The role of endothelial cells in the relaxations induced by 13-hydroxy- and 13-hydroperoxylinoleic acid in canine arteries

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1 One of the major fatty acids in the arterial wall is linoleic acid. It has been shown that its 13-hydroxy metabolite (13-HODE) is generated in significant amounts by cultured endothelial cells. The aim of the present study was to investigate the relaxations to 13-HODE and its hydroperoxy precursor (13-HPODE) and to examine the role of the endothelial cells.

2 Ring segments of canine circumflex and splenic artery were mounted in organ chambers for isometric tension recording. During contractions induced by prostaglandin $F_{2\alpha}$ or noradrenaline, 13-HODE and 13-HPODE evoked dose-dependent relaxations. Removal of the endothelial cells reduced the relaxations to 13-HODE, but had no effect on those elicited by 13-HPODE.

3 Indomethacin and meclofenamate ($0.3\text{ }\mu\text{M}$ to $30\text{ }\mu\text{M}$) blocked the relaxations evoked by 13-HODE and 13-HPODE in endothelium-denuded rings. In segments with endothelium, both cyclo-oxygenase inhibitors again abolished the relaxations to 13-HODE, but only diminished those to 13-HPODE.

4 Prostacyclin biosynthesis, as measured by radioimmunoassay, increased upon incubation with 13-HODE and 13-HPODE ($10\text{ }\mu\text{M}$). Bioassay of the release of nitric oxide (NO) indicated that NO was not involved in the relaxations elicited by either metabolite. Moreover, L- N^G -nitroarginine ($100\text{ }\mu\text{M}$), a specific inhibitor of NO synthesis, did not influence the relaxations to 13-HODE and 13-HPODE. The responses to 13-HPODE were also not altered by superoxide dismutase.

5 In the splenic artery 13-HPODE and 13-HODE induced contractions above $3\text{ }\mu\text{M}$ which were blocked by the thromboxane receptor antagonist, daltroban. In the circumflex artery contractile responses to high concentrations of 13-HODE could be observed only after inhibition of cyclo-oxygenase.

6 We conclude that the vasodilatation induced by 13-HODE and 13-HPODE was due to stimulation of prostacyclin biosynthesis both in the endothelium and smooth muscle cells or other subendothelial structures. An additional, unidentified intermediate, which was neither NO nor a cyclo-oxygenase product nor superoxide anion, contributed to the relaxations to 13-HPODE in arteries with endothelium.

Keywords: 13-Hydroxylinoleic acid (13-HODE); 13-hydroperoxylinoleic acid (13-HPODE); vascular; endothelium; prostacyclin

Introduction

Arachidonic acid can be converted by vascular smooth muscle cells and endothelial cells to 15-hydroperoxyeicosatetraenoic acid (15-HPETE) which, in turn, is reduced by a peroxidase to 15-hydroxyeicosatetraenoic acid (15-HETE). These metabolites are known to evoke vasoconstriction, spontaneous rhythmic activity and relaxations in isolated blood vessels (Asano & Hidaka, 1979; Trachte *et al.*, 1979; Aharony *et al.*, 1981; Koide *et al.*, 1982; Takahashi *et al.*, 1985; Thomas & Ramwell, 1986; Van Diest *et al.*, 1986, 1991; d'Alarcao *et al.*, 1987; Uotila *et al.*, 1987; Lovelady *et al.*, 1988). Besides arachidonic acid, another major fatty acid in the arterial wall is linoleic acid (Simon *et al.*, 1989), which can be (non-)enzymatically converted to 13-hydroperoxyoctadecadienoic acid (13-HPODE). This labile intermediate is further reduced to its 13-hydroxy derivative (13-HODE) (Buchanan *et al.*, 1985; Setty *et al.*, 1987). The presence of 13-HODE has been demonstrated in rabbit, rat and bovine aorta (Funk & Powell, 1985; De Meyer *et al.*, 1991). It has been proposed that 13-HODE maintains the endothelial lining in a non-adhesive state (Buchanan *et al.*, 1985), but does not seem to play a major role in platelet-subendothelium interaction (De Graaf *et al.*, 1989). Cholesterol-induced fatty streak formation is accompanied by a decrease in the endogenous amounts of esterified 13-HODE and 15-HETE.

The reduction of the esterified 13-HODE content correlates with the severity of the lesions (De Meyer *et al.*, 1991).

Since the vascular effects of 13-HODE and its hydroperoxy precursor are poorly documented, we examined their direct and endothelium-dependent effects in canine isolated arteries, which are very sensitive to the actions of the lipoxygenase metabolites of arachidonic acid (Van Diest *et al.*, 1991), and tried to elucidate their mechanism of action.

Methods

Vascular reactivity

Mongrel dogs (15–35 kg) of either sex were anaesthetized with sodium pentobarbitone (30 mg kg^{-1} , i.v.). The heart was isolated and subsequently the splenic artery and the circumflex branch of the left coronary artery were removed. The tissues were immediately immersed in an oxygenated (95% O_2 –5% CO_2) physiological salt solution. The isolated arteries were cleaned of loose connective tissue and ring segments (3 mm long) were mounted in organ chambers filled with 25 ml physiological salt solution, maintained at 37°C and continuously gassed with 95% O_2 –5% CO_2 (Vanhoutte & Verbeuren, 1976). Tension was measured isometrically with a Statham UC₂ force transducer. Before mounting the tissues in the organ chambers some segments were denuded of the endothelium by rubbing the tip of a pair of forceps against the intimal surface. After an equilibration period of 15 min the preparations were gradually stretched and were

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placed at the optimal point of their length-tension relationship with either 50 mM KCl (Vanhoutte & Verbeuren, 1976) or 2 μ M prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). The resting tension amounted to 9.2 ± 0.8 g for splenic artery segments with endothelium, 9.0 ± 0.5 g for splenic artery segments without endothelium, 10.5 ± 1.0 g for circumflex segments with endothelium and 11.5 ± 1.6 g for circumflex segments without endothelium. Subsequently the segments were allowed to equilibrate for 45 min at their optimal length prior to experimentation.

After raising the tone of the vessels with $PGF_{2\alpha}$, noradrenaline (NA) or KCl, cumulative dose-response curves were constructed for prostacyclin (PGI_2), 13-HODE and 13-HPODE. Intact segments and segments without endothelium were tested in parallel to investigate the role of the endothelial cells. The rings with and without endothelium were precontracted to the same tone. The efficacy of the removal of the endothelium was examined in each ring with acetylcholine (Furchgott & Zawadzki, 1980). The thromboxane receptor antagonist daltroban (BM13505; Lefer, 1988), indomethacin, meclofenamate, L- N^G -nitroarginine (L-NNA) and superoxide dismutase (SOD) were administered 40 min before the addition of prostacyclin, 13-HODE or 13-HPODE.

Bioassay of the release of nitric oxide (NO)

A segment of the canine splenic artery (3 cm long) was used as donor vessel and mounted horizontally in a perfusion chamber filled with physiological salt solution gassed with a mixture of 20% O_2 , 5% CO_2 , 75% N_2 and maintained at 37°C (Bult *et al.*, 1988). It was continuously perfused with the same solution at a rate of 3 ml min⁻¹. The perfusate was dripped onto a segment of the rabbit abdominal aorta of which the endothelium had been mechanically removed. This detector segment was mounted vertically over two hooks for isometric tension recording and its initial tension was gradually set at 8 g. Thereafter, it was contracted by an infusion of NA (0.1 μ M). Atropine (0.1 μ M) and daltroban (1 μ M) were infused above the detector tissue to block contractile effects of acetylcholine and the derivatives of linoleic acid respectively. After an equilibration period of 30 min boluses (0.3, 3 and 30 nmol) of acetylcholine, 13-HPODE and 13-HODE were injected in random order and at 20 min intervals into the physiological salt solution which perfused the donor artery.

Biosynthesis of prostacyclin

Mongrel dogs (18–32 kg) of either sex were anaesthetized with sodium pentobarbitone (30 mg kg⁻¹, i.v.). Subsequently the dogs received heparin (100 units kg⁻¹, i.v.) and 5 min later they were killed. The splenic artery was removed and immediately placed in cold physiological salt solution gassed with 95% O_2 , 5% CO_2 . Adjacent tissues were carefully dissected from the adventitial side and thereafter four ring segments of equal length (1 cm) were cut.

These rings were randomly divided into plastic tubes filled with physiological salt solution (1 ml) continuously gassed with 95% O_2 , 5% CO_2 and maintained at 37°C. After an equilibration period of 30 min, the segments were transferred to fresh physiological salt solution. Fifteen min later this incubation fluid was collected into 30 μ M indomethacin. Subsequently, fresh physiological salt solution, containing respectively 0, 1 or 10 μ M 13-HPODE, or 10 μ M 13-HODE was added and the rings were incubated for another 15 min ($n = 7$ –14 dogs) before collecting a second sample. The amount of prostacyclin in each sample was measured by radioimmunoassay (RIA) of 6-keto-prostaglandin $F_{1\alpha}$ (Bult *et al.*, 1982). A correction for the cross-reactivity of the agonists with the assay (0.012%) was made. At the end of the experiment the rings were placed on Whatman No. 1 filter paper in order to remove adhering water and their wet weight was determined.

Preparation and h.p.l.c. analysis of 13-HPODE and 13-HODE

13-HPODE was prepared by incubation of linoleic acid (10 mg in 100 ml Tris buffer 0.2 M; pH = 9 at 4°C) with 126,000 u soybean lipoxidase type I (linoleate: oxygen oxidoreductase; EC 1.13.11.12) at 4°C. After an incubation period of 30 min a second amount of lipoxidase (126,000 u) was added for another 30 min at 4°C. In order to prepare 13-HODE, 100 mg glutathione was added after the first 30 min and the mixture was incubated during the following 30 min at room temperature. Both linoleic acid derivatives were extracted and purified by thin layer chromatography (TLC) as described (Coene *et al.*, 1986; Van Diest *et al.*, 1991). The purity of the metabolites was investigated by means of high performance liquid chromatography (h.p.l.c.) analysis with a Varian 5000 liquid chromatograph. A Nucleosil C₁₈ reversed-phase column (4.6 $\times$ 250 mm, 5 μ m particles, Alltech Europe, Eke, Belgium) was used and elution (1 ml min⁻¹) was carried out with a mixture of tetrahydrofuran-acetonitrile-water-acetic acid (22:40:38:0.05 by volume) (Engels *et al.*, 1986). 13-HPODE and 13-HODE were monitored at 235 nm in a Varian UV50 variable wavelength detector. They were kept in ethanol at -20°C and stored under nitrogen.

Materials

The physiological salt solution had the following composition (mM): NaCl 118, KCl 4.7, CaCl₂ 2.5, KH₂PO₄ 1.2, MgSO₄ 1.2, NaHCO₃ 25, Ca EDTA 0.025 and glucose 11.1. Acetylcholine, atropine sulphate, glutathione, linoleic acid, L- N^G -nitro arginine, (-)-noradrenaline bitartrate and soybean lipoxidase were obtained from Sigma Chemical Corp., St. Louis, MO, U.S.A. Heparin Leo was from Therabel Pharma, Brussels, Belgium, indomethacin sodium tetrahydrate from Merck, Sharp & Dohme, München, Germany, meclofenamate sodium from Parke Davis, Bornem, Belgium, prostaglandin $F_{2\alpha}$ from Upjohn, Puurs, Belgium and sodium pentobarbitone from Psyphac, Brussels, Belgium. Acetic acid and acetonitrile for h.p.l.c. were purchased from Merck, Darmstadt, Germany, tetrahydrofuran for h.p.l.c. from Lab-Scan, Dublin, Ireland and [³H]-6-keto-prostaglandin $F_{1\alpha}$ from New England Nuclear, Boston, MA, USA. Daltroban, prostacyclin (sodium salt) and bovine recombinant superoxide dismutase were gifts from Boehringer Mannheim, Mannheim, Germany, the Wellcome Foundation, Beckenham, Kent, UK and Grünenthal, Aachen, Germany, respectively.

The drugs were dissolved and diluted in distilled water; noradrenaline was dissolved in an aqueous solution of ascorbic acid (0.01%). Daltroban was dissolved in distilled water with equimolar amounts of NaOH and diluted in distilled water. The stock solutions of 13-HPODE and 13-HODE were diluted in distilled water and kept on ice. The final concentration of ethanol in the organ chambers never exceeded 0.2%. The stock solution of prostacyclin was prepared in 0.1 N NaOH and dilutions were made in Tris buffer (pH = 8.5).

Data analysis

In the organ chamber experiments the tension (g) was measured and expressed as a percentage of the initial contraction induced by $PGF_{2\alpha}$, NA or KCl. The negative logarithm of the concentration of agonist (pD_2 or $-\log EC_{50}$) that produced half of the maximal effect obtained with that agonist was calculated for each ring, by linear regression analysis (Tallarida & Murray, 1981). All data are expressed as means $\pm$ s.e.mean. The number of vessels reported (n) equals the number of dogs used. For statistical analysis, Student's t test for paired observations and analysis of variance (ANOVA) (Sokal & Rohlf, 1981) were used; P values less than 0.05 were considered to be significant.

Results

Vascular reactivity

Prostacyclin PGI₂ (1 nM to 1 µM) induced relaxation of splenic artery rings contracted with PGF_{2α} 1 µM or NA, 0.25 µM. Removal of endothelial cells induced a small leftward shift of the dose-relaxation curve (Table 1). The thromboxane receptor antagonist, daltroban, did not have statistically significant effects (Table 1).

13-HODE and 13-HPODE 13-HODE and 13-HPODE (10 nM to 10 µM) induced dose-dependent relaxations of coronary artery segments with endothelium contracted with 1 µM PGF_{2α} (Figure 1a,b). Removal of the endothelium reduced the dilator response to 13-HODE, but had no significant effect on those induced by 13-HPODE (Figure 1a,b).

In splenic artery segments with and without endothelium contracted with NA (0.25 µM), 13-HPODE induced concentration-dependent relaxations, followed by contractions at higher concentrations. Daltroban (7 µM) abolished this additional contraction and unmasked further relaxation (Figure 2).

To investigate the effect of indomethacin and meclofenamate (0.3 µM to 30 µM) on the relaxations induced by 13-HPODE and 13-HODE in the splenic artery, the concentration of NA was reduced to 60 nM in order to obtain an initial level of contraction similar to the controls. Van Diest *et al.* (1991) demonstrated that indomethacin and meclofenamate enhance contractions to NA in canine arteries. In order to block contractile responses of the linoleic acid metabolites, daltroban was added to the organ chambers. Both indomethacin and meclofenamate diminished the endothelium-dependent relaxations induced by 13-HPODE in a dose-dependent way and abolished those induced by 13-HODE (Table 2). Similar results were obtained with segments of the circumflex contracted with PGF_{2α} (Figure 1a,b). In segments without endothelium, indomethacin (30 µM) completely inhibited the relaxations induced by 13-HPODE and 13-HODE (Figure 1a,b). In the circumflex artery with and without endothelium, contractile responses to high concentrations of 13-HODE could be observed, but only after inhibition of cyclo-oxygenase (Figure 1a).

In both the circumflex and splenic artery L-NNA (100 µM) (Moore *et al.*, 1990) did not significantly influence the relaxa-

tions to 13-HODE and 13-HPODE. In contrast, L-NNA induced about 70% inhibition of the maximum relaxation to acetylcholine (Table 3). Incubation of the circumflex and splenic artery rings with superoxide dismutase (SOD, 20 or 40 U ml⁻¹) did not affect the relaxations to 13-HPODE (Table 4).

Bioassay of the release of NO

Stimulation of the canine splenic artery with increasing doses of acetylcholine (0.3–30 nmol) induced a dose-dependent release of NO. In contrast, neither 13-HPODE nor 13-HODE (0.3–30 nmol) evoked a release of NO from this donor vessel (Table 5). Similar results were obtained when the rabbit thoracic aorta was used as donor vessel (results not shown).

Biosynthesis of prostacyclin

Canine splenic artery segments incubated with an aerated physiological salt solution released PGI₂ under basal conditions as indicated by radioimmunoassay of 6-keto-prostaglandin F_{1α}. There were no differences among the rings regarding the baseline 6-keto-PGF_{1α} biosynthesis in the preincubation period. The biosynthesis of PGI₂ increased upon treatment for 15 min with 10 µM 13-HODE and 10 µM 13-HPODE; as little as 1 µM 13-HPODE tended to augment PGI₂ biosynthesis (Figure 3).

Discussion

We investigated the relaxations to 13-HODE and 13-HPODE in canine isolated arteries and compared them to the relaxations induced by prostacyclin. The tone of the vessels was raised with either PGF_{2α} or NA. In addition, the effects of endothelial removal, cyclo-oxygenase inhibitors, inhibition of NO biosynthesis, and SOD on these relaxations were studied.

During NA-induced contractions, 13-HODE and 13-HPODE evoked relaxations of the splenic artery but high concentrations of these linoleic acid derivatives evoked further contraction. The thromboxane receptor antagonist, daltroban (Lefer, 1988) prevented these additional contractions and unmasked further relaxation. Hence, the highest concentrations of 13-HODE and 13-HPODE seem to have a dual effect: in addition to stimulating prostacyclin biosynthesis they induce

Table 1 Effects of contractile agonist, daltroban (7 µM) and removal of the endothelial cells on prostacyclin-induced relaxation of canine splenic artery segments

	Endothelium present	Endothelium absent
<i>EC</i> ₅₀ (–log M)		
(1) Contraction with PGF _{2α} (1 µM)		
Control	7.47 ± 0.14	7.70 ± 0.12*
(2) Contraction with NA (0.25 µM)		
Control	7.48 ± 0.14	7.98 ± 0.15**
Daltroban	7.51 ± 0.12	7.74 ± 0.10**
<i>Maximum relaxation</i> (%)		
(1) Contraction with PGF _{2α} (1 µM)		
Control	88 ± 10 (i.c. = 6.8 ± 1.7 g)	95 ± 5 (i.c. = 6.2 ± 1.7 g)
(2) Contraction with NA (0.25 µM)		
Control	78 ± 14 (i.c. = 3.3 ± 0.3 g)	95 ± 3 (i.c. = 2.5 ± 0.3 g)
Daltroban	93 ± 6 (i.c. = 2.7 ± 0.3 g)	100 ± 0 (i.c. = 2.5 ± 0.3 g)

Values are shown as means ± s.e.mean for 6 dogs. *EC*₅₀: concentration producing half of the maximum relaxation of segments contracted with prostaglandin F_{2α} (PGF_{2α}) or noradrenaline (NA) respectively. i.c.: initial contraction.

P* < 0.05; *P* < 0.01; significantly different from segments with endothelium (ANOVA).

Figure 1 (a) Effect of 30 μM indomethacin on the relaxations to 13-hydroxylinoleic acid (13-HODE) in segments of canine circumflex coronary artery contracted with prostaglandin $\text{F}_{2\alpha}$ ($\text{PGF}_{2\alpha}$, 2 μM). Responses are shown as means with s.e.mean (vertical lines), and expressed as a percentage of the initial contraction. 100% = 3.0 $\text{g} \pm 0.5$ g for control segments with endothelium ($\circ$), 3.5 $\text{g} \pm 0.5$ g for control segments without endothelium ($\square$), 3.3 $\text{g} \pm 0.9$ g for indomethacin treated segments with endothelium ($\bullet$) and 3.2 $\text{g} \pm 1.1$ g for indomethacin-treated segments without endothelium ($\blacksquare$). * $P < 0.05$, (control versus indomethacin-treated), * $P < 0.05$ (with endothelium versus without endothelium), Student's t test (paired, two-tailed), $n = 6$. (b) Effect of 30 μM indomethacin on the relaxations to 13-hydroperoxylinoleic acid (13-HPODE) in segments of canine circumflex coronary artery contracted with $\text{PGF}_{2\alpha}$ (2 μM). Responses are shown as means with s.e.mean (vertical lines) and expressed as a percentage of the initial contraction. 100% = 3.0 $\text{g} \pm 0.4$ g for control segments with endothelium ($\circ$), 3.2 $\text{g} \pm 0.5$ g for control segments without endothelium ($\square$), 3.1 $\text{g} \pm 0.5$ g for indomethacin-treated segments with endothelium ($\bullet$), 3.2 $\text{g} \pm 0.4$ g for indomethacin-treated segments without endothelium ($\blacksquare$). * $P < 0.05$, (control versus indomethacin-treated), Student's t test (paired, two-tailed), $n = 7$.

contraction by activating thromboxane receptors, similar to 15-HETE and 15-HPETE (Van Diest *et al.*, 1991). In the circumflex artery the relaxant responses prevailed and contractile responses to 13-HODE were observed only after treatment with indomethacin. Presumably, this is due to inhibition of endogenous prostacyclin biosynthesis, thereby tipping the balance in favour of the contractile effect of thromboxane receptor activation by 13-HODE. Daltroban could not be used to verify this assumption, since the circumflex arteries were contracted with $\text{PGF}_{2\alpha}$ and thromboxane receptor antagonists suppress the contractile responses to $\text{PGF}_{2\alpha}$ in a competitive manner (Van Diest *et al.*, 1991).

In further experiments, we studied the role of the endothelial cells on relaxations. In both circumflex and splenic artery, removal of the endothelial cells reduced the relaxations in response to 13-HODE. This suggests an involvement of endothelium-derived vasodilators such as PGI_2 (Moncada

Figure 2 Effect of the thromboxane receptor antagonist, daltroban (7 μM) on the relaxations to 13-hydroperoxylinoleic acid (13-HPODE) in segments of canine splenic arteries contracted with noradrenaline (0.3 μM). Responses are shown as means with s.e.mean (vertical lines) and expressed as percentage of the initial contraction. 100% = 3.6 $\text{g} \pm 0.3$ g for control segments with endothelium ($\circ$), 4.0 $\text{g} \pm 0.7$ g for control segments without endothelium ($\square$), 3.3 $\text{g} \pm 0.5$ g for daltroban-treated segments with endothelium ($\bullet$) and 4.5 $\text{g} \pm 1.0$ g for daltroban-treated segments without endothelium ($\blacksquare$). * $P < 0.05$, Student's t test (paired, two-tailed), $n = 6$.

Table 2 Effect of indomethacin (Indo) and meclofenamate (Meclo) on 13-hydroxylinoleic acid (13-HODE) and 13-hydroperoxylinoleic acid (13-HPODE)-induced relaxation of canine splenic artery segments with endothelium

Relaxation (%)	13-HODE (10 μM) ($n = 5$)	13-HPODE (10 μM) ($n = 6$)
Control	43 $\pm$ 5 (i.c. = 5.3 $\pm$ 0.6 g)	63 $\pm$ 5 (i.c. = 6.8 $\pm$ 0.4 g)
Indo 0.3 μM	20 $\pm$ 4* (i.c. = 6.5 $\pm$ 1.2 g)	ND
Indo 3 μM	9 $\pm$ 4* (i.c. = 6.8 $\pm$ 1.2 g)	36 $\pm$ 4* (i.c. = 7.3 $\pm$ 0.7 g)
Indo 30 μM	ND	16 $\pm$ 6* (i.c. = 5.5 $\pm$ 1.0 g)
Meclo 0.3 μM	12 $\pm$ 6* (i.c. = 6.2 $\pm$ 1.0 g)	ND
Meclo 3 μM	9 $\pm$ 9* (i.c. = 5.5 $\pm$ 1.3 g)	31 $\pm$ 10* (i.c. = 8.7 $\pm$ 1.5 g)
Meclo 30 μM	ND	12 $\pm$ 5* (i.c. = 6.7 $\pm$ 0.9 g)

Values are shown as means $\pm$ s.e.mean. The segments were contracted with noradrenaline in the presence of daltroban (7 μM). In the rings treated with a cyclooxygenase inhibitor the concentration of noradrenaline was diminished in order to obtain a level of initial contraction similar to the controls. i.c.: initial contraction; ND: not done. * $P < 0.05$ (ANOVA, Student-Newman-Keuls test).

& Vane, 1979) and/or NO (Furchgott & Zawadzki, 1980; Palmer *et al.*, 1987). It has been reported that in the canine coronary artery, the relaxations induced by 15-HETE and 15-HPETE are impaired by cyclo-oxygenase inhibitors (Takahashi *et al.*, 1985; Van Diest *et al.*, 1991). Likewise, in rings with endothelium, the relaxations were abolished (13-HODE) or diminished (13-HPODE) by indomethacin and meclofenamate. Both cyclo-oxygenase inhibitors also completely inhibited the relaxations to 13-HODE and 13-HPODE in endothelium-free segments. These results suggest that the relaxations to 13-HODE and the major part of the relaxations induced by 13-HPODE were due to stimulation of endothelial and subendothelial biosynthesis of a cyclo-oxy-

Table 3 Effect of L-N^G-nitro arginine (L-NNA) on the amplitude of 13-hydroxylinoleic acid (13-HODE), 13-hydroperoxylinoleic acid (13-HPODE) and acetylcholine (ACh)-induced relaxations of canine splenic artery and circumflex segments with endothelium

Relaxation (%)	13-HODE (10 μ M)	13-HPODE (10 μ M)	ACh (1 μ M) + Indo 3 μ M
<i>Splenic artery</i> (n = 3)			
Control (i.c. = 5.4 $\pm$ 0.8 g)	63 $\pm$ 10	66 $\pm$ 5	99 $\pm$ 1
L-NNA 100 μ M (i.c. = 6.9 $\pm$ 0.9 g)	71 $\pm$ 5	78 $\pm$ 10	28 $\pm$ 2*
<i>Circumflex</i> (n = 3)			
Control (i.c. = 6.4 $\pm$ 1.7 g)	63 $\pm$ 21	59 $\pm$ 10	95 $\pm$ 3
L-NNA 100 μ M (i.c. = 7.2 $\pm$ 1.2 g)	59 $\pm$ 20	50 $\pm$ 11	34 $\pm$ 4*

Values are shown as means $\pm$ s.e.mean. The segments were contracted with noradrenaline 0.3 μ M in the presence of daltroban 7 μ M (splenic artery) or prostaglandin F_{2 α} (PGF_{2 α}) 2 μ M (circumflex). In the inhibitor-treated rings, the concentration of noradrenaline and PGF_{2 α} was diminished in order to obtain a level of initial contraction similar to the controls. i.c.: initial contraction.

* $P < 0.05$ (paired t test).

Table 4 Effect of superoxide dismutase (SOD) on 13-hydroperoxylinoleic acid (13-HPODE)-induced relaxation of canine splenic artery and circumflex segments

	<i>Splenic artery</i> (n = 7)	<i>Circumflex</i> (n = 6)
<i>EC₅₀ (-log M)</i>		
Control	5.86 $\pm$ 0.08	5.91 $\pm$ 0.05
SOD 20 u ml ⁻¹	5.99 $\pm$ 0.06	6.00 $\pm$ 0.08
SOD 40 u ml ⁻¹	5.95 $\pm$ 0.08	6.00 $\pm$ 0.02
<i>Maximum relaxation (%)</i>		
Control	84 $\pm$ 3	58 $\pm$ 5
	(i.c. = 5.7 $\pm$ 0.9 g)	(i.c. = 5.6 $\pm$ 0.8 g)
SOD 20 u ml ⁻¹	75 $\pm$ 8	61 $\pm$ 9
	(i.c. = 5.0 $\pm$ 1.1 g)	(i.c. = 5.3 $\pm$ 0.9 g)
SOD 40 u ml ⁻¹	76 $\pm$ 11	87 $\pm$ 7
	(i.c. = 6.2 $\pm$ 1.2 g)	(i.c. = 4.8 $\pm$ 0.3 g)

Values are shown as means $\pm$ s.e.mean. EC₅₀: concentration producing half of the maximum relaxation of segments contracted with noradrenaline 0.3 μ M in the presence of daltroban 7 μ M (splenic artery) or PGF_{2 α} 2 μ M (circumflex); i.c. = initial contraction. The effect of SOD was not significant (ANOVA, Student-Newman-Keuls test).

Table 5 Bioassay of NO induced by acetylcholine, 13-hydroxylinoleic acid (13-HODE) and 13-hydroperoxylinoleic acid (13-HPODE)

Agonist	Bolus (nmol)	Relaxation (%) (n = 5)
Acetylcholine	0.3	39 $\pm$ 8
	3	45 $\pm$ 9
	30	56 $\pm$ 5
13-HODE	0.3	6 $\pm$ 5
	3	1 $\pm$ 1
	30	2 $\pm$ 2
13-HPODE	0.3	0 $\pm$ 1
	3	1 $\pm$ 1
	30	3 $\pm$ 3

The canine splenic artery was used as donor vessel and the rabbit abdominal aorta denuded of endothelial cells served as detector tissue. Data are given as percentage relaxation $\pm$ s.e.mean of the detector tissue.

genase-derived vasodilator. Incubation experiments indicated that 13-HODE (10 μ M) and 13-HPODE (10 μ M) did indeed stimulate the biosynthesis of PGI₂. This is in agreement with the finding that 13-HODE stimulates the production of PGI₂ in cultured endothelial cells (Setty *et al.*, 1987). It has also

been reported that in human umbilical vein endothelial cell monolayers, 13-HPODE, up to 20 μ M, stimulated the formation of PGI₂, PGI₃ and dihomoprostacyclin from endogenous arachidonate or exogenous eicosapentaenoic acid and docosahexaenoic acid, respectively (Bordet *et al.*, 1988). In our experiments the concentration of 13-HPODE was kept below 20 μ M. Above that level 13-HPODE inhibits PGI₂ biosynthesis, presumably by destroying PGH₂ synthase activity (Hennig *et al.*, 1986; Bordet *et al.*, 1988), the rate limiting step in the formation of PGI₂ from arachidonate (De Witt *et al.*, 1983) and may act as a detergent in cell membranes (Hennig *et al.*, 1986).

It is possible that some of the effects of 13-HPODE may be due to its conversion to 13-HODE in the organ bath. However, 13-HPODE elicited an additional indomethacin- and meclofenamate-resistant, but endothelium-dependent relaxation. The possibility that this part of the relaxation to 13-HPODE was due to stimulation of the release of NO, was ruled out by both the bioassay and the experiments with L-NNA. L-NNA is a potent, L-arginine-reversible inhibitor of endothelial NO biosynthesis (Moore *et al.*, 1990). As expected, 13-HODE did not evoke release of NO either.

Since in arteries with endothelium the 13-HPODE-induced relaxations appeared to be partly due to an intermediate different from a cyclo-oxygenase product or NO, we investigated the possibility that these relaxations were mediated by superoxide anions or superoxide-dependent relaxing factor(s)

Figure 3 Influence of 13-hydroxylinoleic acid (13-HODE) and 13-hydroperoxylinoleic acid (13-HPODE) on the biosynthesis of prostacyclin by rings of the canine splenic artery. Prostacyclin production was determined after 15 min incubation before (left hand open columns) and after addition (right hand columns) of 0 or 10 μM 13-HODE ($n = 14$) or 0, 1 or 10 μM 13-HPODE ($n = 7$). Results are expressed in pg 6-keto-PGF_{2 α} mg⁻¹ tissue as means $\pm$ s.e. mean (vertical bars). * $P < 0.05$; ** $P < 0.01$, Student's t test (paired, two-tailed).

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from endothelial cells (Hong *et al.*, 1989). However, SOD, which destroys superoxide anions (McCord & Fridovich, 1969) failed to diminish the relaxations to 13-HPODE. This argues against a role of superoxide anion in the 13-HPODE induced relaxations, although it cannot be ruled out that SOD failed to diffuse into the intercellular spaces of the tissue.

In conclusion, 13-HODE and 13-HPODE induce relaxations in canine splenic and coronary arteries that are due to stimulation of PGI₂ biosynthesis, both in the endothelium and smooth muscle cells or other subendothelial structures. For 13-HPODE, an additional intermediate contributes to the relaxations of arteries with endothelium. The nature of this mediator, which is neither NO, nor a cyclo-oxygenase product, and is unlikely to be superoxide anion, is at present unknown.

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