Hepoxilins sensitize blood vessels to noradrenaline—stereospecificity of action

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1 The vascular activity of two stereoisomers of hepoxilin A₃ (HxA₃) (8R and 8S) and of its glutathione conjugate, hepoxilin A₃-C (HxA₃-C) (8R and 8S), was investigated on rat heliocidal strips of thoracic aorta and longitudinal strips of portal vein.

2 Neither of the hepoxilins tested had a direct effect on the tone of the aortic strip or on the spontaneous contractions of the portal vein. However, the noradrenaline (NA)-induced response of these vessels, as expressed by the dose required for half maximal contraction, (EC₅₀) was greater in HxA₃ (8S)- and HxA₃-C (8R)-treated aorta. Increased frequency and strength of spontaneous contractions of the portal vein were detected at lower concentrations of NA in the presence of hepoxilins.

3 The threshold dose for both hepoxilins was 10⁻⁸ M and their effect was not dose-related beyond 10⁻⁸ M. The effect of hepoxilin appeared after a 45 min incubation period and could be observed even if the compounds were washed out after 15 min.

4 Stereochemical specificity was observed. The 8S isomer of HxA₃ was active in potentiating the NA-induced contraction of these vessels while the 8R isomer was inactive. In contrast, the 8R isomer of HxA₃-C was active while the 8S isomer was inactive. In both tissues, HxA₃ (8S) was more potent than its glutathione conjugate, HxA₃-C (8R).

5 In calcium-free buffer or in the presence of a calcium channel blocker (nifedipine 1 μM), no potentiation of NA-induced contraction by hepoxilins could be observed, suggesting the involvement of extracellular calcium in the actions of hepoxilins.

6 These experiments suggest that hepoxilins may be involved in the modulation of vascular tone and contractility.

Keywords: Hepoxilin A₃; hepoxilin A₃-C; trioxilin A₃; aorta; portal vein; potentiation; contraction; calcium; noradrenaline

Introduction

Alterations in the reactivity of blood vessels to neurotransmitters and circulating hormones may cause cardiovascular disorders such as hypertension (Mulvany *et al.*, 1980). Different mechanisms have been proposed to explain the enhanced responsiveness of arteries from hypertensive rats to an endogenous vasoconstrictor, noradrenaline. Several hypotheses have been proposed to explain this increase in sensitivity to involve a generalized increase in the contractility of the blood vessels or a reduced relaxation to vasodilators (Winqvist & Bohr, 1983; Cohen & Berkowitz, 1976). Other studies have suggested that the increased sensitivity of hypertensive blood vessels results from an alteration in the regulation of calcium influx in vascular muscle (Shibata *et al.*, 1975; Zsoter *et al.*, 1977). The contractile machinery in muscles is activated by a rise in intracellular calcium. Two sources of calcium may be involved (Bohr, 1963). The release of calcium from intracellular stores, believed to be responsible for the initial phasic component and the influx of extracellular calcium contributes to the tonic component of the contraction (Godfraind *et al.*, 1982; Cauvin & Malik, 1984). The concentration of intracellular free (cytosolic) calcium in vascular smooth muscle cells determines the degree of tension (Bohr, 1963) and is the trigger for muscle contraction (Filo *et al.*, 1965). Elevated intracellular free cytosolic calcium in vascular smooth muscle cells has been suggested in the pathophysiology of hypertension (Kwan, 1985).

Blood vessels respond to various stimuli by releasing free arachidonic acid from membrane phospholipids. The free fatty acid is then rapidly converted by the cyclo-oxygenase pathway to predominantly prostacyclin in endothelial cells and smooth muscle cells (Weksler *et al.*, 1977; Moncada *et al.*, 1977). Alternative pathways for arachidonic acid metabolism have been described in vascular tissue. Lipoxygenases and cytochrome P₄₅₀ epoxigenase metabolize arachidonic acid to various biologically active hydroperoxy-, hydroxy-, dihydroxyicosatetraenoic acids and leukotrienes (Juchau *et al.*, 1976; Greenwald *et al.*, 1979; Larrue *et al.*, 1983). More recently we have described the release of hydroxy epoxide metabolites of arachidonic acid named hepoxilins by vascular tissue (Laneuville *et al.*, 1991a). Hepoxilins are formed through the 12-lipoxygenase pathway via a haemoglobin or other haemoprotein-assisted intramolecular rearrangement of 12(S)-HPETE (Pace-Asciak *et al.*, 1983; Pace-Asciak, 1984a,b; Pace-Asciak & Martin, 1984). Intact pieces of the rat thoracic aorta stimulated by exogenous arachidonic acid release hepoxilin A₃ (HxA₃) (Laneuville *et al.*, 1991a). Homogenates of the rat aorta metabolize HxA₃ via two competing pathways; one involves hydrolysis of the epoxide group to form a trihydroxy metabolite, trioxilin A₃ (TrXA₃), and the second pathway involves conjugation of HxA₃ with glutathione via glutathione S-transferase to form a glutathione conjugate, hepoxilin A₃-C (HxA₃-C) (Laneuville *et al.*, 1991a). The formation of HxA₃-C in blood vessels is dependent on the presence of reduced glutathione and is greatly enhanced in the presence of trichloropropene oxide (TCPO), an epoxide hydrolase inhibitor which blocks utilization of the substrate, HxA₃, via the hepoxilin epoxide hydrolase. We have reported that the glutathione conjugation pathway of HxA₃ was more

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evident in veins than arteries as quantified by HxA₃-C formation (Laneuville *et al.*, 1991a). The aorta from spontaneous hypertensive rats (SHR) was similar to aorta from local normotensive Wistar rats in HxA₃-C formation although the aorta from the normotensive Wistar Kyoto rats (WKy) was much more active than aortae from either of the two other rat types (Laneuville *et al.*, 1991a).

Knowing that hepoxilins are formed by vascular tissue, it was of interest to determine whether they act on this tissue. In this study we report the actions of hepoxilins on isolated helical strips of the thoracic aorta and longitudinal strips of portal vein of the rat.

The following abbreviations are used in the text: hepoxilin A₃, HxA₃, 8(R) and 8(S)-hydroxy, 11(S), 12(S)-epoxy-eicosa-5Z, 9E, 14Z-trienoic acid; trioxilin A₃, TrXA₃, 8(R) and 8(S), 11(R), 12(S)-trihydroxy-eicosa-5Z, 9E, 14Z-trienoic acid; hepoxilin A₃-C, HxA₃-C, 8(R) and 8(S), 12(S)-dihydroxy-11(R)-glutathionyl-eicosa-5Z, 9E, 14Z-trienoic acid; noradrenaline, NA; trichloropropene oxide (TCPO); dimethyl sulphoxide, DMSO; hydroperoxyeicosatetraenoic acid, HPETE; hydroxyeicosatetraenoic acid, HETE; ethylene glycol-bis (β -amino ethyl ether) N,N,N',N'-tetraacetic acid, EGTA; 12-O-tetradecanoylphorbol-13-acetate, TPA.

Methods

Blood vessel preparation

Experiments were performed on male Wistar rats, 200–300 g, purchased from Charles River, St-Constant, Quebec, Canada. Animals were maintained on a standard rat chow diet (Prolab rat chow, Agway, Syracuse, NY, U.S.A.) and tap water *ad libitum*. Animals were killed, thoracic aorta and portal veins were rapidly removed and placed in Krebs solution (composition in mM: NaCl 119.00, KCl 4.70, KH₂PO₄ 0.40, NaHCO₃ 14.90, MgSO₄ 1.17, CaCl₂ 2.50 and glucose 5.50) kept at 37°C and continuously gassed with 95% O₂, 5% CO₂ mixture. Aortae were cleaned of connective tissue and cut helically by the method of Furchgott & Bhadrakom (1953). From each animal, two strips of the following dimensions, 2.0 to 4.0 mm wide and 2.0 to 2.5 cm long, were cut. Portal veins, dissected free from connective tissue, were cut longitudinally by the method of Couture *et al.* (1978) and one entire vessel (0.5 to 1.0 cm) was used per experiment. Tissues were mounted vertically in organ bath containing 10 ml of Krebs buffer maintained at 37°C and equilibrated with a 95% O₂:5% CO₂ gas mixture. A force of 1 g and 0.25 g was applied to the aortae and the portal veins respectively. An equilibration period of 60 min was allowed during which time the bath solutions were replaced every 15 min. Isometric contractions were recorded as changes in grams of tension on a Grass polygraph (model 79D) with a Grass FT03 force displacement transducer.

Effects of hepoxilins on noradrenaline-induced contractile response in the aorta and portal vein

The contractile capacity of the aorta was tested three times on each preparation by constructing cumulative concentration-response curves to NA. Wash out periods of 5 min were allowed between each curve to return to basal tension. The third curve was used for calculations and was identical to the second. When this was not the case, concentration-response curves were repeated until a reproducible response was obtained for both the aortae and the portal veins. The interval time between each cumulative dose-response curve was 15 min. Hepoxilins (at different concentrations) were applied 45 min before the cumulative addition of NA was repeated. In a series of experiments, concentration-response curves to NA were carried out 5 min after the addition of hepoxilins and were repeated for 1 h every 15 min. In control experiments, DMSO was added instead of hepoxilins and concentration-response curves by successive cumulative addition of NA were

obtained over the same period of experimentation as for the hepoxilins.

When the effect of calcium was investigated, a calcium channel blocker (1 μ M nifedipine) was added 20 min before the addition of hepoxilins. Experiments were also performed in a calcium-free Krebs buffer containing 1 mM EGTA. Tissues were dissected and incubated in the calcium free buffer. Calcium (2.5 mM) was added at the end of the experiment and a cumulative concentration-curve to NA was performed.

Expression of results and statistical analysis

Contraction is expressed as the percentage of the maximal response to NA obtained before the addition of hepoxilins. Sensitivity to NA in the aorta was expressed as an EC₅₀ value, EC₅₀ being the concentration of agonist required to give half-maximal response of the vessels. EC₅₀ values were obtained by logit/log regression analysis of each individual preparation and averaged. Contraction of the portal vein was characterized as the frequency of the spontaneous contractions, the basal level of tension and the amplitude of the contractions. All results are expressed as mean $\pm$ s.e.mean of six experiments. The differences between the values, obtained in the same strip, were tested for significance by use of Student's *t* test for paired observations. Unpaired Student's *t* test was used to compare the effect of hepoxilins obtained in different preparations. Probabilities (*P*) smaller than 0.05 were considered to be significant.

Drugs

Noradrenaline (Sigma, MO, U.S.A.) was dissolved in deionized water, containing 0.01% ascorbic acid (Fisher Scientific, Toronto, Canada), to give a stock solution of 10⁻² M. Authentic hepoxilins prepared chemically as described (Corey & Su, 1990) were kindly provided by Prof. E.J. Corey (Harvard University, Cambridge, MA, U.S.A.). HxA₃ and TrXA₃ were dissolved in DMSO (Calbiochem, Toronto, Canada) and HxA₃-C was dissolved in a mixture of phosphate buffer and ethanol (4 to 1). Hepoxilins were diluted with Krebs buffer to a final concentration of DMSO or ethanol not higher than 0.02%. Nifedipine (Sigma) was dissolved in absolute ethanol to give a 5 mM stock solution which was kept in the dark and frozen until use.

Results

Time-related response of the aorta to hepoxilin

NA at a concentration of 0.98 nM weakly contracts the rat aorta and reaches a plateau which persists until drug washout (Figure 1, upper trace). HxA₃ (8S) added at a concentration of 10⁻⁸ M to the bath containing NA had no immediate effect on

Figure 1 Time related induction of noradrenaline (NA) contraction (g tension) of helicoidal strips of rat aorta by hepoxilin A₃ (HxA₃) (8S). NA (0.98 nM) was added at time zero, followed 8 min later by dimethyl sulphoxide (DMSO) vehicle (top panel) or HxA₃ (8S) (lower panel). Note the slow onset but gradual rise in tension in the HxA₃-treated strip as compared to the strip exposed only to vehicle.

the NA-precontracted tissue (Figure 1, lower trace). However, upon leaving the tissue in contact with both NA and HxA₃, a contraction began to develop around 45 min after the initial addition of the hepoxilin. This contraction continued to develop further with time up to 100 min whereupon the bathing fluid was replaced. The tone of the vessel rapidly returned to pre-drug levels after drug washout. In contrast, the NA-precontracted tissue in the absence of hepoxilin did not contract further with vehicle addition only.

Potentialiation by hepoxilins of the noradrenaline-induced contraction of the aorta

Isolated helicoidal strips of the rat contracted in a dose-dependent fashion to increased concentrations of NA (Figure 2). None of the hepoxilins tested had any direct effect on the tone of the aortic strip, but some acted to potentiate the contraction by NA. Figure 2 shows tracings of dose-dependent contractions of the aortic strips to NA before and during the administration of vehicle (DMSO) and 10⁻⁸ M concentrations of HxA₃ (8R), HxA₃ (8S) and TrXA₃ (8S). HxA₃ (8S) significantly potentiated the threshold concentration at which NA caused a contraction (compare Figure 2a for vehicle with 2c for HxA₃ (8S); compare 0.48 nM NA in the presence of HxA₃ (8S) with the corresponding response with DMSO vehicle and with HxA₃ (8R) (Figure 2b) and TrXA₃ (8S) (Figure 2d)).

The dose-response curves to NA were quite reproducible throughout the experiment. Results of a series of experiments are shown in Figure 3. Here, the contractions of the aorta to increasing concentrations of NA are shown in the presence of vehicle, and in the presence of HxA₃ (8S) at two concentrations, 10⁻⁹ and 10⁻⁸ M (Figure 3a). Higher concentrations of HxA₃ were also tested but the effects were not significantly

Table 1 EC₅₀ values for noradrenaline (NA) in the presence and absence of the 8-enantiomers of hepoxilin A₃ (HxA₃), trioxilin A₃ (TrXA₃) and hepoxilin A₃-C (HxA₃-C) in rat thoracic aorta

Treatment	EC ₅₀ (nM) ± s.e.mean
Control	13.1 ± 2.4
HxA ₃ (8S) 10 ⁻⁹ M	12.7 ± 3.7
10 ⁻⁸ M	2.4 ± 0.9***
10 ⁻⁷ M	3.3 ± 0.9***
10 ⁻⁶ M	3.9 ± 0.8***
Control	10.4 ± 0.6
HxA ₃ (8R) 10 ⁻⁶ M	10.5 ± 0.5
Control	8.7 ± 1.5
HxA ₃ -C (8S) 10 ⁻⁸ M	9.3 ± 0.9
10 ⁻⁶ M	7.5 ± 0.6
Control	11.7 ± 0.5
HxA ₃ -C (8R) 10 ⁻⁹ M	12.5 ± 0.3
10 ⁻⁸ M	6.4 ± 0.3**
10 ⁻⁶ M	7.6 ± 0.3*
Control	9.8 ± 1.5
TrXA ₃ (8S) 10 ⁻⁶ M	8.8 ± 1.8

Values are means ± s.e.mean for six separate tissue preparations. Each treatment was compared to the control value which corresponds to the pretreatment value for each tissue by using Student's *t* test for paired samples. **P* < 0.05; ***P* < 0.01 and ****P* < 0.005 with respect to corresponding control value.

different from that observed at 10⁻⁸ M (Table 1). The stable trihydroxy metabolite of HxA₃, was inactive in modulating the vascular reactivity to NA (Figure 2d and Table 1). The EC₅₀ for various compounds is shown in Table 1.

Figure 2 Effect of hepoxilins on contractions of strips of rat thoracic aorta induced by cumulative doses of noradrenaline (NA). Three successive complete dose-response curves to NA (0.48 to 48.85 nM) were obtained (the last one is shown here in each panel) followed by tissue washout. Subsequent application was made of dimethyl sulphoxide (DMSO) vehicle (a), hepoxilin A₃ (HxA₃) (8R) (b), HxA₃ (8S) (c) or trioxilin A₃ (TrXA₃) (8S) (d), followed 45 min later by another cumulative dose-response curve to NA. Note that the presence of HxA₃ (8S) (c) potentiates the contraction of the tissue to NA so that significant contraction takes place at 0.48 nM NA.

Figure 3 Dose-response curves of aortae ($n = 6$) to noradrenaline (NA) in the presence of (a) hepxilin A_3 (HxA_3) (8S) and (b) HxA_3 -C (8R) at two concentrations in normal Krebs solution. Note the left shift of the response curve to NA in the presence of 10^{-8} M concentration of hepxilins. Symbols: (■), vehicle control (dimethyl sulphoxide); (Δ) HxA_3 (8S) 10^{-9} M for (a) and HxA_3 -C (8R) 10^{-9} M for (b); (○) HxA_3 (8S) 10^{-8} M for (a) and HxA_3 -C (8R) 10^{-8} M for (b).

Stereospecificity of the hepxilin-induced potentiation in the contraction of the aorta to noradrenaline

The above results are shown for the 8S isomer of HxA_3 . The 8R isomer is essentially inactive in potentiating the NA-induced contraction of the aorta at the concentrations used here (10^{-9} to 10^{-6} M) (Table 1 and Figure 2b). In contrast, the 8R isomer of the glutathione conjugate of HxA_3 , i.e. HxA_3 -C, was active (Figure 3b) but not the 8S isomer (Table 1). In separate experiments we tested whether the inactive isomer interfered with the potentiating response of the tissue to the active hepxilin isomer. After a cumulative dose-response curve to NA was generated, tissues were exposed first to different concentrations of the inactive isomers, HxA_3 (8R) or HxA_3 -C (8S) (10^{-9} to 10^{-6} M), and then exposed to 10^{-8} M of HxA_3 (8S) or HxA_3 -C (8R) respectively, and after 45 min another cumulative dose-response to NA was generated. In both cases, with either HxA_3 or HxA_3 -C, the inactive isomer neither affected the threshold nor the extent of potentiation of NA contraction by the active isomer.

Effect of hepxilins on noradrenaline-induced contraction of the portal vein

The portal vein displayed spontaneous contractions when stretched with 0.25 g tension (Figure 4a). The tissue returned to the same basal tension between each contraction as opposed to the aorta which kept a plateau of contraction after the tension was applied or after the addition of NA (Figures 1 and 2). NA induced an increase in the frequency of contractions and of the basal tension of the tissue in a dose-

dependent fashion (Figure 4 and Table 2). HxA_3 , (8S) 10^{-8} M increased the sensitivity of the tissue to NA by increasing the frequency of the spontaneous contractions as well as the basal level of tension of the portal vein to doses of NA that were not active before the addition of hepxilins (Figure 4b). The strength of the contractions as reflected by the height of the peaks, was not significantly altered by any of the hepxilins tested. However, the threshold dose of NA required to produce a flurry of spontaneous contractions was lowered when 10^{-8} M hepxilin was added. As observed with the aorta, the hepxilin effect was not dose-dependent beyond the threshold of 10^{-8} M (Table 2) although it was also time-dependent. Only the 8S isomer of HxA_3 was active while its trihydroxy metabolite ($TrXA_3$ (8S)), the 8R isomer of HxA_3 and the 8S isomer of HxA_3 -C were all inactive at a concentration of 10^{-6} M. HxA_3 -C (8R) was also active in potentiating the NA-induced increase of frequency as well as the basal level of tension (Table 2). All the hepxilins tested on the portal vein preparation were inactive on their own at all the doses used (10^{-9} to 10^{-6} M). Control preparations were run in parallel to demonstrate the sensitivity of the portal vein to NA remains unchanged during the time of the experiments (3 h) and the frequency, the basal level of tension and the strength of the contractions obtained after cumulative addition of NA when tested every 30 min were not significantly different from the pre-administration ones (data not shown).

Calcium dependence of the hepxilin A_3 (8S) effect

Contractile responses to cumulative concentrations of NA were measured following incubation of the aorta in Ca^{2+} -free Krebs solution containing 1 mM EGTA. This resulted in a slow phasic contraction of the aortic strip, smaller in magnitude when compared to contractions obtained in a calcium-containing buffer. The EC_{50} value for NA under these conditions was: 93.1 ± 6.2 nm ($n = 6$) in a Ca^{2+} -free buffer and 13.3 ± 3.3 nm ($n = 6$) in a Ca^{2+} -containing buffer. The threshold dose of NA required to cause a contraction was 0.49 nm in the Ca^{2+} -containing buffer and 8.30 nm in Ca^{2+} -free buffer. The amplitude of the maximal contraction was much lower in the Ca^{2+} -free buffer, 0.2 g, compared to that observed in the Ca^{2+} -containing buffer, 1.05 g. This reflects the importance of calcium influx that takes place during the tonic phase of the smooth muscle contraction. Under Ca^{2+} -free conditions, HxA_3 (8S) 10^{-8} M did not significantly reduce the threshold dose of NA (8.30 nm) required to elicit a contraction (Figure 5). The dose-response curve for NA remained unchanged in the presence of HxA_3 (8S) (10^{-8} M). The magnitude of the maximal contractile response obtained with 110.9 nm of NA in the presence of HxA_3 (8S) 10^{-8} M was not significantly different than that observed before administration of hepxilin. Following readdition of Ca^{2+} (2.5 mM), the contractile response of the aorta to NA was comparable to that obtained in Ca^{2+} -containing Krebs solution and a leftward shift (recovery) of the dose-response curve to NA induced by HxA_3 (8S) could be observed ($EC_{50} = 2.6 \pm 2.0$ nm).

The possible role of calcium influx caused by HxA_3 (8S) to explain its role in inducing potentiation of NA was further investigated by using a calcium channel blocker, nifedipine. Experiments were performed in a calcium containing medium and tissues were exposed to nifedipine (1 μ M) or its vehicle ethanol (5 μ L), 20 min before the addition of HxA_3 (8S) 10^{-8} M. As before, a dose-response curve to NA was generated 45 min after the addition of the hepxilin. Nifedipine caused a shift of the NA curve to the right when compared to the ethanol control (Figure 5b). The EC_{50} for NA in the nifedipine plus hepxilin-treated tissue (18.2 ± 1.5 nm) was significantly higher than the value observed prior to administration of ethanol plus hepxilin (10.1 ± 1.0 nm) ($P < 0.005$, $n = 6$). In the presence of HxA_3 (8S) 10^{-8} M, only the ethanol-treated tissue displayed a lower EC_{50} (1.7 ± 0.3 nm) compared to the pre-administration value (9.4 ± 1.6 nm) ($P < 0.005$, $n = 6$).

Figure 4 Effects of hepxilin A₃ (HxA₃) (8S) on the frequency and strength of contractions of strips of rat portal vein to noradrenaline (NA). Cumulative applications of NA are shown before and after addition of (a) dimethylsulphoxide (DMSO) vehicle and (b) HxA₃ (8S). Note the effect of hepxilin in inducing the contraction to NA at 4.88 nm compared to the control at 48.85 nm. Also note that hepxilin alone fails to alter the strength or the frequency of contraction.

Discussion

In this paper we show that hepxilins cause an increase in response of the aorta and of the portal vein to contractions induced by NA. The hepxilins do not display any contractile activity of their own, but potentiate the contractile activity of NA in both vascular tissues. We do not know whether the hepxilin effect is general to other vasoconstrictors or whether the hepxilins affect only the NA-induced contraction. An interesting observation lies in the finding that the action of the hepxilins on the NA-induced contraction is not an immediate response but is delayed in time. It requires about 45 min of contact with the tissue for the hepxilin-induced effect to start. The reason for this delayed action is unknown but we may speculate that the hepxilins 'prime' the cell for NA-contraction. This 'priming' may result from a calcium change in the smooth muscle cells or from the release of some hitherto undefined second messenger. Indeed, hepxilins have been reported to raise intracellular calcium in the neutrophil and this occurs through a mobilization of calcium from intracellular stores as well as through influx mechanisms (Dho *et al.*, 1990). In support of the influx mechanism, hepxilins have been shown to increase the transport of calcium across membranes (Derewlany *et al.*, 1984). Hence a calcium-related mechanism may be used in the priming of the smooth muscle cells for NA-contraction. However, this may not be entirely correct because HxA₃-C, while causing a potentiation of NA-vasoconstriction, as shown in the present study, does not raise intracellular calcium in the neutrophil (unpublished observations). Assuming that similar mechanisms hold for both types of cells (neutrophils and muscle cells), then the

hepxilin 'priming' may not be exclusively through a calcium-mediated mechanism.

An alternate mechanism for hepxilin action as shown in this study, may be due to the release by hepxilins of some second messenger. Indeed, hepxilins have been shown to induce the release of diacylglycerol and arachidonic acid from the human neutrophil (Nigam *et al.*, 1990), but these second messengers are released very quickly (within 5 min). The time-delayed action (onset around 45 min) of the hepxilins seen here must employ some other mechanism. Rasmussen *et al.* (1984) and Forder *et al.* (1985) demonstrated that TPA caused a time-delayed contraction (onset around 60 min) of the rabbit aorta, and they speculated that this action was due to the activation of protein kinase C (Hockin, 1985). Hence our findings with hepxilins may suggest that their action is mediated through a diacylglycerol-induced activation of protein kinase C.

The stereospecificity of hepxilin action warrants further comment. We find in the present study that only the 8S enantiomer of HxA₃ is active in potentiating the contraction by NA of both the aorta and the portal vein. This contrasts with our recent findings on the effect of hepxilins on vascular permeability in the rat skin (Laneuville *et al.*, 1991b). HxA₃ (both enantiomers) are weakly active on their own, but only the 8R enantiomer potentiates at very low doses (threshold 1 ng) the bradykinin-induced response (Laneuville *et al.*, 1991b). With regard to HxA₃-C, the active isomer in potentiating the NA-induced contraction of the aorta and the portal vein in the present study is the 8R isomer, while this isomer is inactive in the vascular permeability studies (unpublished observations). The reasons for the observed differences in isomer specificity

Table 2 Effects of hepoxilin A₃ (HxA₃) (8S and 8R), hepoxilin A₃-C (HxA₃-C) (8S and 8R) and trioxilin A₃ (TrXA₃) (8S) on noradrenaline (NA)-induced increase in frequency and strength of spontaneous contractions and basal tone of the rat portal vein strip

<i>Treatment</i>	<i>NA</i> (nM)	<i>Frequency</i> (contractions min ⁻¹)	<i>Basal tone</i> (g)	<i>Strength</i> (g)
A				
Control	0	3.00 ± 0	0.25 ± 0	0.68 ± 0.04
	0.48	3.80 ± 0.30	0.25 ± 0	0.71 ± 0.04
	5.36	4.30 ± 0.30	0.28 ± 0.03	0.78 ± 0.07
	54.21	8.83 ± 1.20	0.43 ± 0.06	0.90 ± 0.09
	542.71	17.67 ± 2.10	0.98 ± 0.09	1.07 ± 0.09
HxA ₃ (8S) 10 ⁻⁹ M	0	3.20 ± 0.20	0.25 ± 0	0.62 ± 0.06
	0.48	4.33 ± 0.50	0.25 ± 0	0.61 ± 0.06
	5.36	4.67 ± 0.70	0.27 ± 0.02	0.66 ± 0.07
	54.21	9.33 ± 1.00	0.47 ± 0.04	0.92 ± 0.10
	542.71	19.30 ± 2.10	1.09 ± 0.11	1.11 ± 0.13
HxA ₃ (8S) 10 ⁻⁸ M	0	2.83 ± 0.20	0.25 ± 0	0.48 ± 0.07
	0.48	3.67 ± 0.40	0.25 ± 0	0.52 ± 0.07
	5.36	11.83 ± 0.80***	0.46 ± 0.04***	0.76 ± 0.13
	54.21	14.17 ± 1.10***	0.68 ± 0.05**	1.01 ± 0.10
	542.71	19.83 ± 2.20	1.04 ± 0.12	1.25 ± 0.10
HxA ₃ (8S) 10 ⁻⁶ M	0	3.00 ± 0.30	0.25 ± 0	0.43 ± 0.07
	0.48	4.50 ± 0.70	0.25 ± 0	0.45 ± 0.07
	5.36	9.80 ± 1.40***	0.41 ± 0.07**	0.76 ± 0.12
	54.21	11.50 ± 1.50**	0.58 ± 0.09*	1.08 ± 0.16
	542.71	17.50 ± 0.09	0.97 ± 0.09	1.16 ± 0.15
B				
Control	0	3.00 ± 0	0.25 ± 0	0.62 ± 0.03
	0.48	3.00 ± 0	0.25 ± 0	0.68 ± 0.02
	5.36	3.10 ± 0.10	0.25 ± 0	0.65 ± 0.04
	54.21	5.50 ± 0.22	0.40 ± 0.05	0.84 ± 0.04
	542.71	15.30 ± 1.31	0.85 ± 0.06	1.29 ± 0.04
HxA ₃ (8R) 10 ⁻⁶ M	0	3.00 ± 0	0.25 ± 0	0.63 ± 0.03
	0.48	3.00 ± 0	0.25 ± 0	0.65 ± 0.04
	5.36	3.70 ± 0.30	0.25 ± 0	0.65 ± 0.04
	54.21	5.50 ± 0.22	0.39 ± 0.03	0.83 ± 0.04
	542.71	15.20 ± 0.07	0.83 ± 0.04	1.33 ± 0.04
C				
Control	0	3.00 ± 0	0.25 ± 0	0.45 ± 0.02
	0.48	3.20 ± 0.17	0.25 ± 0	0.45 ± 0.05
	5.36	5.80 ± 0.30	0.35 ± 0.03	0.60 ± 0.02
	54.21	6.50 ± 0.60	0.43 ± 0.04	0.79 ± 0.05
	542.71	10.60 ± 0.90	0.71 ± 0.05	0.88 ± 0.05
HxA ₃ -C (8S) 10 ⁻⁶ M	0	3.00 ± 0	0.25 ± 0	0.50 ± 0.04
	0.48	3.00 ± 0	0.25 ± 0	0.61 ± 0.03
	5.36	5.20 ± 0.30	0.35 ± 0.02	0.62 ± 0.03
	54.21	6.80 ± 0.70	0.56 ± 0.07	0.78 ± 0.04
	542.71	11.00 ± 1.20	0.74 ± 0.05	0.90 ± 0.05
D				
Control	0	3.00 ± 0	0.25 ± 0	0.70 ± 0.03
	0.48	3.00 ± 0	0.25 ± 0	0.90 ± 0.03
	5.36	6.50 ± 0	0.33 ± 0.05	1.04 ± 0.05
	54.21	9.00 ± 0.70	0.50 ± 0.04	1.19 ± 0.04
	542.71	14.22 ± 0.10	0.97 ± 0.10	1.42 ± 0.04
HxA ₃ -C (8R) 10 ⁻⁶ M	0	3.00 ± 0	0.25 ± 0	0.69 ± 0.05
	0.48	3.00 ± 0	0.25 ± 0	0.89 ± 0.21
	5.36	9.50 ± 0.60***	0.53 ± 0.08*	1.01 ± 0.02
	54.21	12.30 ± 0.04***	0.68 ± 0.06*	1.28 ± 0.03
	542.71	14.30 ± 1.00	1.01 ± 0.08	1.45 ± 0.06
E				
Control	0	3.00 ± 0	0.25 ± 0	0.51 ± 0.03
	0.48	3.00 ± 0	0.25 ± 0	0.57 ± 0.03
	5.36	4.00 ± 0.30	0.25 ± 0	0.77 ± 0.09
	54.21	6.00 ± 0.40	0.55 ± 0.07	0.99 ± 0.05
	542.71	14.80 ± 1.10	0.95 ± 0.14	1.33 ± 0.04
TrXA ₃ (8S) 10 ⁻⁶ M	0	3.00 ± 0	0.25 ± 0	0.53 ± 0.04
	0.48	3.00 ± 0	0.25 ± 0	0.51 ± 0.03
	5.36	4.00 ± 0.30	0.25 ± 0	0.77 ± 0.07
	54.21	6.30 ± 0.20	0.58 ± 0.08	0.93 ± 0.05
	542.71	15.30 ± 1.30	0.94 ± 0.11	1.27 ± 0.03

Values are means ± s.e.mean for six separate tissues. Each treatment was compared to the control value which corresponds to the pretreatment value by using Student's *t* test for paired samples. * *P* < 0.05; ** *P* < 0.01 and *** *P* < 0.005 with respect to corresponding control value.

Figure 5 Dose-response curves to noradrenaline (NA) ($n = 6$) showing the requirement of calcium for the hepoxilin-induced potentiation of NA contraction. (a) Calcium-free medium, (b) normal Krebs solution in the presence of nifedipine. Note that the hepoxilins are ineffective in potentiating the NA effect in a calcium-free medium (a), and that nifedipine in normal Krebs solution causes an inhibition of the hepoxilin effect. When nifedipine is washed out, hepoxilin potentiation returns in the presence of the vehicle for nifedipine (ethanol). Symbols: (a) (■) control pre-hepoxilin A₃ (HxA₃); (□) HxA₃ (8S) 10^{-8} M; (●) control pre-dimethylsulphoxide (DMSO); (○) DMSO vehicle for HxA₃; (b) (■) control, pre-nifedipine and HxA₃; (□) nifedipine ($1 \mu\text{M}$) + HxA₃ (8S) (10^{-8} M); (●) control, pre-ethanol and HxA₃; (○) ethanol + HxA₃ (8S) 10^{-8} M.

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are not yet known. We have shown in neutrophils that the hepoxilin-mediated actions on calcium and second messenger release are inhibited by pertussis toxin (Dho *et al.*, 1990), suggesting that hepoxilin action may be under receptor control coupled to G-protein activation. Further work needs to be done to explore whether the vasculature has receptors for the hepoxilins.

It has been shown that 12-HETE potentiates the vasoconstrictor effect of phenylephrine in rabbit thoracic aortic rings (Masferrer & Mullane, 1988). These authors further showed that the effect of HETE was stereospecific in that only the 12R-isomer was active. It should be noted that the hepoxilins used in this study possess either an epoxide group with a 12S stereochemistry (HxA₃), or a 12S-hydroxyl group (HxA₃-C). Hence the stereochemistry at carbon 12 of the hepoxilins is opposite to that in the active enantiomer of 12-HETE, i.e. 12R. It is interesting that while 12S-HETE was found to be inactive in modulating vascular contraction (Masferrer & Mullane, 1988), the hepoxilins with the 12S stereochemistry were indeed active as long as the hydroxyl group at carbon 8 was in the proper stereochemistry, i.e. in the HxA₃ series, the 8S enantiomer is the active compound while in the HxA₃-C series in which a 12S-hydroxyl group is present, the 8R isomer is the active species. Further correlation of the responses between 12R-HETE and the hepoxilins will be derived by comparing the activity of the hepoxilins with the 12R stereochemistry. This would be worth investigating to determine which part of the molecule is responsible for the biological activity shown in this study.

Hepoxilin action requires calcium. This was shown in the aorta by the lack of effect of hepoxilins in potentiating the NA-induced contraction in calcium-free medium as well as blockade of the hepoxilin-induced potentiation of the NA-induced contraction in the presence of nifedipine. This supports our concept that the hepoxilins affect the smooth muscle cells through a calcium-mediated mechanism, either making calcium available in stores for mobilization by vasoconstrictors or through the compartmentalization and/or release of second messengers for immediate activation by vasoconstrictors. Further studies are in progress to investigate these possibilities.

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