the reaction. The reaction was halted by adding 0.94 ml of ice-cold chloroform/methanol (1:2 v/v) and 0.31 ml of chloroform, and then 0.31 ml of water was added to separate the phases. After disrupting the tissue by ultrasound sonication and subsequent centrifugation at 1,000 X g for 10 min, 0.75 ml of the upper aqueous phase was diluted to 3 ml with water and added to 1 ml of a slurry (50% w/w) of Dowex-1 resin. The column was washed with 30 ml of 5 mM myo-inositol, and then the ³H-IPs fraction was eluted with 1 ml of 0.1 M formic acid/1.0 M ammonium formate. A portion (0.8 ml) of this eluate was added to 10 ml of scintillation fluid and counted for radioactivity by a Packard Tri-Carb scintillation spectrometer (Model 3255) at a counting efficiency of 40%.

In some experiments, following separation of the phases, 200 µl of the lower organic phase was removed, dried overnight and counted for the radioactivity to determine the total incorporation of [³H]inositol into phospholipids.

After evaporation of the organic phase, proteins were dissolved in 0.5 M NaOH and measured according to the method of Lowry et al. (14).

All the values shown in the text are means ± S.E.M. from n determinations, each done in triplicate. Statistical analysis was done by analysis of variance (ANOVA).

RESULTS

In our assay conditions, STA₂ stimulated ³H-IPs accumulation in canine mesenteric artery strips, but not in cerebral (basilar) artery
strips (Fig. 1). Therefore, all of the subsequent experiments were done on mesenteric artery strips. Because STA2 caused a linear accumulation of $^3$H-IPs up to 30 min incubation (data not shown), the reaction time was set to 30 min in the subsequent experiments. The effect of STA2 was dose-dependent with an EC$_{50}$ value of 1.6 ± 0.2 µM (n = 3; Fig. 2). The results were essentially the same when the endothelial layer was disrupted by manual rubbing (data not shown). The presence of 10 µM STA2 caused no significant change in the radioactivity incorporation into phospholipids at the end of the 30-min reaction time (8,253 ± 678 or 7,891 ± 501 dpm/200 µl of CHCL$_3$ layer in the presence or absence of STA2, respectively. n = 3). The presence of equimolar concentrations of TXA2 receptor antagonists, either ONO-3708 or S-1452, completely blocked the effect of STA2 (Fig. 3). The inhibition curves gave IC$_{50}$ values of 4.2 ± 0.9 and 1.9 ± 0.4 µM for ONO-3708 and S-1452, respectively (n = 3, Fig. 4).

Fig. 1. STA2 stimulated the accumulation of $^3$H-IPs in canine mesenteric artery strips, but not in cerebral (basilar) artery strips. Each strip was incubated for 30 min in the presence (solid bar) or absence (empty bar) of 10 µM STA2. $^3$H-IPs accumulation in each strip was determined as described in “Materials and Methods”. Each bar represents the means ± S.E.M. of three determinations, each done in triplicate. *P < 0.01, significantly different from the basal value.

Fig. 2. Dose-response curves for STA2-stimulated $^3$H-IPs accumulation in canine mesenteric artery strips. Mesenteric artery strips were processed as described in the legend for Fig. 1, with increasing concentration of STA2. Data are the means ± S.E.M. of three determinations, each done in triplicate.
DISCUSSION

This paper is the first report of the effect of STA2 on IPs accumulation in canine artery strips. As far as the data on mesenteric artery is concerned, the observed effect of STA2 was clearly a receptor-mediated event because it was completely blocked by the specific TXA2 receptor antagonist ONO-3708 (15) or S-1452 (16). There are three possible primary sites of action for STA2: 1) endothelium 2) smooth muscle and 3) nerve endings that are supposed to be located outside the smooth muscle layer.

First, the endothelium-dependent effect of STA2 seemed unlikely because the effects were essentially the same when the endothelial layer had been disrupted by manual rubbing.

Second, if we assume that STA2 has a direct effect on smooth muscles, the observed results suggest the involvement of the PI turnover pathway in the vasoconstrictive effect of TXA2. However, we cannot tell whether the effect is the primary event of the receptor...
activation leading to phospholipase C activation or an event secondary to the enhanced Ca\(^{2+}\) influx caused by a still unknown mechanism(s). Preliminary experiments have shown that STA\(_2\) in micromolar concentrations caused a significant increase in \(^3\)H-IPs accumulation in mesenteric artery strips even when Ca\(^{2+}\) was absent from the incubation medium (data not shown). These results, however, might simply imply that the adequate Ca\(^{2+}\) influx can be achieved even in low extracellular Ca\(^{2+}\) concentrations. The possible coupling of TXA\(_2\) receptors on mesenteric artery strips with PI turnover regardless of whether it is primary or secondary to Ca\(^{2+}\) influx, seemed not to be the main signal transduction mechanism for the vasoconstrictive effect of TXA\(_2\) because 1) the observed EC\(_{50}\) value of micromolar order for STA\(_2\)-stimulated accumulation of \(^3\)H-IPs is much higher than the previously reported EC\(_{50}\) value for STA\(_2\)-stimulated contraction of mesenteric artery strips which is in the nanomolar range (12) and 2) the effect of STA\(_2\) on \(^3\)H-IPs accumulation was not observed in canine basilar artery strips. Previous studies have shown STA\(_2\)-evoked constriction of canine basilar artery strips, and the observed EC\(_{50}\) value for the constrictive effect of STA\(_2\) on basilar artery strips was compatible with that on mesenteric artery strips (12). These discrepancies between the effect of STA\(_2\) on \(^3\)H-IPs accumulation presented here and that on constriction previously reported indicate that if we assume the direct effect of STA\(_2\) on smooth muscles, there must be another, probably more vital, signal transduction system(s) for the TXA\(_2\)-receptor mediated vasoconstriction.

Third, if we assume that STA\(_2\) acts nerve terminals, the observed effect of STA\(_2\) may reflect either the STA\(_2\)-stimulated PI turnover in the nerve endings or the effect of some other substance(s), the release of which was evoked by STA\(_2\). In this case, the observed differences in the effects of STA\(_2\) between mesenteric and cerebral artery strips may reflect the differential nervous innervation between these two arteries. Another experimental approach such as denervation experiments is necessary to examine this possibility.

In summary, we found that STA\(_2\) stimulates IPs accumulation in canine mesenteric artery strips. Further study is necessary to determine the exact site of action of the drug and to examine the possible involvement of the PI turnover pathway for the vasoconstrictive effect of STA\(_2\).

Acknowledgments

This work was supported by a Grant-in Aid for Special Project Research (01641007) from the Ministry of Education, Science and Culture, Japan and by a Grant-in-Aid for Fellowships of the Japan Society for the Promotion of Science for Japanese Junior Scientists.

REFERENCES

1. Arita, H., Nakano, T. and Hanasaki, K.: Thromboxane A\(_2\): Its generation and role in platelet activation. Prog. Lipid. Res. 28, 273–301 (1989)

2. Hirata, M., Hayashi, Y., Ushikubi, F., Yokota, Y., Kageyama, R., Nakanishi, S. and Narumiya, S.: Cloning and expression of cDNA for a human thromboxane A\(_2\) receptor. Nature 349, 617–620 (1991)

3. Mihara, S., Doteuchi, S., Hara, M., Ueda, M., Ide, M., Fujimoto, M. and Okabayashi, T.: Characterization of \(^3\)H-U46619 binding in pig aorta smooth muscle membranes. Eur. J. Pharmacol. 151, 59–65 (1988)

4. Hanasaki, K., Nakano, K., Kasai, H., Arita, H., Ohtani, K. and Doteuchi, M.: Specific receptors for thromboxane A\(_2\) in cultured smooth muscle cells of rat aorta. Biochem. Biophys. Res. Commun. 150, 1170–1175 (1988)

5. Morinelli, T.A., Mais, D.E., Oatis, J.E., Jr., Crumbley A.J., III and Halushka, P.V.: Characterization of thromboxane A\(_2\)/prostaglandin H\(_2\) receptors in human vascular smooth muscle cells. Life Sci. 46, 1765–1772 (1990)

6. Lefer, A.M., Smith, E.F., III, Arata, H., Smith, J.B., Aharony, D., Claremon, D.A., Magolda, R.L. and Nicolau, K.C.: Dissociation of vasoconstrictor and platelet aggregating activities of thromboxane by carbocyclic thromboxane A\(_2\), a stable analog of thromboxane A\(_2\). Proc. Natl. Acad. Sci. U.S.A. 77, 1706–1710 (1980)

7. Akbar, H., Mukhopadhyay, A., Anderson, K.S., Navran, S.S., Romstedt, K., Miller, D.D. and Feller, D.R.: Antagonism of prostaglandin-mediated responses in platelets and vascular
smooth muscle by 13-azaprostanoic acid analogs. Biochem. Pharmacol. 34, 641–647 (1985)

8 Armstrong, R.A., Jones, R.L., Peesapati, V., Will, S.G. and Wilson, N.H.: Competitive antagonism at thromboxane receptors in human platelets. Br. J. Pharmacol. 84, 595–607 (1985)

9 Mais, D.E., Dunlap, C., Hamanaka, N. and Halushka, P.V.: Further studies on the effects of epimers of thromboxane A₂ antagonists on platelets and veins. Eur. J. Pharmacol. 111, 125–128 (1985)

10 Mais, D.E., Saussy, D.L., Chaikhouni A., Kochel, P.J., Knapp, D.R., Hamanaka, N. and Halushka, P.V.: Pharmacologic characterization of human and canine thromboxane A₂/prostaglandin H₂ receptors in platelets and blood vessels: evidence for different receptors. J. Pharmacol. Exp. Ther. 233, 418–424 (1985)

11 Swayne, G.T.G., Maguire, J., Dolan, J., Raval, P., Dane, G., Greener, M. and Owen, D.A.A.: Evidence for homogeneity of thromboxane A₂ receptor using structurally different antagonists. Eur. J. Pharmacol. 152, 311–319 (1988)

12 Toda, N., Nakajima, M., Okamura, T. and Miyazaki, M.: Interactions of thromboxane A₂ analogs and prostaglandins in isolated dog arteries. J. Cardiovasc. Pharmacol. 8, 818–825 (1986)

13 Ninomiya, H., Taniguchi, T. and Fujiwara, M.: Phosphoinositide breakdown in rat hippocampal slices. Sensitivity to glutamate induced by in vitro anoxia. J. Neurochem. 55, 1001–1007 (1990)

14 Lowry, O.H., Rosebrough, N.J., Farr A.L. and Randall, R.J.: Protein measurement with the Folin phenol regent. J. Biol. Chem. 193, 265–275 (1951)

15 Fujioka, M., Nagao, T. and Kuriyama, H.: Actions of the novel thromboxane A₂ antagonists, ONO-1270 and ONO-3708, on smooth muscle cells of the guinea-pig basilar artery. Naunyn Schmiedebergs Arch. Pharmacol. 334, 468–474 (1986)

16 Hanasaki, K. and Arita, H.: Characterization of a new compound, S-145, as a specific TXA₂ receptor antagonist in platelets. Thromb. Res. 50, 365–376 (1988)