Biochemical Pharmacology, Vol. 34, No. 7, pp. 933–936, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

LIPID PEROXIDATION MODIFIES THE EFFECT OF PHENOLIC ANTI-INFLAMMATORY DRUGS ON PROSTAGLANDIN BIOSYNTHESIS

MASAHIKO HIRAFUJI* and YASUMI OGURA
Department of Pharmacology, Tohoku University School of Dentistry, Sendai 980, Japan

(*Received* 3 *July* 1984; *accepted* 6 *September* 1984)

Abstract—The effects of phenolic anti-inflammatory drug, MK-447, on prostaglandin (PG) I_2 and thromboxane (TX) A_2 biosynthesis by rat dental pulp tissue were evaluated in the presence of 10 mM mannitol (MA) or 1 mM ascorbic acid with 0.3 mM Fe^{2+} (A + F). Although MK-447 alone at 1 and 10 μM had no significant effects, MK-447 at 100 μM stimulated both PGI_2 and TXA_2 biosynthesis, and suppressed the lipid peroxidation in the pulp tissue as estimated by thiobarbituric acid method. MA also reduced the lipid peroxidation, but had no effect on PG and TX production. However, in the presence of MA, the stimulatory effect of MK-447 was potentiated, and the significant effects were observed at concentrations higher than 1 μM. In contrast, A + F remarkably stimulated the lipid peroxidation, and inhibited both PG and TX biosynthesis. In the presence of A + F, MK-447 showed no stimulatory effect, and contrary, at 100 μM inhibited PG and TX production. These results suggest that the cellular levels of lipid peroxidation exert a significant influence on the effects of phenolic anti-inflammatory drugs like MK-447 on PG biosynthesis. The possible mechanism of action for such drugs has been discussed in view of the significance of lipid peroxidation in inflammatory condition.

Since the discovery that aspirin-like anti-inflammatory drugs inhibit the cyclooxygenase-catalyzed biosynthesis of prostaglandins (PGs), the inhibition of PGs has been accepted as the mechanism of action for these drugs [1]. However, the apparent contradictory actions of phenolic compounds such as MK-447 (2-aminomethyl-4-*t*-butyl-6-iodophenol) have been reported. MK-447 is an effective anti-inflammatory agent *in vivo* [2–5], whereas it stimulates PG biosynthesis *in vitro* [2–4, 6].

Kuehl *et al.* [2] showed that MK-447 facilitated the conversion of PGG_2 to PGH_2, acting as a scavenger of oxygen-derived free radicals, presumably [.OH], released from PGG_2. From these results, the authors proposed that PGG_2 itself and the free radicals were mediators of inflammation, and that the action of MK-447 was to reduce these concentrations. However, Harada *et al.* [5] have recently demonstrated that MK-447 decreases the pleural level of PGs in rat carrageenin-induced pleurisy. MK-447 has been also shown to inhibit the production of PG endoperoxides in certain *in vitro* conditions [6, 7]. Therefore, these facts suggest that MK-447 may exert its anti-inflammatory property by inhibiting PG biosynthesis in certain *in vivo* conditions.

Lands and Hanel [7] demonstrated that MK-447 acted to inhibit cyclooxygenase activity dose-dependently in purified PGH synthase (PG endoperoxide synthetase) when the concentration of hydroperoxides was decreased by glutathion peroxidase. Hence, they suggested that the ambient tissue levels of lipid peroxides play a very significant role in modifying the effect of phenolic anti-inflammatory drugs like MK-447.

The formation of lipid peroxides is a significant chemical event associated with the appearance of phagocytic cells such as macrophages and leukocytes at the inflamed sites [8]. In the present study, therefore, we have investigated whether the alteration of tissue level of lipid peroxidation modifies the effect of phenolic anti-inflammatory drug MK-447 on PG biosynthesis by isolated rat dental pump. The pulp tissue is an abundantly vascularized connective tissue, and recently has been found to predominantly synthesize PGI_2 and thromboxane (TX) A_2 [9].

MATERIALS AND METHODS

Materials. [^{3}H]6-keto-$PGF_{1\alpha}$ was obtained from the Radiochemical Centre, Amersham, and [^{3}H]-TXB_2 from New England Nuclear (Boston, MA). Anti-6-keto-$PGF_{1\alpha}$ rabbit serum was purchased from Seragen Inc. (Boston, MA). Anti-TXB_2 rabbit serum and authentic 6-keto-$PGF_{1\alpha}$ and TXB_2 were gifts from Ono Pharmaceutical Co. (Osaka, Japan). D-mannitol, ascorbic acid, $FeSo_4$ and thiobarbituric acid were obtained from Wako Pure Chemical Indust. (Osaka, Japan). MK-447 was a gift from Merck and Co. (Rahway, NJ).

Methods. Adult male Wistar rats weighing 200–250 g were sacrificed by decapitation, and the mandibles and maxilla were excised. The dental pulp tissues were carefully removed in one piece from pulp cavities of incisors as described previously [10], and maintained in ice-cold physiological saline until use (30–60 min). The isolated pulp tissues (approx. 20 mg) were incubated for 30 min at 37° in 1 ml of 50 mM Tris–HCl buffer (pH 7.4) containing 0.9% NaCl in the presence or the absence of test agents.

* To whom all correspondence should be addressed.

PGI_2 and TXA_2 released into the incubation medium were determined by specific radioimmunoassay as 6-keto-$PGF_{1\alpha}$ and TXB_2, respectively [9].

After the incubation, the pulp tissues were removed from the medium. Because of the small size of pulp tissue, the tissues of six individual samples were pooled (approx. 100 mg) and homogenized in 2 ml of Tris–HCl buffer at 0°. Lipid peroxides in the homogenates were estimated as malondialdehyde by thiobarbituric acid (TBA) method [11]. The values were expressed as TBA value (O.D. at 530 nm/g tissue).

Statistical analysis was made by Student *t*-test.

RESULTS

As shown in Fig. 1, when dental pulp tissue was incubated in Tris–HCl buffer with 10 mM mannitol, the tissue level of lipid peroxides was reduced to 50% of the control level as estimated by the TBA method. In contrast, when the pulp tissue was incubated in the presence of 1 mM ascorbic acid and 0.3 mM $FeSO_4$ (A + F), the level of lipid peroxides was increased to about 2-fold of the control. MK-447 (100 μM) itself inhibited the lipid peroxidation in the pulp tissue by 34%. MK-447 also reduced the A + F-promoted lipid peroxidation by 33%, but did not further reduce the mannitol-suppressed lipid peroxidation.

Figure 2 shows the effects of mannitol and A + F on 6-keto-$PGF_{1\alpha}$ and TXB_2 production by dental pulp. Mannitol did not stimulate PG or TX formation. Contrarily, A + F significantly inhibited 6-keto-$PGF_{1\alpha}$ and TXB_2 production by 53% and 46%, respectively.

The effect of MK-447 on 6-keto-$PGF_{1\alpha}$ and TXB_2 biosynthesis by rat dental pulp is shown in Fig. 3. MK-447 at concentrations of 1 and 10 μM had no significant influences on PG and TX production, but at a higher concentration of 100 μM significantly stimulated the formation of both.

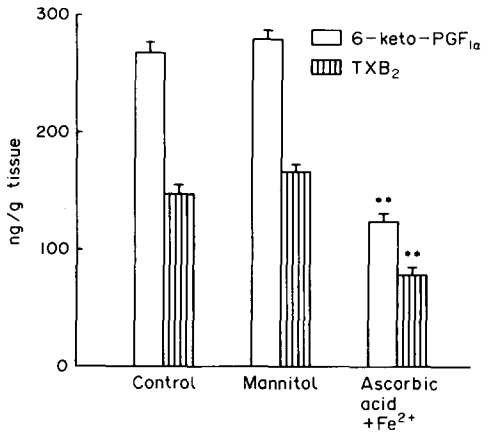

Fig. 2. Effects of mannitol and ascorbic acid with Fe^{2+} on 6-keto-$PGF_{1\alpha}$ and TXB_2 biosynthesis by isolated rat dental pulp. Incubation conditions were the same as Fig. 1. After the incubation, 6-keto-$PGF_{1\alpha}$ and TXB_2 released into the medium were determined by radioimmunoassay. Each column represents mean ± S.E. (N = 6). **P < 0.01 vs control.

The effects of MK-447 on PG and TX production in the presence of mannitol are shown in Fig. 4. In the presence of 10 mM mannitol, the significant stimulatory effect of MK-447 was observed at concentrations higher than 1 μM. Furthermore, 6-keto-$PGF_{1\alpha}$ production at 10 and 100 μM of MK-447 in the presence of mannitol were significantly (both P < 0.05) higher than those in the absence of mannitol. On the other hand, the effect of MK-447 at lower concentrations of 1 and 10 μM on TXB_2 production was not influenced by the presence of mannitol. However, TXB_2 synthesis at 100 μM of MK-447 in the presence of mannitol was significantly (P < 0.05) higher than that in the absence of mannitol.

The effects of MK-447 on 6-keto-$PGF_{1\alpha}$ and TXB_2 biosynthesis in the presence of A + F are shown in

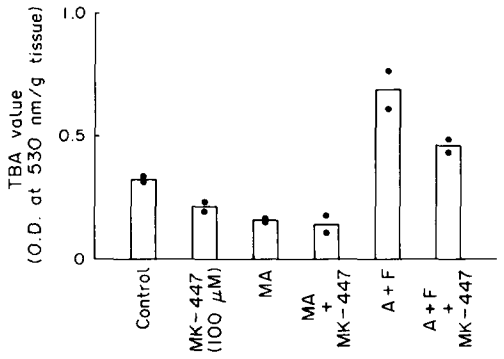

Fig. 1. Effects of MK-447, mannitol and ascorbic acid with Fe^{2+} on lipid peroxidation of isolated rat dental pulp. The pulp tissue was incubated in Tris–HCl buffer (pH 7.4) for 30 min in the absence (control) or the presence of 100 μM MK-447, 10 mM mannitol (MA) or 1 mM ascorbic acid with 0.3 mM Fe^{2+} (A + F). After incubation, the lipid peroxidation in the pulp tissue was estimated by TBA method. Each column represents mean of two experiments of pooled samples. Points indicate the individual value.

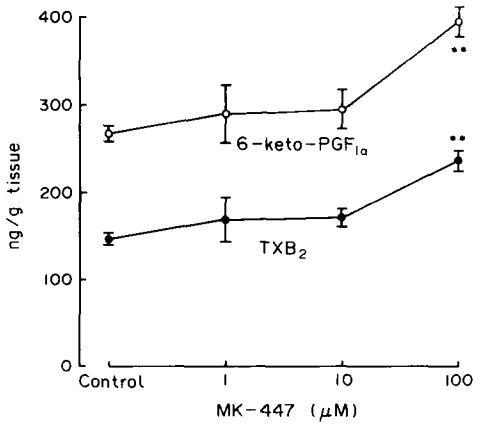

Fig. 3. Effects of MK-447 concentrations on 6-keto-$PGF_{1\alpha}$ and TXB_2 biosynthesis by isolated rat dental pulp. The pulp tissue was incubated in Tris–HCl buffer (pH 7.4) for 30 min in the absence (control) or the presence of MK-447. Each points represents mean ± S.E. (N = 6). **P < 0.01 vs control.

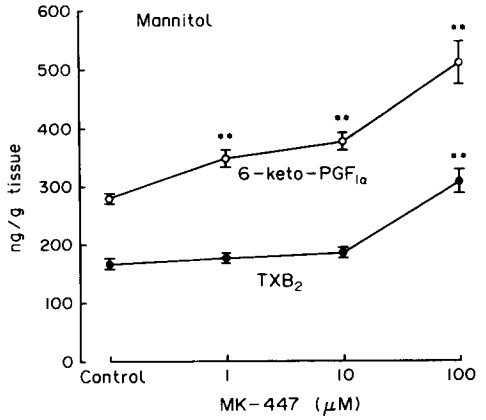

Fig. 4. Effects of MK-447 concentrations on 6-keto-$PGF_{1\alpha}$ and TXB_2 biosynthesis by dental pulp in the presence of mannitol. The pulp tissue was incubated in the presence of 10 mM mannitol without (control) or with MK-447. Each points represents mean ± S.E. (N = 6). **P < 0.01 vs control.

Fig. 5. As shown in Fig. 2, A + F itself inhibited the PG and TX production. In the presence of A + F, MK-447 showed no stimulatory effects, and contrarily, at a higher concentration of 100 μM significantly inhibited both 6-keto-$PGF_{1\alpha}$ and TXB_2 production by 27%.

DISCUSSION

The present study has demonstrated that MK-447 stimulates PGI_2 and TXA_2 biosynthesis by rat dental pulp tissue, although significant increase is observed at higher concentration (100 μM). This compound also suppressed the tissue level of lipid peroxides. MK-447 also has been reported to enhance PG endoperoxide formation in seminal vesicle microsomes [2–4, 6]. Kuehl *et al.* [2] suggested that MK-447 facilitates the conversion of PGG_2 to PGH_2 by acting as a scavenger of free radicals, presumably [.OH], released during the conversion. Since [.OH] is the main initiator of lipid peroxidation [8], our results are in support of the above concept. However, the scavenging of [.OH] may not always result in the stimulation of cellular PG biosynthesis, since mannitol, a typical scavenger of [.OH], had no effect on PG and TX production by the pulp tissue whereas it reduced the lipid peroxidation more effectively than MK-447.

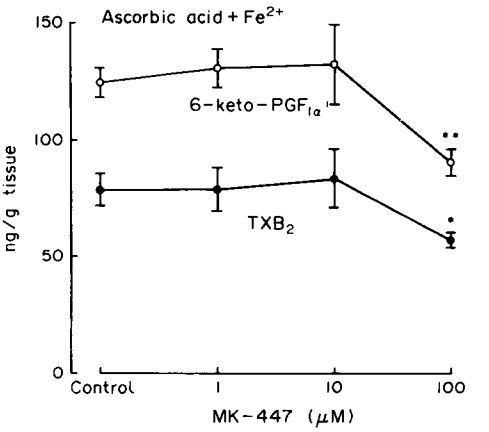

Fig. 5. Effects of MK-447 concentrations on 6-keto-$PGF_{1\alpha}$ and TXB_2 biosynthesis by dental pulp in the presence of ascorbic acid and Fe^{2+}. The pulp tissue was incubated in the presence of 1 mM ascorbic acid and 0.3 mM Fe^{2+} without (control) or with MK-447. Each points represents mean ± S.E. (N = 6). *P < 0.05, **P < 0.01 vs control.

In the presence of mannitol, the stimulatory effects of MK-447 on PGI_2 production by the pulp tissue were potentiated. The enzymes of the PG biosynthetic pathway, PGI_2 synthetase in particular, are sensitive to oxidative deactivation by lipid peroxides or the resultant free radicals [12, 13]. Therefore, mannitol may facilitate the stimulatory effect of MK-447 by protecting the enzyme against such deactivation.

On the other hand, ascorbic acid and ferrous ions are important initiators of non-enzymic lipid peroxidation, which may be promoted by [.OH] formation [8, 14]. The lipid peroxidation induced by ascorbic acid and Fe^{2+} inhibits PGE generation in kidney medulla slices [15]. The present study also demonstrated that this combination markedly stimulated the cellular lipid peroxidation, and inhibited both PGI_2 and TXA_2 production in dental pulp tissue. Since TXA_2 synthetase is resistant to the oxidative deactivation [12], our result indicates that the lipid peroxidation inhibited PG endoperoxides formation in the pulp tissue. MK-447 also suppressed the elevation of tissue lipid peroxidation induced by ascorbic acid and Fe^{2+}. However, in the presence of these agents, MK-447 inhibited PGI_2 and TXA_2 production. Similarly to MK-447, paracetamol (4-acetamidophenol) at 100 μM stimulated PG and TX production in the pulp tissue, but in the presence of ascorbic acid and Fe^{2+} inhibited the synthesis of both (data not shown).

MK-447 [2–4, 6] and paracetamol [3, 16] stimulate PG synthesis at the lower concentrations, probably acting as phenolic cosubstrate of PG endoperoxide synthetase [17], and inhibit at the higher concentrations in seminal vesicle microsomes. Furthermore, the large amount of tryptophan or hydroquinone, which also act as the cosubstrate [17], alters the stimulatory effect of these drugs to inhibition [6, 16]. These facts suggest that the *in vitro* effect of phenolic compounds on PG production depends on the concentration of the cosubstrate-like activities in the reaction mixture. Therefore, it is conceivable that the inhibition of PG biosynthetic enzymes by ascorbic acid with Fe^{2+} in the pulp tissue increased the ratio of endogenous cosubstrates of PG endoperoxide synthetase to the enzyme. Accordingly, the addition of MK-447 may have resulted in the inhibition of the enzyme, i.e. the inhibition of PGI_2 and TXA_2 biosynthesis. Thus, the present investigations suggest that the cellular levels of lipid peroxidation exert a crucial influence on effects of phenolic anti-inflammatory drugs like MK-447 or paracetamol on PG biosynthesis.

In contrast to our results, Lands and Hanel [7] have reported that MK-447 stimulates cyclooxygenase activity in purified PGH synthase prep-

aration, but inhibits the activity when the concentration of peroxides is decreased by glutathione peroxidase. The difference may be attributed to the different system used between the purified enzyme preparation with exogenous stimulators of PG endoperoxide synthetase and the isolated intact tissue containing natural cytoplasmic substance(s). Otherwise, the lipid peroxides involved may be different between those formed enzymically in their system and those induced non-enzymically by [.OH].

However, in agreement with our result, MK-447 has been reported to stimulate PG biosynthesis in isolated mouse ovaries, rat kidney slices [18], and rat aorta [19]. Further, MK-447 has diuretic property, which is also suggested to be mediated by enhancing PG production *in vivo* [20]. These results suggest that normal tissues contain sufficient levels of peroxides for MK-447 to stimulate PG synthesis, although they have appreciable levels of hydroperoxidase activities.

During the inflammatory process, phagocytic cells migrate to the inflammed site, and generate [$.O_2^-$], which subsequently is converted to other oxidizing species like [.OH] [8]. Therefore, it is likely that the abnormal amounts of lipid peroxides induced by [.OH] gradually deactivate the PG biosynthetic enzymes at the inflammatory site. In support of this concept may be the finding that the levels of PGs, (PGI_2 in particular), in exudate from rat carrageenin-induced pleurisy showed an increase of short duration in the early phase [21]. Under such conditions, the ratio of endogenous cosubstrate(s) of PG endoperoxide synthetase to the enzyme would be altered gradually in favour of the former. Hence, the treatment with phenolic anti-inflammatory drugs such as MK-447 or paracetamol would inhibit PG production at the inflamed sites [6, 22]. Some lipid peroxides are known to be potent chemotactic factors [8, 12]. Therefore, the property of such drugs to suppress lipid peroxidation may be further enhancing their anti-inflammatory efficacy. At present, we have no data to verify this hypothesis. The relationship between the local levels of lipid peroxides and PG synthesizing activity at the site of inflammation remains to be investigated intensively.

Acknowledgement—This work was supported in part by Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.

REFERENCES

1. J. R. Vane, *Nature new Biol.* **231**, 232 (1971).
2. F. A. Kuehl, Jr., J. L. Humes, R. W. Egan, E. A. Ham, G. C. Beveridge and C. G. Van Arman, *Nature, Lond.* **265**, 170 (1977).
3. J. Robak, E. Kostka-Trąbka and Z. Duniec, *Biochem. Pharmac.* **29**, 1863 (1980).
4. T. G. Payne, B. Dewald, H. Siegl, H. U. Gubler, H. Ott and M. Baggiolini, *Nature, Lond.* **296**, 160 (1982).
5. Y. Harada, K. Tanaka, K. Yamashita, M. Ishibashi, H. Miyazaki and M. Katori, *Prostaglandins* **26**, 79 (1983).
6. Y. Harada, K. Tanaka and M. Katori, *Japan. J. Pharmac.* **30**, 549 (1980).
7. W. E. M. Lands and A. M. Hanel, *Prostaglandins* **24**, 271 (1982).
8. F. Hertz and A. Cloarec, *Life Sci.* **34**, 713 (1984).
9. M. Hirafuji and Y. Ogura, *Biochem. Pharmac.* **32**, 2983 (1983).
10. M. Hirafuji, K. Terashima, S. Satoh and Y. Ogura, *Archs. Oral Biol.* **27**, 961 (1982).
11. K. M. Wilbur, F. Bernheim and O. W. Shapiro, *Arch. biochem.* **24**, 305 (1949).
12. F. A. Kuehl, Jr. and R. W. Egan, *Science* **210**, 978 (1980).
13. M. E. Hemler and W. E. M. Lands, *J. biol. Chem.* **255**, 6253 (1980).
14. E. D. Wills, *Biochem. J.* **113**, 315 (1969).
15. Y. Fujimoto and T. Fujita, *Biochim. biophys. Acta* **710**, 82 (1982).
16. J. Robak, A. Więckowski and Glyglewski, *Biochem. Pharmac.* **27**, 393 (1978).
17. S. Ohki, N. Ogino, S. Yamamoto and O. Hayaishi, *J. biol. Chem.* **254**, 829 (1979).
18. F. A. Kuehl, Jr., R. W. Egan, J. L. Humes, G. C. Beveridge and C. G. Van Arman, in *Biochemical Aspects of Prostaglandins and Thromboxanes* (Eds. N. Kharasch and J. Fried), p. 55. Academic Press, New York (1977).
19. Y. Harada, K. Tanaka and M. Katori, *Japan. J. Pharmac.* **31**, 845 (1981).
20. A. Scriabine, L. S. Watson, H. F. Russo, C. T. Ludden, C. S. Sweet, G. M. Fanelli, Jr., N. R. Bohidar and C. A. Stone, *J. Pharmac. exp. Ther.* **208**, 148 (1979).
21. Y. Harada, K. Tanaka, Y. Uchida, A. Ueno, S. Ohishi, K. Yamashita, M. Ishibashi, H. Miyazaki and M. Katori, *Prostaglandins* **23**, 881 (1982).
22. G. A. Higgs, E. A. Harvey, S. H. Ferreira and J. R. Vane, in *Advances in Prostaglandin and Thromboxane Research*, (Eds. B. Samuelsson and R. Paoletti), Vol. 1, p. 105. Raven Press, New York (1976).