PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 168, 399–402 (1981)

Contraction of the Canine Basilar Artery following Linoleic, Arachidonic, 13-Hydroperoxylinoleic, or 15-Hydroperoxyarachidonic Acid (41294)

TOHRU KOIDE, YUKIFUMI NODA, SHUN'ICHI HATA, KATSUAKI SUGIOKA,* SHOHEI KOBAYASHI,* AND MINORU NAKANO*,[1]

*Research Laboratories, Chugai Pharmaceutical Company, Ltd., 3-chome, Takada, Toshima-ku, Tokyo, and *College of Medical Care and Technology, Gunma University, Maebashi, Gunma, Japan*

Abstract. The contractile activity of linoleic acid (LA), arachidonic acid (AA), 13L-hydroperoxy-*cis*-9-*trans*-11-octadecadienoic acid (13HPLA), or 15L-hydroperoxy-*cis*-5-*cis*-8-*cis*-11-*trans*-13-eicosatetraenoic acid (15HPAA) was tested on canine basilar artery segments in a small chamber, using serotonin as the reference vasoconstrictor. The cumulative dose–response contraction was approximately 400 times that of serotonin. On the other hand, either 15HPAA or 13HPLA at 10^{-6} *M* induced a contraction almost equal to the maximum attained with serotonin (1×10^{-6} *M*) contraction. The maximal artery response to each of the hydroperoxy fatty acids was almost 1.4 times stronger than that obtained with serotonin, LA, or AA.

When platelets aggregate, free fatty acids are released from phospholipids by the action of phospholipase A_2. Thus arachidonic acid can be released and oxygenated to prostaglandin H_2 and 12L-hydroperoxy-5,8,10,14-eicosatetraenoic acid by a cyclooxygenase (1) and a lipoxygenase (2, 3), respectively. Furthermore prostaglandin H_2, an endoperoxide, is converted to thromboxane A_2 which is an extremely potent vasoconstrictor (4, 5). On the other hand, autoxidation of hemoglobin, probably during bleeding, produces $O_2^{-}\cdot$ and $\cdot OH$ (an active oxidant) (6) or other oxidants (7), which may attack linoleic acid (LA) and arachidonic acid (AA) released from phospholipids, yielding the corresponding hydroperoxides.

Little is known, however, of the action of hydroperoxy fatty acids on the arteries. The present work was undertaken to test the contractile activity of hydroperoxy fatty acids (which were prepared from LA and AA by soybean lipoxygenase) on the canine basilar artery.

Materials. LA (99.5% purity) and AA (99.0% purity) were purchased from Nakarai Chemical Ltd. and Sigma Chemical Company, respectively. The 13L-hydroperoxy-*cis*-9-*trans*-11-octadecadienoic acid (13HPLA) was prepared by aerobic incubation of linoleic acid with soybean lipoxygenase (type 1, Sigma Chemical Co.) and purified by preparative thin-layer chromatography on silica gel G (8). The 15L-hydroperoxy-*cis*-5-*cis*-8-*cis*-11-*trans*-13-eicosatetraenoic acid (15HPAA) was prepared by a modification of the methods described by Bild *et al.* (9) and Ohki *et al.* (10). A 37-mg portion of arachidonic acid was dissolved in 300 ml of 0.1 *M* NH_3-NH_4Cl buffer (pH 9.0), and incubated with 36 mg of soybean lipoxygenase (type 1, Sigma Chemical Co.) for 1 min at 25°, and the solution was occasionally agitated. At the end of the reaction 100 ml of ethanol was promptly added to the reaction mixture. After acidification to pH 2.0 with 80 ml of 0.4 *M* citric acid, the reaction mixture was extracted three times with 450 ml of ethyl ether. The pooled ether extract was then washed twice with 100 ml of water and the ether layer was collected. After removal of the organic solvent under reduced pressure, the hydroperoxy acids in the residue were isolated by thin-layer chromatography on silica gel G (Merck, $60F_{254}$) with the solvent system, ethyl ether–petroleum ether–acetic acid (85:15:0.1, by volume). Silica gel corresponding to the major band which was revealed by spraying *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine–dihydrochloride in ethanol or

[1] To whom reprint requests should be addressed.

0037-9727/81/110399-04$01.00/0

under ultraviolet light was scraped off and extracted with chloroform–methanol (2:1, by volume). The purified hydroperoxy acid in chloroform–methanol was stored at −80° until ready for use. These compounds demonstrated a positive reaction with peroxide reagent (KI in acidic methanol) (11) and were used for the experiments within 5 days of their preparation. The concentrations of 13HPLA and 15HPAA were determined spectrophotometrically by using a molar extinction coefficient at 234 nm of 25,000 $M^{-1}cm^{-1}$ (in 0.1 M NH_3-NH_4Cl buffer, pH 9.0) (8) and at 237 nm of 30,000 $M^{-1}cm^{-1}$ (in ethanol) (9), respectively. Just before the experiment, unsaturated fatty acid or the hydroperoxy fatty acid in chloroform–methanol was transferred to a round-bottom flask and the organic solvent was removed under reduced pressure. The residue was then dissolved in a minimum amount of bicarbonate–NaOH buffer (pH 9.1) with or without indomethacin.

Methods. The contractile activity of the agents on the canine basilar artery was tested in a chamber using, with slight modifications, the method described by Allen *et al.* (12). Beagles of both sexes weighing about 20 kg were anesthetized by the intravenous administration of sodium pentobarbital (30 mg/kg) and killed by rapid exsanguination. The brain was removed and placed in Krebs–Ringer solution. The basilar artery was dissected out under magnification and sectioned at 2- to 3-mm intervals. The segments were mounted in the chamber (maintained at 37 ± 1°) containing 5 ml of a buffer solution (NaCl, 120 mM; KCl, 4.5 mM; $CaCl_2$, 2.5 mM; $MgCl_2$, 1.0 mM; $NaHCO_3$, 27.0 mM; KH_2PO_4, 1.0 mM; Na_4EDTA, 0.01 mM; and glucose, 10.0 mM) bubbled with 95% O_2 and 5% CO_2 (pH 7.4 ± 0.1). The segment was initially stretched at a tension of 400 mg with a force-displacement transducer (Nihon Kohden, SB-1T) for 1 hr during which time the buffer was changed several times. Experiments were started after the segments were stretched at a final resting tension of 3 g. The total volume of the agents never exceeded 250 μl or 5% of the chamber volume. Under our experimental conditions, the addition of the agent(s) in the bicarbonate–NaOH buffer (pH 9.1) did not change the pH of the bath. The agents tested were added in a cumulative log-dose manner. After each addition of the agents, the artery was allowed to reach a stable contracted state. This was continued until subsequent additions did not further increase the degree of contraction.

Results. The cumulative dose–response curves of the basilar artery contraction induced by unsaturated fatty acids, their hydroperoxy analogs and serotonin are shown in Fig. 1. The most consistent vasoconstrictor, serotonin, induced a detectable contraction (3–5% of maximum attainable serotonin contraction) at a concentration as low as 10^{-10} M, whereas other agents required higher concentrations, i.e., 10^{-6} M for 15HPAA, 3×10^{-8} M for 13HPLA and AA, and 3×10^{-7} M for LA. The cumulative concentration of LA or AA required for the maximal contraction, 2×10^{-4} M, was approximately 400 times that of serotonin. On the other hand, either 15HPAA or 13HPLA at a cumulative concentration of

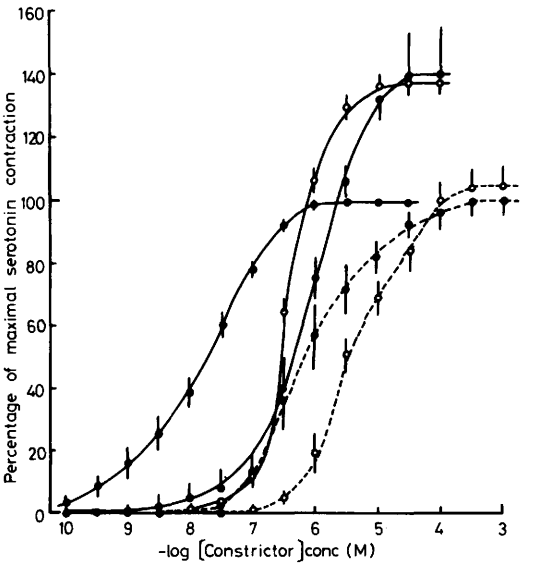

FIG. 1. Cumulative dose–response curves of the isolated canine basilar artery contraction induced by serotonin (⊙——⊙, n = 5), 13HPLA (○——○, n = 15), 15HPAA (●——●, n = 5), AA (●– – –●, n = 5), and LA (○– – –○, n = 8). Values are expressed as the mean ± SEM of the maximal contraction of serotonin. n represents the number of experiments.

10^{-6} *M* induced a contraction almost equal to the maximum attainable with serotonin at 10^{-6} *M*. Furthermore the maximal artery response to each of the hydroperoxy fatty acids was about 1.4 times stronger than that obtained with serotonin or the unsaturated fatty acid. Judging from the data obtained under comparable conditions (12), the cumulative concentration for the maximal contraction attainable with each of the hydroperoxy fatty acids is almost equal to that obtained with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). It has also been reported that the maximal canine basilar artery and bovine cerebral artery responses to $PGF_{2\alpha}$ are 1.1 and 1.8 times (respectively) stronger than the maximal response to serotonin (5, 12). After the artery was contracted by 10^{-5} *M* hydroperoxy fatty acid and then rinsed with buffer, the artery responded normally to the addition of 10^{-6} *M* serotonin. This indicates that the acid does not damage the contractile elements in the artery during our experiments.

As shown in Fig. 2, the dose–response curve obtained with either 15HPAA or AA was not significantly affected by the presence of indomethacin, a potent inhibitor of cyclooxygenase (3). Under similar conditions imidazole at 1×10^{-3} *M*, an inhibitor of thromboxane synthetase (13), had no effect on the 15HPAA- and AA-induced contraction. Furthermore, neither $PGF_{2\alpha}$, PGE_2, thromboxane B_2 (a metabolite of thromboxane A_2) nor the hydroxy fatty acids were detected when the canine basilar artery was incubated with ^{14}C-labeled AA (3×10^{-6}–2×10^{-5} *M*) in the presence or absence of indomethacin (1×10^{-5} *M*) according to the method by Salzman *et al.* (14) (unpublished results). However, 6-keto-$PGF_{1\alpha}$, a metabolite of PGI_2 present in tracer amounts, was detected as a product of [^{14}C]AA in the absence of indomethacin.

From these results, it seems likely that AA-induced canine basilar artery contraction observed here is caused by AA itself, and not by the cyclooxygenase or lipoxygenase products of AA.

Discussion. The *in vitro* data presented here show that free unsaturated fatty acids will directly induce the contraction of the canine basilar artery provided that these agents are present in a relatively high concentration. Since only small amounts of free unsaturated fatty acids are present in serum, these acids could not act as vasoconstrictors under normal conditions. However, if LA or AA freed from serum or other organs is oxidized in an enzymatic or nonenzymatic process by some event such as hemorrhage or clotting of blood, these acids could be converted to several hydroperoxy fatty acids including 13HPLA and 15HPAA with a contractile effect on the artery. An isomer of 15HPAA, 12L-hydro-

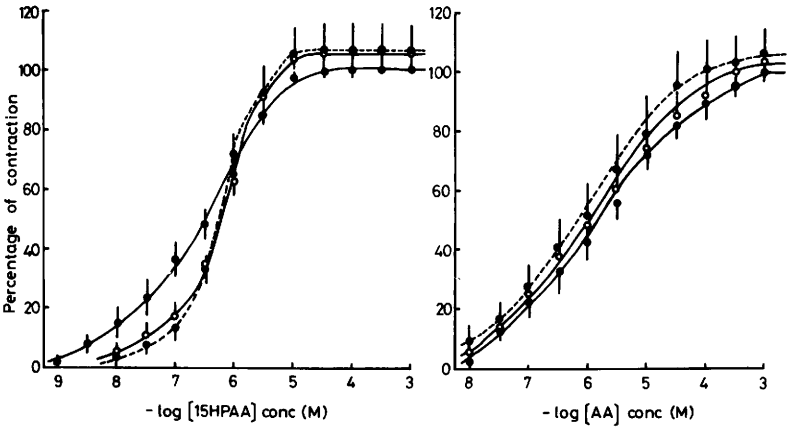

FIG. 2. Effect of indomethacin on the contractile responses of canine basilar artery induced by 15HPAA or AA. Each point represents the mean ± SEM of five determinations. ●——●, control; ○——○, indomethacin (1×10^{6} *M*); ⊙– – –⊙, indomethacin (1×10^{-5} *M*).

peroxyarachidonic acid, which may also behave like 15-hydroperoxy acid with respect to the basilar artery, has been found to be generated enzymatically in platelets (3). Furthermore, the conversion of arachidonic acid to the 15L-hydroxy analog in polymorphonuclear leukocytes, which suggests the formation of 15HPAA as an intermediate, has been reported (15). It is likely that several chemical factors in blood, including serotonin, prostaglandins such as $PGF_{2\alpha}$ and thromboxane A_2, and others act as the mediators of the spasm associated with basilar artery hemorrhage. Several hydroperoxy fatty acids may also be included in the mediators of the vasospasm. In contrast to the vasoconstrictors derived from prostaglandin H_2 ($PGF_{2\alpha}$ and thromboxane A_2), the formation of the hydroperoxy fatty acids including 13HPLA and 15HPAA would not be inhibited by indomethacin which inhibits only the cyclooxygenase enzyme (3).

1. Samuelsson, B., Hamberg, M., Malmsten, C., and Svensson, J., *in* "Advances in Prostaglandin and Thromboxane Research" (B. Samuelsson and R. Paoletti, eds.), Vol. 12, pp. 737–784. Raven Press, New York (1976).
2. Nugteren, D. H., Biochim. Biophys. Acta **380**, 299 (1975).
3. Hamberg, M., and Samuelsson, B., Proc. Nat. Acad. Sci. USA **71**, 3400 (1974).
4. Gryglewki, R. J., Burting, S., Moncada, S., Flower, R. J., and Vane, J. R., Prostaglandins **12**, 685 (1979).
5. Ellis, E. F., Nies, A. S., and Oates, J. A., Stroke **8**, 48 (1977).
6. Misra, H. P., and Fridovich, I., J. Biol. Chem. **247**, 6960 (1972).
7. Tappel, A. L., Arch. Biochem. Biophys. **44**, 378 (1953).
8. Garssen, G. S., Vliegenthart, J. F., and Boldingh, J., Biochem. J. **122**, 327 (1971).
9. Bild, G. S., Ramadoss, C. S., Lim, S., and Axelrod, B., Biochem. Biophys. Res. Commun. **74**, 949 (1977).
10. Ohki, S., Ogino, N., Yamamoto, S., and Hayaishi, O., J. Biol. Chem. **254**, 829 (1979).
11. Khan, A. U., Science **168**, 476 (1970).
12. Allen, G. S., Menderson, L. M., Chou, S. N., and French, L. A., J. Neurosurg. **40**, 433 (1974).
13. Fitzpatrick, F. A., and Gorman, R. R., Biochim. Biophys. Acta **539**, 162 (1978).
14. Salzman, P. M., Salmon, J. A., and Moncada, S. J., Pharmacol. Exp. Ther. **215**, 240 (1980).
15. Borgeat, P., and Samuelsson, B., Proc. Nat. Acad. Sci. USA **76**, 2148 (1979).

Received October 13, 1981. P.S.E.B.M. 1981, Vol. 168.