DUAL EFFECTS OF A BASIC ANTI-INFLAMMATORY AGENT,
2-AMINOMETHYL-4-*t*-BUTYL-6-IODOPHENOL
HYDROCHLORIDE (MK-447), ON BIOSYNTHESIS OF
PROSTAGLANDIN ENDOPEROXIDES

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Abstract—Effects of MK-447 on prostaglandin (PG) endoperoxide formation from arachidonic acid by bovine seminal vesicle microsomes in the presence of cofactors (hemoglobin and tryptophan) were studied by cascade superfusion on rabbit aorta and mesenteric artery and rat stomach and colon. In the presence of hemoglobin (0.2 μ M), MK-447 (up to 30 μ M) accelerated PG endoperoxide formation, as tryptophan did, whereas higher concentrations of MK-447 lost the acceleration effect and finally inhibited the PG endoperoxide formation. Increased concentration of hemoglobin (2 μ M) shifted the dose of MK-447 for peak generation from 30 to 100 μ M. Thus, MK-447 shows dual action, acceleration and inhibition, on the PG endoperoxide formation.

A basic anti-inflammatory agent, 2-aminomethyl-4-*t*-butyl-6-iodophenol hydrochloride (MK-447), was reported not to inhibit, but to stimulate biosynthesis of prostaglandin (PG) endoperoxides from arachidonic acid using ram seminal vesicle microsomes in the absence of cofactors (1). On the other hand, purified PG endoperoxide synthetase obtained from bovine seminal vesicle microsomes required hemoglobin for generation of PGG₂ from arachidonic acid and tryptophan with hemoglobin for conversion of PGG₂ to PGH₂ (2).

In the present studies, effects of MK-447 on PG endoperoxide generation were determined using bovine seminal vesicle microsomes, since these microsomes do not generate PG endoperoxides from arachidonic acid, unless cofactors are added. In addition to a preliminary communication (3), this paper presents precise results indicating that MK-447 showed dual actions, acceleration and inhibition, on biosynthesis of PG endoperoxides.

MATERIALS AND METHODS

Enzyme source: Bovine and sheep seminal vesicle microsomes (BSVM and SSVM, 105,000 $\times$ g pellet), prepared by the method of Takeguchi et al. (4), and stored at -80°C after lyophilization, were used as sources of PG endoperoxide synthetase.

Generation of PG endoperoxides: BSVM (200 μ g protein) suspended in 20 μ l of 50 mM Tris-HCl buffer (pH 7.4) were pre-incubated with MK-447 (or buffer) (20 μ l) for 2 min at 22°C . Following the addition of various concentrations of hemoglobin (Sigma, St. Louis, Mo.) and tryptophan (Kyowa Hakko, Tokyo) (40 μ l), as cofactors, the mixture was left for another 1 min at 22°C . Then, arachidonic acid (5.26 nmoles/120 μ l, Applied Science Lab.,

Pa.) was added and incubation was carried out for 2 min to generate PG endoperoxides. Hydroquinone (Wako Pure Chemical, Tokyo) was also used as a cofactor. Total volume of the incubation mixture was 200 μ l and the concentrations were expressed as final amounts. The products generated after incubation of BSVM with arachidonic acid in the presence of cofactors were considered to be PG endoperoxides from the evidence described in results and as previously reported (5).

SSVM (80 μ g protein/20 μ l) were also used as an enzyme source to determine the effect of MK-447 on the PG endoperoxides generation.

Assay of PG endoperoxides: Immediately after the incubation, PG endoperoxides were assayed by the cascade superfusion (6). Spirally cut strips of rabbit aorta (RA) and mesenteric artery (RMA), rat stomach strip (RSS) and rat colon (RC) were superfused in cascade at 10 ml/min with Krebs solution at 37°C, gassed with 5% CO₂ and 95% O₂. The following antagonists (7) were added to the solution (concentrations are expressed as bases): atropine sulfate (0.1 μ g/ml, Wako Pure Chemical, Tokyo), phenoxybenzamine hydrochloride (0.1 μ g/ml, Tokyo Kasei, Tokyo), propranolol hydrochloride (0.1 μ g/ml, Ono Pharmaceutical Co. Ltd., Osaka), mepyramine maleate (0.1 μ g/ml, May & Baker, Essex), methysergide hydrogen maleate (0.2 μ g/ml, Sandoz, Basel). Indomethacin (1 μ g/ml, Merck, N.J.) was also added to prevent endogenous PG production by the assay tissues (8).

The responses of the smooth muscles were recorded with isotonic transducers (ME Commercial Co. Ltd., Tokyo) connected to a multi-pen recorder (Rikadenki Kogyo Co. Ltd., Tokyo). The initial tension on the muscles was 3.0 g for RA, 1.5 g for RMA, 2.5 g for RSS and 1.0 g for RC. The amount of PG endoperoxides in the incubation mixture was estimated from a dose-response curve of rabbit aorta against authentic PGH₂ (Ono Pharmaceutical Co. Ltd., Osaka).

Protein was estimated by the method of Lowry et al. (9) with bovine serum albumin (Miles Lab. Inc., Ind.) as a standard.

RESULTS

As shown in Fig. 1, incubation of bovine seminal vesicle microsomes (BSVM) with arachidonic acid, in the presence of hemoglobin (0.2 μ M) and tryptophan (5 mM), caused generation of products which induced a stronger contraction of rabbit aorta and relaxation of rabbit mesenteric artery. These products were considered to be mainly PG endoperoxides because of the above mentioned typical actions on isolated smooth muscles, of an inhibition of the generation by indomethacin, of a half life (5–6 min) in incubation at 37°C (data not shown), and of conversion to more active and labile substance (thromboxane A₂) by horse platelet microsomes (5).

Furthermore, Figure 1 shows that the amounts of PG endoperoxides generated from arachidonic acid depend on the presence of the cofactors above mentioned. In the absence of these cofactors, an incubation of BSVM with arachidonic acid did not generate appreciable amounts of products to contract the rabbit aorta, although small contractions of rat stomach strip and rat colon and relaxation of rabbit mesenteric artery were seen, and such responses

FIG. 1. Requirement of hemoglobin (Hb) and tryptophan (Trp) for the generation of PG endoperoxides by BSMV, assayed by a cascade superfusion technique. RA; rabbit aorta, RMA; rabbit mesenteric artery, RSS; rat stomach strip, RC; rat colon.

may be due to small amounts of endogenous prostaglandins already formed from arachidonic acid in BSMV. Addition of hemoglobin ($0.2 \mu\text{M}$) induced contraction of the rabbit aorta and, in the presence of hemoglobin and tryptophan, the contraction of rabbit aorta and the relaxation of rabbit mesenteric artery were further increased. This further increase in the contraction of rabbit aorta and the relaxation of mesenteric artery after addition of tryptophan with hemoglobin may suggest the conversion of PGG_2 to PGH_2 (2).

These results strongly indicate that the bovine seminal vesicle microsomes used in the experiments produce detectable amounts of PG endoperoxides, whenever hemoglobin and tryptophan were added as cofactors.

The accelerating effects of hemoglobin and tryptophan on the generation of PG endoperoxides were dose-dependent, as shown in Fig. 2. Without these cofactors, the rabbit aorta did not contract, but, in the absence of tryptophan, increasing doses of hemoglobin accelerated the formation of PG endoperoxide, and a maximum was reached with doses of hemoglobin over $2 \mu\text{M}$. Addition of tryptophan to hemoglobin further increased the contraction of rabbit aorta and 5 mM of tryptophan with $2 \mu\text{M}$ of hemoglobin induced the maximal generation of PG endoperoxide. The maximal generation of the PG endoperoxide was equivalent to $150\text{--}160 \text{ ng}$ of PGH_2 , when calculated from a dose-response curve of rabbit aorta against authentic PGH_2 (data not shown).

Figure 3 shows that MK-447 can play a similar role to that of tryptophan in the stimulation of the PG endoperoxides generation. In the absence of tryptophan, MK-447 ($30 \mu\text{M}$) stimulated the generation with increase in the concentrations of hemoglobin. Even at the higher concentrations of hemoglobin, the generation was not saturated, but tended to increase further, as seen in Fig. 3. This does not mean that MK-447 has a cofactor action

FIG. 2. Acceleration of the PG endoperoxide generation by cofactors. Ordinate shows the contraction heights of rabbit aorta (RA) (open symbols) and rat stomach strip (RSS) (closed symbols) responded to PG endoperoxide generated. Three doses (0, 1 and 5 mM) of tryptophan (Trp) (circles, triangles and squares) accelerated the generation dose-dependently at each dose of hemoglobin (Hb) (abscissa). Addition of the doses of hemoglobin (1 μ M) and tryptophan (5 mM) caused the nearly maximal generation of PG endoperoxides.

FIG. 3. Stimulatory effect of MK-447 (30 μ M) on the PG endoperoxide generation by BSVM in the presence of hemoglobin. Ordinate shows the contraction heights of rabbit aorta. Abscissa indicates the concentration of hemoglobin. Open or closed circles indicate the responses to PG endoperoxides generated in the absence or presence of MK-447, respectively.

similar to that of hemoglobin. By contrast, as shown in the upper panel of Fig. 4, in the presence of fixed concentration of hemoglobin (1 μ M), acceleration of the PG endoperoxide generation by MK-447 (30 μ M) was evident when the concentration of tryptophan was low, but with increasing doses of tryptophan, the generation of PG endoperoxide was saturated in the presence of MK-447. Furthermore, as shown in the lower panel of Fig. 4, when the hemoglobin concentration was reduced from 1 μ M to 0.2 μ M, MK-447 (30 μ M) accelerated the generation of PG endoperoxides at the lower concentration of tryptophan (less than 1 mM), whereas at concentrations of tryptophan above 1 mM, this drug suppressed the generation of the PG endoperoxide. These results indicate that MK-447 acts in a similar manner to tryptophan, but not to hemoglobin, in the generation of PG endoperoxides.

As shown in Fig. 5, in the absence of tryptophan, the stimulatory effect of MK-447 on the PG endoperoxide (PGH₂) generation was dose-dependent, up to 30 μ M of the drug at a lower concentration of hemoglobin (0.2 μ M), whereas the higher doses of MK-447 reduced the acceleration and finally inhibited the PG endoperoxides generation at 300 μ M, as seen in the reduced height of contraction at 300 μ M of MK-447. When the hemoglobin concen-

FIG. 4. Effects of MK-447 on PG endoperoxide generation in the presence of tryptophan and hemoglobin. Ordinate shows the height of contraction of rabbit aorta in the absence (open circles) or presence (closed circles) of 30 μM of MK-447. Abscissa indicates the concentration of tryptophan. At over 1 mM of tryptophan, the stimulatory effect of MK-447 was saturated at higher concentration of hemoglobin (1 μM) (top), whereas this effect was changed to inhibition at a lower concentration of hemoglobin (0.2 μM) (bottom).

FIG. 5. Dual effects of MK-447 on the PG endoperoxides generation. Ordinate indicates the height of contraction of rabbit aorta (RA) (open symbols) and rat stomach strip (RSS) (closed symbols). Circles and triangles show the responses to PG endoperoxide generated at 0.2 and 2 μM of hemoglobin, respectively. The concentration of MK-447 producing the maximal stimulation shifted from 30 μM to 100 μM by increasing the concentration of hemoglobin (Hb) from 0.2 to 2 μM .

tration was increased to 2 μM , the peak generation of PG endoperoxide by MK-447 was shifted to the higher dose of MK-447, that is from 30 to 100 μM . The maximal heights due to the PG endoperoxide generated at 0.2 and 2 μM of hemoglobin were equivalent to 60 and 160 ng of PGH_2 , when calculated from a dose-response curve of rabbit aorta against authentic PGH_2 (data not shown).

Tryptophan did not exert an inhibitory action on the PG endoperoxide synthesis up to 10 mM. By contrast, as shown in Fig. 6, hydroquinone stimulated the PG endoperoxide generation up to 100–300 μM , but further increase to over 300 μM induced less stimulation and finally the inhibition of the PG endoperoxide generation (Fig. 6). This is exactly the same pattern seen with MK-447. Thus, MK-447 resembles hydroquinone rather than tryptophan in the acceleration and inhibition of the PG endoperoxide generation. There

FIG. 6. Dual effects of hydroquinone (HQ) in the PG endoperoxide generation. Ordinate indicates the contraction heights of rabbit aorta (RA) (open symbols) and rat stomach strip (RSS) (closed symbols). Abscissa shows the concentrations of hydroquinone (HQ). The concentration ($100 \mu\text{M}$) which produced the maximal generation of PG endoperoxides at lower concentration ($0.2 \mu\text{M}$) of hemoglobin (Hb) was not significantly different from that at a higher concentration ($2 \mu\text{M}$) of hemoglobin.

FIG. 7. Effects of cofactors on the acceleration of the PG endoperoxide formation by MK-447 in sheep seminal vesicle microsomes (SSVM). SSVM ($80 \mu\text{g}$ protein) was incubated with arachidonic acid with or without MK-447 in the presence of hemoglobin (Hb) ($0.4 \mu\text{M}$) or tryptophan (Trp) (5 mM) or in their absence. Details of the procedure were the same as in the case of BSVM. Ordinate shows the height of contraction of rabbit aorta. Abscissa indicates the concentrations of MK-447.

was, however, a difference between MK-447 and hydroquinone, since the concentration of the latter, which was required to yield the peak stimulation, was not shifted by an increment in hemoglobin concentration.

The effects of MK-447 on the PG endoperoxide generation on bovine seminal vesicle microsomes were essentially the same as those seen with sheep seminal vesicle microsomes. As shown in Fig. 7, addition of hemoglobin ($0.4 \mu\text{M}$) also accelerated the PG endoperoxide generation by MK-447 up to $100 \mu\text{M}$, whereas tryptophan (5 mM) alone showed only the gradual suppression with increasing doses of MK-447. The addition of both hemoglobin and tryptophan induced a maximal generation of the PG endoperoxide and MK-447 maintained a maximal formation up to $30 \mu\text{M}$. Further increase (above $30 \mu\text{M}$) of the dose of MK-447 suppressed the PG endoperoxide formation.

DISCUSSION

It has been reported that purified PG endoperoxide synthetase from bovine seminal vesicle microsomes contained two enzyme activities; fatty acid cyclooxygenase and PG hydroperoxidase activity (2). The former catalyzed bis-oxygenation of unsaturated fatty acids to produce PGG. Hemoglobin and other heme compounds such as myoglobin and hematin are required for this reaction. The latter catalyzed the reduction of the hydroperoxy group at position 15 of PGG to produce PGH. This reaction also required hemoglobin and is stimulated by tryptophan and other aromatic compounds such as indole, serotonin, melatonin, tyrosine, epinephrine, hydroquinone and benzoquinone.

In the present experiments, addition of more than 2 μ M of hemoglobin to the bovine seminal vesicle microsomes did not induce a further increase in heights of the contraction of rabbit aorta and rat stomach strip (Fig. 2), which means the maximal generation of PGG₂ from arachidonic acid. In the same way, further addition of tryptophan increased the contraction heights of the smooth muscles which again reached the maximum with 5 mM of tryptophan and 2 μ M of hemoglobin. This increase in heights could be considered as the conversion of most of PGG₂ to PGH₂, since the relative potencies of PGH₂/PGG₂ were reported to be 2-4/1 for rabbit aorta (10) and 2.6 for rabbit aorta and 1.74 for rat stomach strip (11).

It was reported by Kuehl et al. (1) that MK-447 accelerated the conversion of PGG₂ to PGH₂ as did phenol, and both may act as a free radical scavenger. The present study indicates that tryptophan could be replaced by a lower concentration of MK-447, as seen in Figs. 4 and 5. In fact, MK-447 exerted the stimulatory action by itself (Fig. 5) or additively to tryptophan (Fig. 4) in the presence of hemoglobin. Accordingly, the findings by Kuehl et al. (1) with sheep seminal vesicle microsomes were confirmed with bovine seminal vesicle microsomes.

The stimulatory effect of MK-447 on the PG endoperoxide generation, however, was changed to inhibition, when the hemoglobin concentration was reduced from 1 to 0.2 μ M, in the presence of more than 1 mM of tryptophan (Fig. 4, lower panel). Similarly, the increasing concentrations of MK-447 alone more than 100 μ M lost the stimulatory effect and finally suppressed the generation at the reduced concentration of hemoglobin (0.2 μ M) (Fig. 5). Thus, it can be concluded that MK-447 exerted a dual action on the PG endoperoxide formation.

Nugteren et al. reported that hydroquinone stimulated PG formation (12). Since then, it has been found that hydroquinone could be replaced to some extent by a wide variety of compounds in the overall production of PGE (4, 13, 14). It was also confirmed using purified enzyme from bovine seminal vesicles that similar compounds including tryptophan and tyrosine stimulate the conversion of PGG to PGH in the presence of hemoglobin (2).

Hydroquinone, however, was also reported to inhibit the PGE production above 5×10^{-4} M (12) and this was confirmed qualitatively by Wallach and Daniels (15) and Schwartzman et al. (16), although enzyme preparations and the concentrations which inhibited the PGE production differed.

The present experiments confirmed that hydroquinone accelerated PG endoperoxide biosynthesis up to 0.1 mM and inhibited it above 1 mM. Thus, the dual action of MK-447 was not exceptional.

Different from hydroquinone, the concentration of MK-447 required to produce the maximal stimulatory effect was shifted to the left, when the hemoglobin concentration was reduced from 2 to 0.2 μ M. This suggests the possibility that MK-447 might exert both stimulatory and inhibitory actions, depending on the concentrations of the hemoglobin-like cofactors in cells.

The failure in observation of the inhibition of the PG endoperoxide synthesis by MK-447 in the previous report of Kuehl et al. (1) may be due to the difference in the nature of bovine and sheep microsomes. As shown in Fig. 1, BSVM did not generate appreciable amounts of PG endoperoxides after incubation with arachidonic acid without any cofactor, whereas addition of these cofactors to BSVM produced a generation without arachidonic acid. This indicates that BSVM contained endogenous non-esterified arachidonic acid, but most of cofactors had been washed away during preparation of the microsomes. By contrast, sheep seminal vesicle microsomes do not require cofactors to generate PG endoperoxides, indicating that cofactors are present in microsomes from sheep seminal vesicles, but these microsomes contain no arachidonic acid. However, as shown in Fig. 7, when cofactors were added to the reaction mixture of the sheep seminal vesicle microsomes, the higher doses of MK-447 inhibited the PG endoperoxide formation, essentially in the same pattern as in BSVM.

Thus, it can be concluded that MK-447 acts as a tryptophan- or hydroquinone-like cofactor in the PG endoperoxide formation, and exerts the dual action of stimulation and inhibition on the PG endoperoxide formation, depending on the concentration of hemoglobin and tryptophan.

Precise mechanisms in the requirement of cofactors for this enzyme and the interaction of the cofactors with enzyme on molecular levels remain to be studied.

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