原 著

抗動脈硬化薬に関する研究 (第10報)

EG-626 (7-ethoxycarbonyl-4-hydroxymethyl-6, 8-dimethyl-1 (2H)-phthalazinone) の血小板凝集抑制に関する研究

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Studies on anti-atherosclerotic agents. X.

Mode of action of EG-626 (7-ethoxycarbonyl-4-hydroxymethyl-6, 8-dimethyl-1 (2H)-phthalazinone) on platelet aggregation

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TXA₂

EG-626 (7-ethoxycarbonyl-4-hydroxymethyl-6, 8-dimethyl-1 (2H)-phthalazinone) is a newly synthesized anti-atherosclerotic and anti-aggregatory agent. The mode of inhibitory action of EG-626 on arachidonic acid (AA)-induced rabbit platelet aggregation was studied in relation to its antagonistic effect on the action of thromboxane (TX) A₂, and the following results were obtained.

EG-626 in a dose of 3 to 100 μ g/ml inhibited sodium arachidonate (SAA)-, ADP-, or collagen-induced rabbit platelet aggregation in a concentration dependent manner, 8 and 50 μ g/ml inhibited rabbit platelet aggregation induced by TXA₂ generated by prostaglandin (PG) H₂ and platelet microsomes and 10 and 60 μ g/ml markedly inhibited rabbit platelet aggregation induced by labile aggregation-stimulating substance (LASS). However, a dose of 100 μ g/ml had no discernible inhibitory effect on the conversion of AA to its metabolites using washed platelet suspension. In addition, EG-626 inhibited the contractile response of aortic strips to TXA₂, without affecting TXA₂-formation.

These results suggest that EG-626 inhibits AA-induced rabbit platelet aggregation primarily through an antagonistic effect on the actions of TXA₂ and other active metabolites of AA, without affecting their biosynthesis in platelets.

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要 約

EG-626 は新規に合成された 抗動脈硬化および抗凝集薬である。EG-626 の家兎血小板におけるアラキドン酸凝集抑制作用の機序をその抗 thromboxane(TX) A_2 作用と関連して検討し、以下の結果を得た。

EG-626 ($3\sim 100\mu\text{g/ml}$) は濃度依存的に、アラキドン酸ナトリウム、ADP もしくはコラーゲンによる家兎血小板凝集を抑制し、またこの化合物 (8 および $50\mu\text{g/ml}$) は prostaglandin H_2 と血小板マイクロリームによって生成された TXA_2 による 家兎血小板凝集を抑制した。さらに、EG-626 (10 および $60\mu\text{g/ml}$) は LASS (labile aggregation-stimulating substance) に

よる家兎血小板凝集も抑制した。しかしながら、洗滌血小板によるアラキドン酸の代謝物への変換に関しては、何ら影響をおよぼさなかった。また、EG-626 は家兎大動脈標本の TXA_2 による収縮を抑制したが、 TXA_2 の生成を抑制しなかった。

これらの結果は、この化合物の家兎血小板におけるアラキドン酸凝集抑制の作用機序が、主として、 TXA_2 をはじめとするアラキドン酸から生成された凝集物質に対する拮抗作用に基づき、それらの血小板での生合成に対する阻害作用に基づくものではないことを示唆するものである。

Introduction

Thromboxane (TX) A_2 , one of the metabolites of arachidonic acid (AA) in platelets, is a labile substance which induces platelet aggregation and vasoconstriction¹⁾ and can trigger fatal events such as stroke and myocardial infarction²⁾.

It has been reported that EG-626 inhibits platelet aggregation induced by AA, ADP, collagen, etc.³⁾, and other findings on the mechanism of anti-aggregatory effect of the compound have also been reported^{4) 5)}. However, to our knowledge, there has been no report on the relationship between its inhibitory effect on AA-induced platelet aggregation and the antagonistic effect on the action of TXA_2 .

We now report that EG-626 has a potent inhibitory effect on TXA_2 -induced aggregation, and that the inhibitory effect on AA-induced aggregation is primarily due to its antagonistic effect on the actions of TXA_2 and other active metabolites of AA.

Materials and Methods

1) Materials,

AA (Sigma Chemical Co. Grade I) was used as sodium salt or free acid. TXB_2 was kindly provided by Dr. Y. Nakamoto of Fuji Pharmaceutical Co. Ltd. EG-626 was synthesized in our laboratory⁶⁾. Indomethacin (donated by Nippon Merck-Banyu Co. Ltd.) was used as sodium salt. Other materials used in experiments were as follows: [$1\text{-}^{14}\text{C}$] AA (60 mCi/mmol) (New England Nuclear), imidazole (Nakarai Chemicals Ltd.), adenosine 5'-diphosphate sodium salt (ADP) (Sigma Chemical Co. Type I), collagen (from equine tendon) (Hormon Chemie), L-tryptophan (Sigma Chemical Co.), bovine hemoglobin (Sigma Chemical Co.

Table 1 Effects of EG-626 and indomethacin on platelet aggregation induced by ADP, SAA and collagen.

Compounds ($\mu\text{g/ml}$)	Inhibition Percent		
	ADP(10 μM)	SAA(137 μM)	Collagen(40 $\mu\text{g/ml}$)
EG-626			
3	—	6*(7)	—
10	5 (2)	35 (7)	9 (4)
30	26 (5)	67 (6)	21 (3)
100	76 (4)	—	—
Indomethacin			
8	—	100 (2)	43 (4)
80	0 (2)	—	—

* Mean value; number of determinations in parenthesis.

Type I), prostaglandin E_2 and Prostaglandin $\text{F}_{2\alpha}$ (Sigma Chemical Co.), precoated silica gel 60 F254 plate (E. Merck).

2) Preparation of Platelet Rich Plasma (PRP) and Platelet Poor Plasma (PPP).

Blood was collected into plastic tubes containing 1/10 volume of sodium citrate solution (3.8 % (w/v)) from the carotid artery of rabbits (2 to 3 kg) anesthetized with ether. The citrated blood was spun at 150 g for 20 min. PRP was carefully separated from the bottom layer of erythrocytes and leucocytes using a plastic pipette. The platelet counts in the PRP samples were determined to be 3 to $5 \times 10^5/\mu\text{l}$ by means of a Coulter Counter Model Z-B (Coulter Electronics Inc.). PPP was prepared by centrifugation of PRP at 1,500 g for 10 min.

3) Preparation of Washed Platelet Suspension (WPS).

WPS was prepared by the method of Svensson et al.⁷⁾ The platelet counts of WPS samples were determined to be 6 to $10 \times 10^5/\mu\text{l}$, as described above.

4) Preparation of Prostaglandin (PG) H_2 , and Generation of TXA_2 .

Bovine platelet microsomes (PM) were prepared according to Needleman et al.⁸⁾ PGH_2 was prepared by the method of Yoshimoto et al.⁹⁾ TXA_2 was generated as follows: PGH_2 (0.5 or 1 μg) was transferred into a cuvette and dried under a stream of N_2 . PM (0.8 or 1.6 mg protein) suspended in 50 mM Tris buffer pH 7.8 (400 μl) were preincubated with a solution of each test compound (100 μl) or saline at 22° for 3 min. The mixture was transferred into the cuvette containing PGH_2 , and then incubated at 0° for 2 min.

5) Generation of Labile Aggregation-Stimulating Substance (LASS).

LASS^{1) 10)} was generated as follows: WPS (1 ml) was preincubated with saline (100 μl) at 37° for 3 min. The mixture was then incubated with a solution of sodium arachidonate (SAA) (100 μl , final conc. 34 μM) at 37° for 1 min.

6) Bioassay Using Rabbit Aortic Strips.

Spirally cut strips of rabbit aorta, 30 to 40 mm in length and 3 to 4 mm in

Fig. 1 (A) Aggregation profiles by the addition of PGH₂ and PM alone or combined. PGH₂ (1 μg) and PM (1.6 mg) were incubated as described in Materials and Methods. Alternatively, PGH₂ or PM was incubated alone. An aliquot (80 μl) of the incubation mixture was transferred into a cuvette containing PRP (440 μl).
 a: PGH₂+PM; b: PM alone; c: PGH₂ alone.

(B) Lability of the active substance by incubation at 37°.

a: PGH₂ was incubated with PM at 0° for 2 min before testing; b: the incubation mixture was further incubated at 37° for 5 min before testing.

(C) Effect of EG-626 on TXA₂-induced platelet aggregation. PRP (400 μl) was preincubated with a solution of test compound (40 μl) or saline (control) at 37° for 3 min, and platelet aggregation was then initiated by the addition of the incubation mixture (80 μl).
 a: control; b: EG-626 8 μg/ml; c: EG-626 50 μg/ml.

(D) Effect of indomethacin on TXA₂-induced platelet aggregation.

a: control; b: indomethacin 140 μg/ml.

width were suspended under a tension of 1.5 g and superfused with 10 ml/min of Krebs-Henseleit bicarbonate medium containing a mixture of combined antagonists¹¹⁾ and indomethacin (1 μg/ml). The changes in tension of the preparation were recorded on a recticorder (RJG-3024, Nihonkoden Kogyo Co. Ltd.) through a force-displacement transducer (SB-1T, Nihonkoden Kogyo).

7) Measurement of Platelet Aggregation.

Measurement of platelet aggregation was carried out at 37° by the modified method of Born¹²⁾ using a dual sample aggregometer (Sienco Model DP-247E). The light transmission was adjusted to 0 and 100 % with PRP and PPP, respectively. PRP was preincubated with a solution of each test compound or saline at 37° for 3 min. Platelet aggregation was then initiated by the addition of an aggregating agent.

8) Radiochemical Experiments.

WPS (2 ml) was preincubated with the test compound (or saline) at 37° for 3 min, and then incubated with [¹⁴C] AA (2 μCi, 33 nmoles) at 37° for 20 min. The reaction mixture was diluted with

- Fig. 2** (A) Lability of the active substance generated by incubation of SAA with WPS. The active substance was generated as described in Materials and Methods. An aliquot (40 μ l) of the incubation mixture was transferred into a cuvette containing PRP (440 μ l).
 a: SAA and WPS were incubated at 37° for 1 min before testing; b: the incubation mixture was further incubated at 37° for 5 min before testing.
- (B) Effects of EG-626 and indomethacin on LASS-induced platelet aggregation. PRP (400 μ l) was preincubated with a solution of each test compound (40 μ l) or saline (control) at 37° for 3 min, and platelet aggregation was then initiated by the addition of the incubation mixture (40 μ l).
 a: control; b: indomethacin 120 μ g/ml; c: EG-626 10 μ g/ml; d: EG-626 60 μ g/ml.
- (C) Effect of EG-626 on LASS-formation. WPS (1 ml) was preincubated with a solution of test compound (100 μ l) or saline (control) at 37° for 3 min. The mixture was then incubated with a solution of SAA (100 μ l, final conc. 34 μ M) at 37° for 1 min, and platelet aggregation was initiated by the addition of an aliquot (40 μ l) of the incubation mixture to a cuvette containing PRP (440 μ l).
 a: control; b: EG-626 120 μ g/ml.
- (D) Effect of indomethacin on LASS-formation.
 a: control; b: indomethacin 8 μ g/ml.

water, and 5 volumes of methanol were then added. The mixture was acidified to pH 3.5 with 1 N HCl and extracted twice with ethyl acetate. The organic phases were pooled and washed twice with water, and dried over anhydrous

sodium sulfate. After evaporation of the solvent, the residue was dissolved in ether (2 ml), and 100 μ l aliquot was spotted on a silica gel plate. The plate was developed in a solvent system (isooctane-ethyl acetate-acetic acid-water (5 : 11 : 2 : 10, v/v)). Authentic TXB₂, AA, PGE₂ and PGF_{2 α} were also placed on the plate. The spots on the plate were revealed by exposure to iodine vapour and the radioactivity was assessed using a scanner (Packard Model 7220).

Results

1) Effects of EG-626 and Indomethacin on Platelet Aggregation Induced by ADP, SAA and Collagen.

As shown in **Table 1**, EG-626 caused a concentration dependent inhibition of platelet aggregation induced by ADP (10 μ M), SAA (137 μ M) or collagen (40 μ g/ml). The inhibitory effect of EG-626 on SAA-induced aggregation was more potent than that on ADP-or collagen-induced aggregation. Though indomethacin markedly inhibited SAA-and collagen-induced aggregation, the compound showed no discernible inhibition of ADP-induced aggregation in a high concentration of 80 μ g/ml.

2) Effects of EG-626 and Indomethacin on TXA₂-Induced Aggregation.

As shown in **Fig. 1**, PGH₂ or PM alone did not induce a discernible platelet aggregation, at the concentration tested. On the other hand, a potent aggregating substance was generated by the incubation of PGH₂ with PM. The active substance was scarcely detected following a further 5 min incubation at 37°. Therefore, it seems likely that the aggregation was related to the generation of TXA₂⁸⁾. EG-626 (8 and 50 μ g/ml) caused a marked inhibition of aggregation induced by the incubation mixture of PGH₂ with PM, presumably caused by TXA₂, in a concentration dependent manner. On the other hand, indomethacin in a high concentration of 140 μ g/ml showed only a slight inhibition.

3) Effects of EG-626 and Indomethacin on LASS-Induced Aggregation and LASS-Formation.

As shown in **Fig. 2**, a potent aggregating substance was generated by incu-

Fig. 3 The thin layer chromatogram scan of the metabolites produced by incubation of [¹⁴C] AA with WPS in the presence of EG-626 or indomethacin. In the control experiment, saline (200 μ l) was added instead of a solution of the test compound. The experimental conditions are described in Materials and Methods.

Fig. 4 Effects of EG-626 and imidazole on the conversion of PGH_2 to TXA_2 by PM. The vasoactive substance was generated by incubation of PGH_2 (0.5 μg) with PM (400 μl , 0.8 mg protein) preincubated with a solution of each test compound (100 μl) or saline as described in Materials and Methods. Alternatively PGH_2 was incubated alone. Rabbit aorta contractile activity was assayed immediately after termination of the incubation.
 a: PGH_2 alone; b: $\text{PGH}_2 + \text{PM}$; c: $\text{PGH}_2 + \text{PM}$ (pretreated with imidazole 200 $\mu\text{g}/\text{ml}$); d: $\text{PGH}_2 + \text{PM}$; e: $\text{PGH}_2 + \text{PM}$ (pretreated with EG-626 200 $\mu\text{g}/\text{ml}$); f: $\text{PGH}_2 + \text{PM}$.

bation of SAA with WPS. The substance was scarcely detected following a further 5 min incubation at 37° , thereby suggesting that the substance was LASS^{1) 10)}. Preincubation of PRP with EG-626 (10 and 60 $\mu\text{g}/\text{ml}$) caused a marked inhibition of the aggregation induced by the incubation mixture of SAA with WPS, presumably caused by LASS, whereas preincubation of WPS with a high concentration of EG-626 (120 $\mu\text{g}/\text{ml}$) resulted in only a slight inhibition of platelet aggregation, suggesting that the compound inhibited LASS-induced aggregation without affecting LASS-formation. In contrast, preincubation of WPS with indomethacin (8 $\mu\text{g}/\text{ml}$) completely abolished LASS-formation, however, the compound showed only a slight inhibition of LASS-induced aggregation in a high concentration of 120 $\mu\text{g}/\text{ml}$.

4) Effects of EG-626 and Indomethacin on the Metabolic Pathway of AA.

Effects of EG-626 and indomethacin on the metabolic pathway of AA were also assessed by radiochemical methods. **Fig. 3** shows a representative thin layer radiochromatogram scan of the metabolites produced by the incubation of [$1\text{-}^{14}\text{C}$] AA with WPS: four main peaks, P_1 , P_2 , P_3 and P_4 were detected. Typical R_f values for the peaks were: P_1 , 0.30; P_2 , 0.67; P_3 , 0.74 and P_4 , 0.86. P_1 and P_4 comigrated with authentic TXB_2 and AA, respectively. The R_f values for P_2 and P_3 in the solvent system were similar to those reported by others¹³⁾ for 12L-hydroxy-5, 8, 10-heptadecatrienoic acid (HHT) and 12L-hydroxy-5, 8, 10, 14-eicosatetraenoic acid (HETE), respectively. Therefore, it seems likely that P_1 , P_2 , P_3 , P_4 include TXB_2 , HHT, HETE and unchanged AA, respectively. When WPS was treated with indomethacin (8 $\mu\text{g}/\text{ml}$), the peaks corresponding

to TXB_2 and HHT disappeared, whereas that corresponding to HETE was increased. In contrast, EG-626 ($100 \mu\text{g/ml}$) did not affect the biosynthesis of these metabolites from AA.

5) Effects of EG-626 and Imidazole on the Conversion of PGH_2 to TXA_2 by PM.

PGH_2 was incubated with PM or alternatively incubated alone. The biological activity of the reaction mixture was assayed using strips of rabbit aorta. As shown in **Fig. 4**, contraction by PGH_2 was significantly increased by incubation with PM, suggesting that PGH_2 was converted to a more vasoactive substance, namely TXA_2 ⁸⁾. The contractile response caused by TXA_2 was markedly reduced by preincubation of PM with imidazole ($200 \mu\text{g/ml}$) but only slightly with EG-626 ($200 \mu\text{g/ml}$). However, when the same concentration of EG-626 was perfused with preformed TXA_2 , the contractile response was also slightly reduced (data not shown). Thus, the inhibitory effect of EG-626 on the contractile response is probably not due to the inhibition of the conversion to TXA_2 , such as is the case with imidazole¹⁴⁾, but rather to its antagonistic effect on the action of TXA_2 , as previously reported¹⁵⁾.

Discussion

EG-626 markedly inhibited platelet aggregation induced by ADP as well as SAA- or collagen-induced aggregation. On the other hand, indomethacin which is an inhibitor of cyclooxygenase remarkably inhibited SAA- or collagen-induced aggregation, but did not inhibit the aggregation induced by ADP. These results indicate differences in the mode of actions between EG-626 and indomethacin. EG-626 did not affect LASS-formation from AA and TXA_2 -formation from PGH_2 , and such were effectively inhibited by indomethacin and imidazole, respectively. Thus, unlike indomethacin or imidazole, EG-626 probably inhibits SAA-induced aggregation without affecting TXA_2 - or LASS-formation.

We found that EG-626 remarkably inhibited TXA_2 - or LASS-induced aggregation without affecting their biosynthesis. Therefore, EG-626 may inhibit AA-induced aggregation through an antagonistic effect on the actions of TXA_2 and LASS. As it has been reported that collagen-induced aggregation is mediated through LASS-formation¹⁶⁾, the inhibitory effect of EG-626 on collagen-induced aggregation may be partially due to an antagonistic action on the activity of LASS.

Recently, other workers^{17) 18)} have presented a hypothesis regarding the role of TXA_2 , PG endoperoxides and ADP in platelets: these substances activate platelet functions through mobilization of Ca^{2+} from storage sites within the platelets, whereas cyclic AMP enhances Ca^{2+} uptake into the sites, inhibiting the activation of platelet contractile proteins by calcium. Since EG-626 is a

potent inhibitor of cyclic AMP phosphodiesterase¹⁹⁾, it may be reasonable to presume that this agent inhibits TXA₂-or LASS-induced aggregation through an accumulation of cyclic AMP. Furthermore, EG-626 inhibited ADP-induced aggregation as well. The anti-aggregatory activity may be also related to an accumulation of cyclic AMP.

Gerrad et al. reported that agents which induced an accumulation of intracellular cyclic AMP had no effect on the conversion of AA to its metabolites¹⁷⁾. In our present radiochemical studies, conversion of AA to its metabolites such as TXB₂, HHT or HETE was unaffected by EG-626. This would suggest the possible accumulation of intracellular cyclic AMP as induced by EG-626. Malmsten et al. have reported the inhibition of cyclooxygenase by dibutyl cyclic AMP or theophylline²⁰⁾. We could not detect such inhibition of cyclooxygenase in platelets. We are unable to explain the reason for the differences of these results.

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