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Endothelium is required for 12-hydroperoxyeicosatetraenoic acid-induced vasoconstriction

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Abstract

The pharmacological effects of 12-hydroperoxyeicosatetraenoic acid (12-HPETE) were examined using isolated canine basilar artery segments and isometric tension recording. 12-HPETE produced transient contraction of the artery segment while arachidonic acid or 12-hydroxyeicosatetraenoic acid (12-HETE) had a much lower potency. 12-HPETE-induced contraction which showed a requirement of a functional endothelium and a rapid insensitivity to re-administered 12-HPETE, was completely inhibited by the potassium channel blocker, glibenclamide. Other hydroperoxides did cross-desensitize the 12-HPETE-induced contraction, however, arachidonic acid or 12-HETE did not affect markedly. Here, we present that 12-HPETE is involved in the regulation of vascular tension via its effects on the endothelium. © 1998 Elsevier Science B.V.

Keywords: 12-HPETE (12-hydroperoxyeicosatetraenoic acid); 12-Lipoxygenase; Arachidonic acid; Endothelium-dependent contraction; Basilar artery; (Dog)

1. Introduction

12-Lipoxygenase converts arachidonic acid to 12-hydroperoxy-5,8,10,14-eicosatetraenoic acid (12-HPETE), which is then metabolized mainly to 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE) as well as to 12-keto-5,8,10,14-eicosatetraenoic acid (12-KETE), hepoxilins (epoxy alcohol compounds) and other minor substances. The enzyme is present in various mammalian tissues, including human platelets (Hamberg and Samuelsson, 1974) and porcine leukocytes (Yoshimoto et al., 1982). Metabolism related to this enzyme is one of the major pathways in the arachidonic acid cascade. In the nervous system, 12-lipoxygenase metabolites such as 12-HPETE, 12-KETE and hepoxilin A_3, modulate S-type K^+ channels, producing the presynaptic inhibition of neurotransmitter release (Piomelli et al., 1987, 1988, 1989; Belardetti et al., 1989; Buttner et al., 1989). However, the biological role of these metabolites is not fully understood.

12-HETE was detected in the medium of primary cultured endothelial cells of bovine aorta (Nakao et al., 1982) and more recently, 12-lipoxygenase activity was detected in canine platelets (Nishiyama et al., 1992), polymorphonuclear leukocytes and macrophages (unpublished data). Furthermore, the enzyme was demonstrated immunohistochemically in endothelial cells of a dog (Nishiyama et al., 1993). It is, therefore, possible that 12-lipoxygenase metabolites play a physiological role in vasomotor regulation via the endothelium itself and via cross-talk between blood cells and the endothelium. We now examined the effects of 12-HPETE on vascular tension in the isolated basilar artery, in the presence or absence of endothelium, using isometric tension measurement techniques. Our results suggest a potential role for 12-HPETE in the endothelium-dependent regulation of cerebrovascular tone.

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2. Materials and methods

2.1. Purification of 12-HPETE and HETE

12-HPETE and -HETE were prepared as described elsewhere (Kitamura et al., 1988) with some modifications. Briefly, 12-lipoxygenase was partially purified from porcine leukocyte cytosol fractions. The enzyme extract (6 ml) in 60 ml of 0.1 M Tris–HCl buffer (pH 7.4) containing 0.03% Tween 20, was cooled on ice for 5 min. 30 μmol of arachidonic acid (Nu-Chek, Elysian, MN) were incubated 4°C for 20 min in the reaction mixture, with stirring under a stream of oxygen. At the end of the reaction, 120 ml of ice-cold methanol was promptly added to the reaction mixture, which was kept at -20°C for 30 min and then centrifuged at $10{,}000 \times g$ for 15 min. After acidification to pH 2.5 with 6 N HCl, the supernatant of the reaction mixture was extracted with 360 ml of diethyl ether. The extract was then washed three times with distilled water (240, 120 and 30 ml saturated with NaCl, successively) and the ether-layer was collected. After evaporation of the organic solvent under reduced pressure, synthesized 12-HPETE and 12-HETE were isolated by straight-phase high performance liquid chromatography (HPLC) on a silica column (Nucleosil 50-5, 10×300 mm) with the solvent system: *n*-hexane/*n*-isopropanol/acetic acid (99:1:0.01, v/v, 5 ml/min at room temperature), as the mobile phase. The purified eicosanoids in methanol were stored at -80°C until use. The concentration of 12-H(P)ETE was measured spectrophotometrically as a molar extinction coefficient at 235 nm of 27,000 M^{-1} cm^{-1} in methanol. Preparation of 15-HPETE was as described elsewhere (Van Os et al., 1981). Prior to the experiments, almost all the methanol was removed with a stream of nitrogen, and eicosanoids (1 to 10 μmol) were dissolved in 1 ml of 100 mM $NaHCO_3$ buffer (pH 9.1; the final concentration of methanol was less than 5%, v/v).

2.2. Artery preparation and measurement of tension

Mongrel dogs of either sex, each weighing 8 to 15 kg were deeply anesthetized with an intravenous injection of sodium pentobarbital (30 mg/kg) and were then rapidly exsanguinated. The dogs were cared for in compliance with standards relating to the care and management of experimental animals recommended by the Japanese Prime Minister's Office and the Japan Animal Welfare Society.

After the brainstem containing the basilar artery was removed, the artery segment was dissected free of surrounding tissue, under magnification, and was sectioned at 2 to 3 mm intervals. In some preparations, the endothelium of basilar artery segments was mechanically removed by drying with a stream of gas (95% O_2/5% CO_2) and gentle rubbing of the endothelial surface with a Teflon catheter, before the basilar arterial rings were prepared (Katusic et al., 1984). The basilar arteries without endothelium were then sectioned in the same manner. The arterial rings, with or without endothelium were mounted in chambers (which were kept at 37°C with a water bath) containing 10 or 20 ml of a standard buffer solution (modified Krebs bicarbonate solution) (in mM: NaCl, 120; KCl, 4.5; $MgSO_4$, 1.0; $NaHCO_3$, 27.0; KH_2PO_4, 1.0; $CaCl_2$, 2.5; dextrose, 10.0; pH 7.4 adjusted with NaOH) bubbled with a gas (95% O_2/5% CO_2). Each segment was gradually stretched and was allowed to equilibrate at 37°C for 90 min before use. Experiments were started after the arterial rings had stretched at final basal tensions of 1.5 or 2.0 g. The total volume of agents added to the experimental chamber never exceeded 200 μl (1% of the chamber volume) and the final concentration of methanol was also less than 0.05%. Tension of the arterial ring was isometrically recorded using a force-displacement transducer (SB-1T, Nihon Koden, Tokyo, Japan). Details of the arterial preparation and tension measurement were as described elsewhere (Allen et al., 1974). At the start of experiment, the contractile responses to 40 mM KCl and 10^{-5} M 5-hydroxytryptamine (5-HT) were tested for each arterial ring, which was then washed repeatedly in fresh standard buffer solution. At the end of the experiment, the presence of contractile responses to KCl and 5-HT was verified. For rings with endothelium, we confirmed the presence of a functional endothelium, as determined by endothelium-dependent relaxation using thrombin as an indicator (Nakagomi et al., 1988).

To estimate the dose-dependence of effects on rings, eicosanoids, including arachidonic acid, 12-HPETE and 12-HETE, were added directly to the chamber in a cumulative manner (10^{-9} to 10^{-5} M). To confirm the stability of 12-HPETE in the chamber medium, the aliquots were taken out immediately when the sample was added and again 6 min after. Then, the samples were analyzed by reverse-phase HPLC (solvent system; acetonitrile/methanol/water = 35:15:25) using a multiscan UV detector (Multi 320, Nihon Bunko, Japan).

2.3. Statistical analysis

The data were expressed as means $\pm$ S.E.M. Statistical comparisons between groups were made using the Mann–Whitney analysis. *P*-values less than 0.05 were considered to be statistically significant.

3. Results

3.1. 12-HPETE-induced contraction via endothelium

As shown in Fig. 1, 12-HPETE induced contractile responses in the intact basilar artery in a dose-dependent manner, whereas arachidonic acid and 12-HETE induced less strong contraction. The isometric tension elicited by 12-HPETE, arachidonic acid and 12-HETE at 10 μM was

49.6 ± 5.2% ($n = 7$), 24.9 ± 7.3% ($n = 5$) and 6.9 ± 1.9% ($n = 5$), respectively, of that seen with 10^{-5} M 5-HT. The contractile tensions induced by 12-HPETE and arachidonic acid were transient in the case of a high dose and decreased to baseline within 3 to 10 min (Fig. 1A).

In contrast, 12-HPETE (10^{-5} M)-induced contraction was completely abolished in the denuded arterial rings. In some cases, 12-HPETE induced a slight vasodilatation as shown in Fig. 2A (lower panel). The 12-HPETE-induced response was −2.1 ± 1.1% of the 5-HT (10^{-5} M)-induced contractile force without endothelium ($n = 5$), in contrast to 48.8 ± 10.7% ($n = 5$) of that seen in 10^{-5} M 5-HT in the presence of endothelium (Fig. 2B). The loss of endothelial function was confirmed by the absence of thrombin-induced endothelium-dependent relaxation in the 5-HT contracted muscle (Nakagomi et al., 1988).

Using HPLC, we tested the stability of 12-HPETE in the recording chamber, as 12-HPETE is an unstable product (Nishiyama et al., in press). The transiency of 12-HPETE-induced contraction could have been due to rapid reduction of 12-HPETE to 12-HETE in the recording chamber. HPLC, however, showed that more than 95% of the 12-HPETE administered was stable in the chamber medium for at least 6 min (Fig. 3).

3.2. Desensitization of 12-HPETE-induced contraction in intact rings

Another reason for the transient nature of 12-HPETE-induced contraction could have been rapid desensitization to the same agonist administered repeatedly. Following the 12-HPETE (10^{-5} M)-induced transient contraction, the second application of this eicosanoid at the same concentration induced very little tension even after 10 min, regardless of wash-out at least five times with fresh medium (13.2 ± 3.7% of the initial contraction by 12-HPETE; 8.0 ± 2.4% of that seen with 10^{-5} M 5-HT, $n = 4$) (Fig. 4 upper panel). The contractile response to 10^{-5} M 12-HPETE was also reduced to 12.6 ± 2.8% of that seen with 10^{-5} M 5-HT, after the contraction induced by 10^{-5} M 15-HPETE (Fig. 4 lower panel). Pretreatment with 15-HPETE reduced 12-HPETE (10^{-5} M)-induced contractions to about 25% of the usual responses. On the other hand, pretreatment with either arachidonic acid or 12-HETE reduced the contractile force induced by 12-HPETE by about half (23.9 ± 5.6% and 26.5 ± 5.2% of that seen with 10^{-5} M 5-HT, respectively, $n = 4$).

In all these cases, 12-HPETE did not reduce the 5-HT and KCl-induced contractions, but did enhance those in the

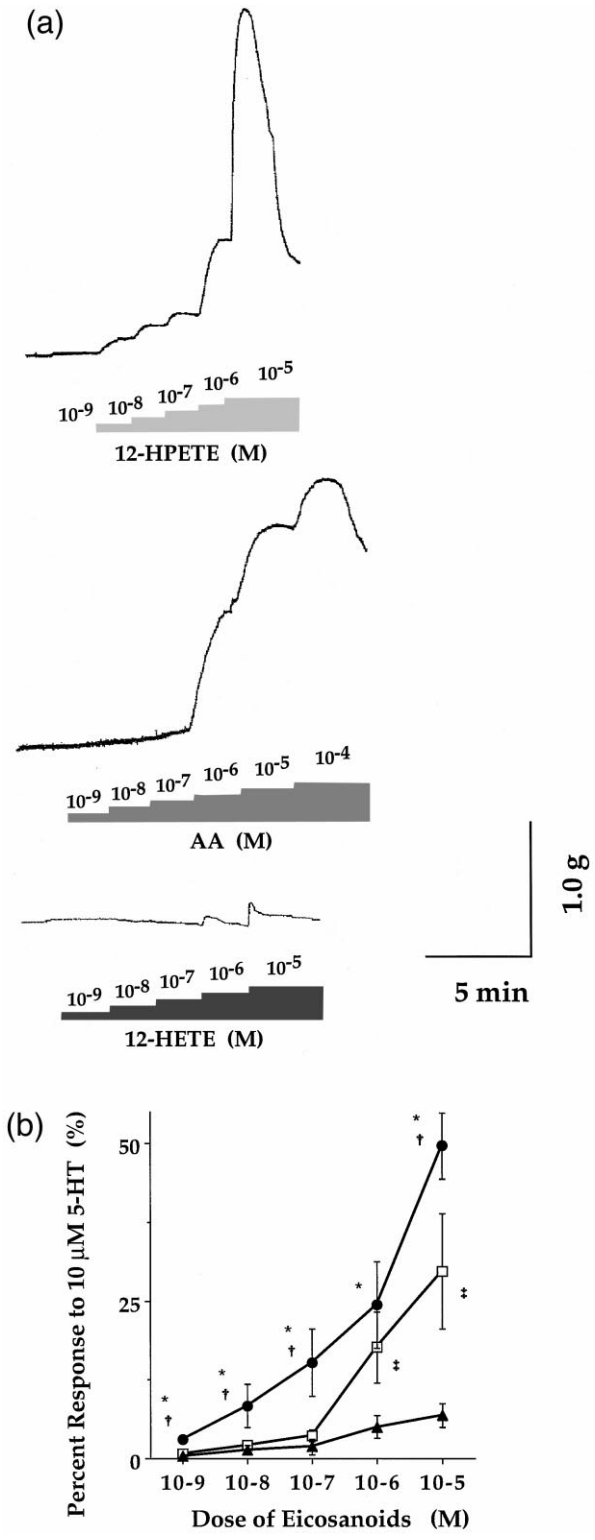

Fig. 1. Eicosanoids induced contraction of the intact basilar artery. (A) Representative tension profile induced by 12-HPETE, arachidonic acid and 12-HETE in intact rings. 12-HPETE (upper) and arachidonic acid (middle) induced transient contraction in arterial rings with the endothelium intact. On the other hand, 12-HETE (lower) elicited little response. The step-shaped bars denote the application of eicosanoids for each dose. (B) Effects of arachidonic acid (open squares, $n = 5$), 12-HPETE (filled circles, $n = 7$) and 12-HETE (filled triangles, $n = 5$) on intact arterial rings. 12-HPETE induced-contractile response in the intact basilar artery, in a dose-dependent manner. In contrast, arachidonic acid and 12-HETE induced a lesser degree of contractile force. Dose–response curves were obtained by cumulative additions. Vertical bars represent S.E.M. *, † and ‡ indicate the difference from 12-HETE, arachidonic acid and 12-HETE, respectively ($p < 0.05$).

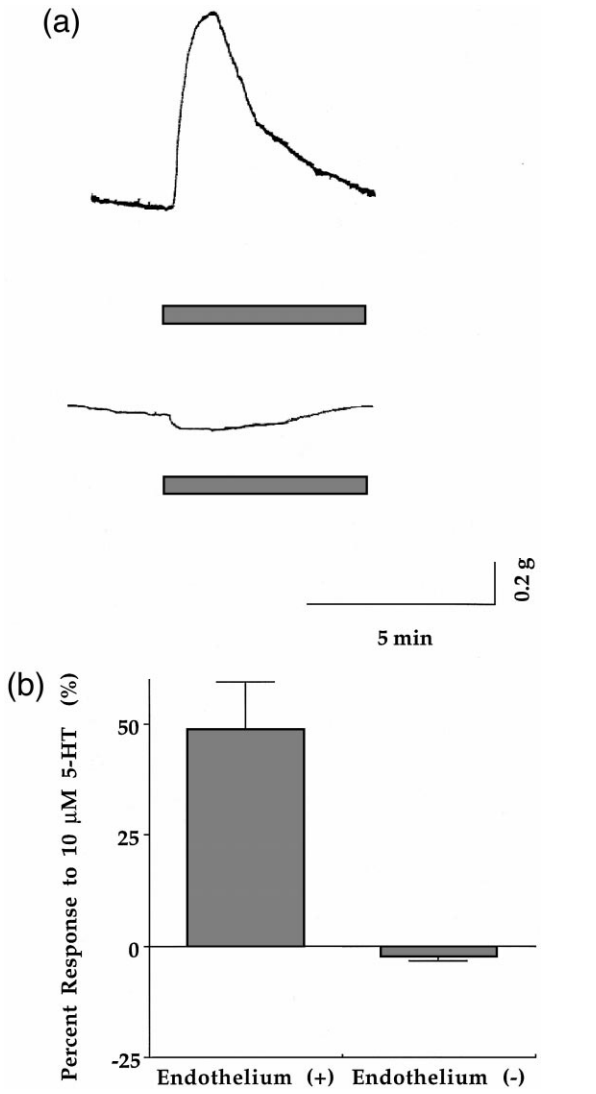

Fig. 2. 12-HPETE induced transient vasoconstriction via endothelium. (A) Representative tension profile induced by 12-HPETE (10^{-5} M) in intact arterial rings (upper panel) and rings without endothelium (lower). Note that 12-HPETE did not induce any contractile force in denuded arterial rings (lower panel). Bars denote the administration of 12-HPETE. (B) Tension induced by 12-HPETE (10^{-5} M) in arterial rings with ($n = 5$) or without endothelium ($n = 5$). Tensions was expressed as a percentage of the contraction induced by 10 μM 5-HT. Vertical bars represent S.E.M.

intact rings (178.3 ± 17.7% and 171.0 ± 13.8% of control responses, respectively, $n = 5$). The enhancing effects of 12-HPETE also required functional endothelium (104.6 ± 6.0% and 110.9 ± 6.9% of control responses without endothelium, respectively, $n = 5$).

3.3. Glibenclamide inhibited the 12-HPETE-induced contraction in intact rings

The last possibility for explaining the transiency of 12-HPETE-induced contraction was activation of K^+ channels by 12-HPETE. To address this possibility, we administered 12-HPETE in the presence of K^+ channel

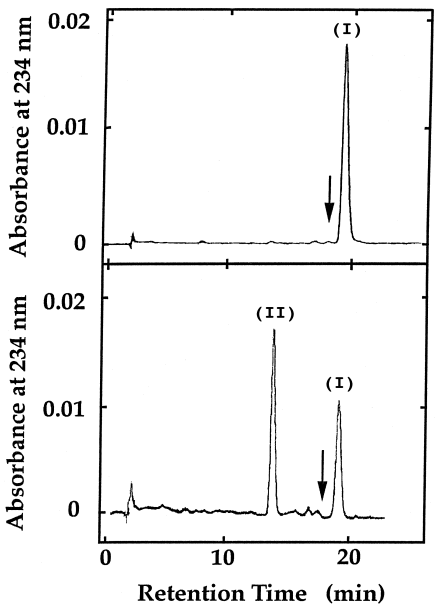

Fig. 3. The stability of 12-HPETE in the chamber medium. To confirm the stability of 12-HPETE in the chamber medium, the aliquots taken immediately after (upper panel) and 6 min after (lower panel) 12-HPETE administration were analyzed by reverse-phase HPLC. 13-Hydroxy-9-*cis*-11-*trans*-octadecadienoic acid (II) was added as an internal standard (0.5 nmol; equivalent to 10 μM) to the latter aliquot. Note that 12-HPETE (I) was stable in the bath medium 6 min after administration (lower panel) and 12-HETE (its position indicated by the arrows) was rarely detected.

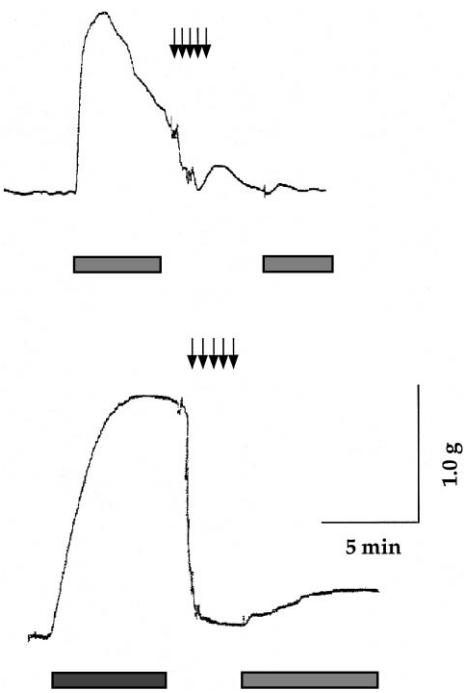

Fig. 4. Desensitization of 12-HPETE-induced contraction in intact rings. This contraction showed strong self-desensitization (upper panel). Other lipid peroxides such as 15-HPETE also showed cross-desensitization (lower panel). Bars denote the application of eicosanoids (10^{-5} M) and arrows indicate wash-out with fresh standard medium.

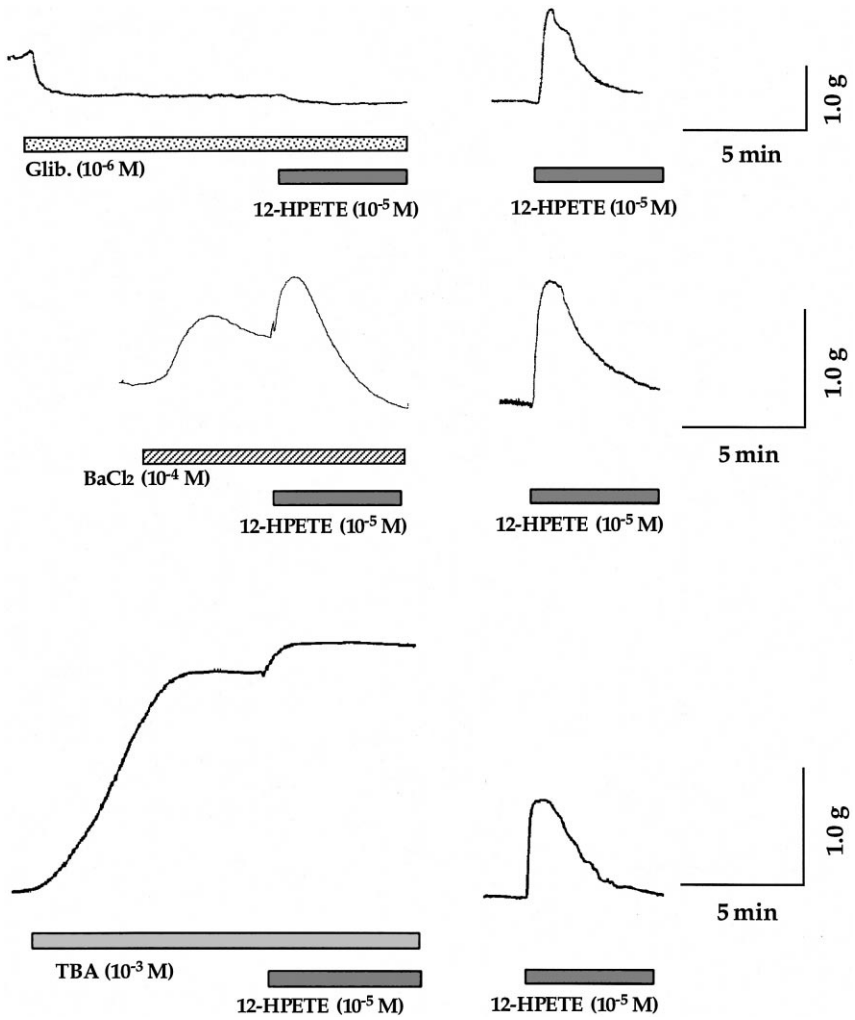

Fig. 5. Effects of K^+ channel blockers such as glibenclamide, $BaCl_2$ and tetrabutylammonium on 12-HPETE-induced contraction. Glibenclamide (Glib., 10^{-6} M) fully inhibited 12-HPETE-induced contraction (upper panel). 10^{-4} M $BaCl_2$ had no effect on this contraction (middle panel). However, 10^{-3} M tetrabutylammonium successfully modified 12-HPETE-induced contraction from a transient to a sustained one (lower panel). The bars below the traces denote the application of K^+ channel blockers and 10^{-5} M 12-HPETE, respectively. The ordinary contraction profiles induced by 12-HPETE are shown on the right, as standards. The same results were obtained from at least three different rings.

blockers such as $BaCl_2$, tetrabutylammonium and glibenclamide. Under such conditions, 12-HPETE should induce sustained contraction in the presence of K^+ channel blockers. However, pretreatment with the non-selective K^+ channel blockers, $BaCl_2$ (10^{-4} M) (Fig. 5, middle panel) and tetrabutylammonium (10^{-4} M) (data not shown) which slightly increased basal tension, did not affect the 12-HPETE-induced contraction. On the other hand, a high concentration of tetrabutylammonium (10^{-3} M) (Fig. 5, lower panel), which produced a marked increase of the basal tension, successfully changed the 12-HPETE-induced contraction from a transient to a sustained one. Interestingly, the ATP-sensitive K^+ channel blocker, glibenclamide (10^{-6} M), reduced the basal tension and inhibited the contraction in response to 12-HPETE (Fig. 5, upper panel). Pretreatment with K^+ channel blockers (10^{-4} M $BaCl_2$; 10^{-4} M tetrabutylammonium; 10^{-6} M glibenclamide) did not affect significantly the contractile response to KCl ($93.6 \pm 6.1\%$; $95.2 \pm 5.9\%$; $98.0 \pm 5.0\%$, respectively, $n = 4$) or 5-HT ($107.4 \pm 1.6\%$; $108.9 \pm 5.9\%$; $104.5 \pm 2.8\%$, respectively, $n = 4$).

4. Discussion

The present results demonstrated that the fundamental effect of 12-HPETE on the isolated basilar artery is to produce transient vasoconstriction that requires the presence of a functional endothelium. Lipid peroxides impair cellular functions in both endothelium and smooth muscle cells (Kontos et al., 1980; Sasaki et al., 1981). The effects of 12-HPETE on our arterial ring preparations would seem to be a physiological response, not a pathological one owing to tissue damage, since the endothelium-dependent relaxation was preserved in all segments which could be examined, and is considered a most fragile function

(Nakagomi et al., 1987; Kim et al., 1988). It has also been found that 12-HPETE is unstable in vivo and is promptly metabolized, mainly to 12-HETE (about 80 to 90%) and to other compounds. However, 12-HPETE in the chamber was reasonably stable in our experiments and was not metabolized to other compounds, at least for 6 min (Fig. 3). Therefore, the relaxation induced by 12-HPETE may not have been due to a prompt reduction to 12-HETE, but self-desensitization may have occurred (Fig. 4 upper panel). The reduced responsiveness to 12-HPETE was also observed after pretreatment with another lipid hydroperoxide, 15-HPETE, but much less so with arachidonic acid or 12-HETE. Glibenclamide, i.e. an ATP-sensitive K^+ channel blocker, which is also a prostanoid receptor blocker (Zhang et al., 1991, 1992), completely inhibited the 12-HPETE-induced contraction (Fig. 5 upper panel). Thus, 12-HPETE may either enhance the release of an endothelium-derived contracting factor (EDCF) or reduce the release of the endothelium-derived relaxing factor (EDRF), in a receptor-operated fashion. It is reported that O_2^- generation is dependent on 12-lipoxygenase activity in human platelets (Jahn and Hansch, 1990). This enzyme does exist in canine endothelial cells (Nishiyama et al., 1993). Therefore, the oxygen radical is one candidate for EDCF (Vanhoutte and Shimokawa, 1989), as induced by 12-HPETE. It is not likely that 12-HPETE reduces the release of EDRF, since soluble guanylate cyclase is activated by 12-HPETE (Brune and Ullrich, 1991).

Arterial rings denuded of endothelium had no contractile response to 12-HPETE, but vasodilator reactions were observed instead in some cases (Fig. 2A, lower panel). It has been reported that arachidonic acid applied to the brain surface induces cerebral arteriolar dilatation (Kontos et al., 1980). We had examined earlier the effects of 12-HPETE applied intrathecally on canine basilar artery in vivo (Watanabe et al., 1990, 1992). Just after 0.5 mg of 12-HPETE was injected into the canine cisterna magna, a mild vasodilatation in the canine basilar artery was evidenced angiographically. Based on these findings, we suspected that 12-HPETE has a vasodilator effect on arterial smooth muscle itself, possibly because of the membrane hyperpolarization via K^+ channel opening (Belardetti et al., 1989; Buttner et al., 1989). K^+ channel blockers such as $BaCl_2$ and a low dose of tetrabutylammonium, failed to sustain the 12-HPETE-induced contraction in intact rings, so general K^+ channels might not be involved in the reduction phase of 12-HPETE-induced contraction. A high dose of tetrabutylammonium, however, sustained this contraction (Fig. 5, lower panel). The possibility remains that Ca^{2+}-activated K^+ channels participate in the reduction phase of 12-HPETE-induced contraction. The possible involvement of the ATP-sensitive K^+ channel in the actions of 12-HPETE was also suggested by the potent effects of glibenclamide. However, glibenclamide itself produced relaxation of the basilar artery. Therefore, it is unclear whether the inhibition by glibenclamide of the 12-HPETE response was directly related to inhibition of the ATP-sensitive K^+ channel or indirectly to the vasoconstrictor action of thromboxane A_2 (Cocks et al., 1990).

Arachidonic acid and lipoxygenase metabolites have vasoconstrictive effects on isolated rabbit aortic strips. These contractions are not due to the formation of prostaglandin endoperoxides, since they are not affected by indomethacin or aspirin (Asano and Hidaka, 1979). Moreover, the contractile response to 15-HPETE, which inhibited the 12-HPETE-induced contraction (Fig. 4 lower panel), is significantly reduced by eicosatetraynoic acid (an oxygenase inhibitor) (Koide et al., 1982). These same authors stressed that enhancement of lipoxygenase activity was involved in eliciting contractile responses to 15-HPETE, since 15-HPETE enhanced the synthesis of lipoxygenase products. It is well known that lipid peroxides activate arachidonate lipoxygenases (Rouzer et al., 1985; Yokoyama et al., 1986; Shimizu et al., 1988). The activity of 12-lipoxygenase purified from the canine brain is also enhanced by lipid hydroperoxides (12- and 15-HPETE) (Nishiyama et al., 1992). Lipoxygenase metabolites increases the level of diacylglycerol, one of the sources of free arachidonic acid via diacylglycerol kinase inhibition (Setty et al., 1987), which leads to continuous production of lipid hydroperoxides in endothelial cells inducing vasoconstrictive agents. In contrast to other second messengers such as cyclic nucleotides and Ca^{2+}, 12-HPETE migrates from its cells of origin and act on neighbouring cells, as a first messenger (see for review; Piomelli and Greengard, 1990; Shimizu and Wolfe, 1990). Furthermore, leukocytes, and especially platelets, possess high amounts of 12-lipoxygenase (Hamberg and Samuelsson, 1974; Yokoyama et al., 1986; Nishiyama et al., 1992). Therefore, it is reasonable to suspect that 12-HPETE originated from blood cells or endothelial cells modulates the cerebral blood flow.

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