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A SPECIFIC PROSTAGLANDIN I_2 SYNTHETASE INHIBITOR, 3-HYDROPEROXY-3-METHYL-2-PHENYL-3H-INDOLE

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SUMMARY: Inhibitory effects of 3-hydroperoxy-3-methyl-2-phenyl-3H-indole(HPI) on prostaglandin endoperoxide synthase(EC 1.14.99.1) and prostaglandin I_2(PGI_2) synthetase were compared with those of 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid, namely, 15-hydroperoxyarachidonic acid(15-HPAA) and tranylcypromine (TCP). Sheep seminal vesicle microsomes were used as a source of prostaglandin endoperoxide synthase and bovine aortic microsomes as that of PGI_2 synthetase. 15-HPAA and HPI inhibited PGI_2 synthetase with IC_{50}s of 5×10^{-7} and 3.5×10^{-6} M, respectively, whereas neither compound had effect on prostaglandin endoperoxide synthase at the concentration inhibiting PGI_2 synthetase by 90%. TCP was a weak($IC_{50} = 5 \times 10^{-4}$M) PGI_2 synthetase inhibitor with low specificity.

INTRODUCTION

A new aspect in the prostaglandin(PG) research has been developing since the discovery of thromboxane A_2(TXA_2) and prostacyclin(PGI_2) (1). Specific inhibitors for the generation of either TXA_2 or PGI_2 may be useful as a tool to assess the physiological roles of both substances. There are several reports of the TXA_2 synthetase inhibitor (2-4), but few reports of the PGI_2 synthetase inhibitor (5). 15-Hydroperoxy-5,8,11,13-eicosatetraenoic acid, namely, 15-hydroperoxyarachidonic acid(15-HPAA) and tranylcypromine(TCP) have usually been used as the specific inhibitor of PGI_2 synthetase. However, 15-HPAA failed to inhibit the activity of this enzyme in isolated, perfused heart and kidney preparation (6) and TCP had only a weak inhibitory activity (IC_{50} = 160 μg/ml) on this enzyme (5). Furthermore, the effects of both substances on PG endoperoxide synthase(EC 1.14.99.1) have not fully been examined.

Abbreviations used are : AA, arachidonic acid; BAM, bovine aortic microsomes; 15-HPAA, 15-hydroperoxyarachidonic acid; HPI, 3-hydroperoxy-3-methyl-2-phenyl-3H-indole; PG, prostaglandin; SSV, sheep seminal vesicle microsomes; TCP, tranylcypromine; TXA_2, thromboxane A_2.

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The inhibitory action of test agents on PG endoperoxide synthase was estimated from the contractile response of the aortic strip by the following procedures. 1) 4 μg of arachidonic acid in 20 μl of the Tris buffer was mixed with an SSV solution(100 μg/10 μl) at 25°C for 2 min and 10 μl of the mixture was then added to the superfusion fluid. 2) An SSV solution(200 μg/10 μl) was mixed with 10 μl of the Tris buffer containing varying concentrations of test agents. After leaving the mixture standing at 25°C for 5 min, 10 μl of the mixture was added to 4 μg of arachidonic acid(20 μl). After standing at 25°C for further 2 min, 10 μl of the mixture was added to the superfusion fluid. The percent inhibition of the PG endoperoxide synthase activity by test agents was calculated from the following formula;

$$\% \text{ Inhibition} = 100 - \frac{\text{Contraction(cm) by drug-treated SSV + AA}}{\text{Contraction(cm) by SSV + AA}} \times 100,$$

where AA means arachidonic acid. The SSV solution alone had no significant effect on the aortic contraction. Standing of SSV solution(100 μg/10 μl) at 25°C for 5 min did not affect the activity of PG endoperoxide synthase.

The inhibitory action of test agents on PGI_2 synthetase was estimated from the rate of disappearance of PGH_2 seen as decreasing contractions of the aortic strip (5) as follows. 1) 50 μl of 50 mM Tris buffer(pH 7.5) was added to a glass tube containing 100 ng of dried PGH_2. After mixing fully, the solution was left standing at 25°C for 2 min and an aliquot(40 μl) was added to the superfusion fluid. 2) A BAM solution(60 μg/30 μl) was mixed with 30 μl of the Tris buffer containing test agents. After leaving the mixture standing at 25°C for 3 min, 50 μl of the mixture was added to the tube containing 100 ng of dried PGH_2. After standing at 25°C for further 2 min 40 μl of the mixture was added to the superfusion fluid. The percent inhibition of the PGI_2 synthetase activity by test agents was calculated from the following formula;

$$\% \text{ Inhibition} = \frac{\text{Contraction(cm) by drug-treated BAM + } PGH_2}{\text{Contraction(cm) by } PGH_2} \times 100$$

The BAM solution alone and PGH_2(80 ng) incubated with BAM had no effect on the aortic contraction. Standing of a BAM solution(50 μg/50 μl) at 25°C for 3 min did not reduce the activity of PGI_2 synthetase.

Evaluation of PGI_2 synthetase inhibition by platelet aggregation study

Blood was collected by cardiac puncture from the male conscious rabbit weighing about 3 kg. Clotting of the blood was prevented by adding 1/9 volume of 3.15% sodium citrate. Platelet rich plasma(PRP) was obtained by centrifuging the blood at 300 x g for 10 min at room temperature. Aggregation of platelet in 250 μl PRP was monitored in a Born-type aggregometer(RIKA DENKI, Japan), in which the final concentration of 280 to 560 μM of arachidonic acid (25 μl) was added to PRP as an aggregatory inducer. To assess the antiaggregatory action of PGI_2, probably generated by mixing PGH_2 with BAM, 20 μl of the reaction mixture of 100 ng PGH_2 with 50 μg of BAM(50 μl) at 25°C for 2 min was added to a tube containing the PRP 2 min before adding arachidonic acid. Inhibitory action of test agents on the generation of PGI_2 was estimated by using BAM solution(50 μg/25 μl) treated with varying concentrations of test agents(25 μl) at 25°C for 3 min before mixing with 100 ng PGH_2.

RESULTS AND DISCUSSION

Figure 2 shows the inhibitory action of indomethacin on the PG endoperoxide synthase activity and that of 15-HPAA on the PGI_2 synthetase activity. The contraction of rabbit aorta induced by PG endoperoxides(PGG_2 and PGH_2),

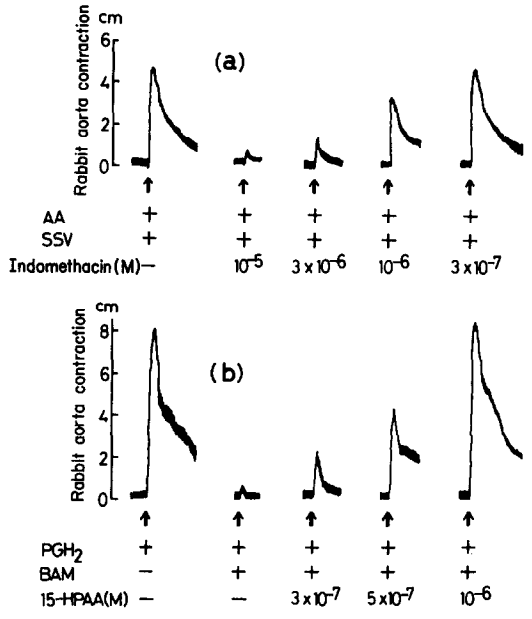

Fig. 2. Evaluation of enzyme inhibition by the superfusion method. (a) PG endoperoxide synthase: 4 μg of arachidonic acid(AA) in 20 μl of 50 mM Tris buffer(pH 7.5) was mixed with an SSV solution(100 μg/10 μl) at 25°C for 2 min and 10 μl of the mixture was then added to the superfusion fluid. When indomethacin was used, an SSV solution(200 μg/10 μl) was mixed with 10 μl of the Tris buffer containing varying concentrations of indomethacin. After leaving the mixture standing at 25°C for 5 min, 10 μl of the mixture was added to 4 μg of AA(20 μl). After standing at 25°C for further 2 min, 10 μl of the mixture was added to the superfusion fluid. (b) PGI_2 synthetase: 50 μl of 50 mM Tris buffer(pH 7.5) was added to 100 ng of dried PGH_2. The solution was left standing at 25°C for 2 min and an aliquot(40 μl) was added to the superfusion fluid. When PGH_2 was mixed with BAM, the BAM solution(50 μg/50 μl) instead of the Tris buffer was added to the dried PGH_2. When 15-HPAA was used, a BAM solution(60 μg/30 μl) was mixed with 30 μl of the Tris buffer containing varying concentrations of 15-HPAA. After leaving the mixture standing at 25°C for 3 min, 50 μl of the mixture was mixed with 100 ng of dried PGH_2. After standing at 25°C for further 2 min, 40 μl of the mixture was added to the superfusion fluid.

which were generated by incubating arachidonic acid with SSV, was dose-dependently inhibited by pretreating SSV with varying concentrations(10^{-5} to 3×10^{-7} M) of indomethacin(Fig. 2-a). On the other hand, the rabbit aorta contracting action induced by 80 ng of PGH_2 almost disappeared after mixing PGH_2 with BAM (40 μg), whereas this effect of BAM was prevented by pretreating BAM with 15-HPAA(10^{-6} to 3×10^{-7}M) in a dose-related manner(Fig. 2-b).

Figure 3 shows the comparison of the inhibitory effects of test agents on the activity of both enzymes bioassayed as described in Figs. 2-a and b. The inhibitory activity on PGI_2 synthetase was most potent with 15-HPAA, more

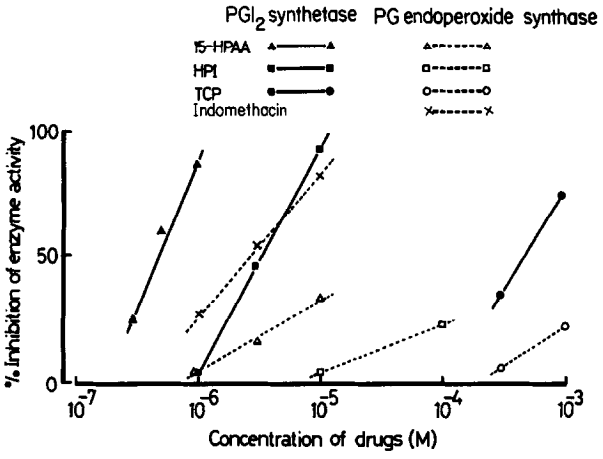

Fig. 3. Comparison of the inhibitory action of 15-HPAA, HPI, TCP and indomethacin on PG endoperoxide synthase or PGI_2 synthetase. The inhibitory activity was estimated by the methods described in Figs. 2-a and b. Each point shows a mean ± S.E.M. in 6 to 10 experiments.

potent with HPI and less potent with TCP, IC_{50}s of which were 5×10^{-7}, 3.5×10^{-6} and 5×10^{-4}M, respectively. On the other hand, 15-HPAA, HPI and TCP showed weak PG endoperoxide synthase inhibiting activity. Their specificity as a PGI_2 synthetase inhibitor was higher in 15-HPAA and HPI than in TCP. Indomethacin markedly inhibited the PG endoperoxide synthase activity with IC_{50} of 3×10^{-6}M.

Figure 4 shows the antiaggregatory action of enzymatically generated PGI_2 and inhibitory effects of test agents on the generation of PGI_2. The addition of the reaction mixture of PGH_2 with BAM to PRP inhibited completely the arachidonic acid-induced platelet aggregation. This inhibitory activity diminished with $t_{1/2}$ of about 5 min by leaving the mixture standing at 37°C for 3 to 10 min(Fig. 4-a), indicating that PGI_2 had been produced in the mixture (5). Figures 4-b, c and d show that pretreatment of BAM with each of 15-HPAA, HPI and TCP, in that decreasing order of potency, inhibited the generation of PGI_2 by the reaction of PGH_2 with BAM, resulting in the restoration of aggregatory activity by arachidonic acid. The results in the platelet aggregation study agreed with those in the superfusion study(Fig. 3). Each agent alone at the concentrations used in the present experiments had no effect on platelet aggregation.

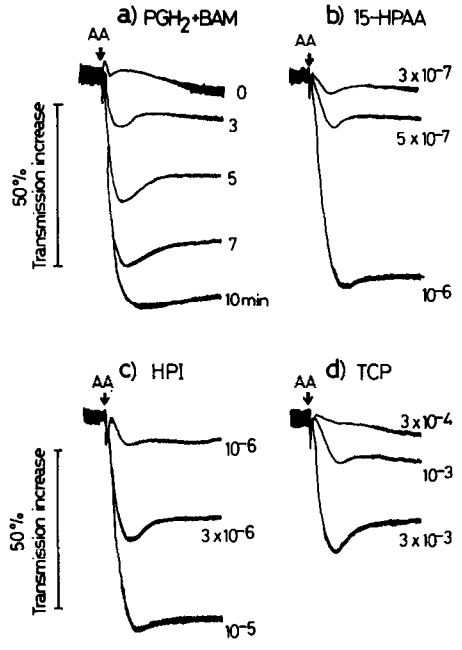

Fig. 4. Evaluation of the inhibitory action of 15-HPAA, HPI and TCP on PGI_2 synthetase by platelet aggregation study. (a): The mixture of 100 ng of PGH_2 with 50 μg of BAM in 50 μl of 50 mM Tris buffer(pH 7.5) was left at 25°C for 2 min before adding arachidonic acid(AA). To assess $t_{1/2}$ of the antiaggregatory activity of the substance which was generated by mixing PGH_2 with BAM, the mixture of PGH_2 with BAM was left at 37°C for 3, 5, 7 and 10 min before adding the mixture to the aggregometer tube. (b, c and d): 50 μg of BAM in 25 μl of the Tris buffer was treated with 25 μl of varying concentrations of 15-HPAA, HPI or TCP at 25°C for 3 min before mixing with 100 ng of PGH_2. AA was used at concentrations of 280 to 560 μM.

Neither 3-methyl-2-phenylindole, the starting material of HPI, nor 3-hydroxy-3-methyl-2-phenyl-3H-indole, a main product derived from HPI, at a concentration of 10^{-4}M had significant effect on PGI_2 synthetase and PG endoperoxide synthase, suggesting an important role of 3-hydroperoxy group in HPI molecule in the inhibition of PGI_2 synthetase. It is of interest that the hydroperoxide except those of unsaturated fatty acids is a potent inhibitor of PGI_2 synthetase.

HPI is stable in a crystalline form. In addition, its inhibitory activity on PGI_2 synthetase did not reduce at a concentration of 2×10^{-5}M in 50 mM Tris buffer(pH 7.5) at least for 3 hr at room temperature although the activity reduced to about 50% 22 hr later.

HPI may be useful in inhibiting PGI_2 synthetase in the perfused organ system, since in our preliminary experiments HPI was confirmed to reduce the angiotensin-II-stimulated generation of PGI_2 in isolated, perfused kidneys of spontaneously hypertensive rats, which have been reported to release PGI_2 (9).

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