Biochemical Pharmacology, Vol. 42, No. 2, pp. 279–283, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

EARLY ATHEROSCLEROSIS IS ACCOMPANIED BY A DECREASED RATHER THAN AN INCREASED ACCUMULATION OF FATTY ACID HYDROXYDERIVATIVES

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(*Received* 31 *October* 1990; *accepted* 3 *March* 1991)

Abstract—The content of 13-hydroxylinoleic acid (13-HODE) and 15-hydroxyarachidonic acid (15-HETE) in the rabbit thoracic aorta was measured using high performance liquid chromatography after chronic exposure to cholesterol and a high dose of molsidomine, a donor of nitric oxide (NO). Cholesterol-induced fatty streak formation was accompanied by a decrease in the amounts of esterified 13-HODE and 15-HETE. The reduction of the esterified 13-HODE content correlated significantly with the severity of the lesions. These results do not support the hypothesis that fatty acid hydroperoxides accumulate in the arterial wall during atherosclerosis. On the other hand, the quantity of esterified 13-HODE and 15-HETE was increased markedly after exposure to molsidomine. The high dose of this agent could have initiated radical reactions (via liberation of NO and production of superoxide anions) thereby leading to a raise of the 13-HODE and 15-HETE content of the vessel.

During early fatty streak formation endothelial prostacyclin biosynthesis augments [1], whereas at later stages endothelial biosynthesis of prostacyclin and the vasodilating endothelium-derived relaxing factor (EDRF) becomes progressively suppressed [1, 2]. This decreased prostacyclin biosynthesis has been attributed to the presumed accumulation of lipid hydroperoxides [3] and the detection of malondialdehyde positive material in a few atherosclerotic lesions [4] has led to the generalized assumption that lipid hydroperoxides accumulate in atherosclerotic vessels (e.g. Ref. 3).

If fatty acid or lipid hydroperoxides indeed accumulate in atherosclerotic vessels, this should be reflected by increased amounts of the hydroxymetabolites derived from these unstable intermediates. One of the major fatty acids in the arterial wall is linoleic acid [5], which can be (non)enzymatically converted to 13-hydroperoxylinoleic acid (13-HPODE). This labile intermediate is further reduced to its 13-hydroxy derivative (13-HODE). Likewise, 15-hydroxyeicosatetraenoic acid (15-HETE) is derived from arachidonic acid via a 15-hydroperoxyprecursor. Although the synthesis of hydroxy fatty acids by hypercholesterolaemic aortae has been studied after incubation of the arteries with linoleic or arachidonic acid [5–7], no data are available about the amount of hydroxy fatty acids derived from endogenous substrates in atherosclerotic vessels. In the present study the content of 13-HODE and 15-HETE in normal aortae and aortae from cholesterol-fed rabbits was determined using high pressure liquid chromatography (HPLC). Measurement of these hydroxy fatty acids may provide a good indication of the levels of their possibly noxious hydroperoxyprecursors [8].

Since the formation of EDRF, which is most likely NO or a nitrosoderivative releasing NO [9, 10], is suppressed in atherosclerotic vessels [2] the effect of supplementation with a donor of NO on the 13-HODE and 15-HETE content in aorta was also investigated. Molsidomine was selected as a source of NO, because its hepatic metabolite 3-morpholinosydnonimine (SIN-1) releases NO without the development of tolerance [11].

MATERIALS AND METHODS

Induction and evaluation of fatty streaks. Male New Zealand white rabbits (2.34 ± 0.02 kg, N = 40) were divided randomly in four groups. These received standard chow (group 1), a diet supplemented with 0.3% cholesterol (group 2), a diet with 0.02% molsidomine (group 3) or a diet containing both molsidomine and cholesterol (group 4). The animals received 150 g of diet daily.

After 16 weeks the rabbits were anaesthetized with sodium pentobarbital (30 mg/kg, i.v.). A blood sample was taken for determination of plasma cholesterol and triglyceride levels. Subsequently the rabbits were killed. The thoracic aorta was carefully removed and immediately placed in a gassed (95% oxygen–5% carbon dioxide) physiological salt solution. The vessel was cleaned of adhering connective tissue and the distal part (about 2.5 cm long) was opened longitudinally and the occurrence of fatty streaks on its luminal surface was evaluated on a scale from zero to five, using binocular magnification. A score of zero was attributed to segments showing no visible lesions, a segment completely covered by fatty streaks received a score of five. This macroscopic method correlates significantly with planimetrical evaluation of the lesions [1, 2]. Each segment was evaluated by two persons blinded to experimental conditions. Next

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the wet weight of the segment was determined. During the tissue preparation care was taken to preserve the endothelial cells. The aortae were stored at −20° under nitrogen in small sealed vials containing 1.5 mL methanol with 0.05% butylated hydroxytoluene (BHT) as antioxidant [8].

Extraction and quantification of esterified and non-esterified 13-HODE and 15-HETE. After addition of the internal standard [2 nmol 15-hydroxy-11,13-eicosadienoic acid (15-HEDE)] [12] the tissues were homogenized with a Tissue Tearer™ (Biospec Products, Bartlesville, OK, U.S.A.) at 25,000 rpm in 2 mL of ice-cold hexane:isopropanol (3:2) containing 0.05% BHT [8, 13]. The lipids were extracted three times with hexane:isopropanol (3:2). The extracts were centrifuged (800 *g*, 5 min). The supernatants and the 1.5 mL methanol in which the tissues had been stored were combined and divided into two equal volumes. Subsequently, the solvent was evaporated under argon. The residue of one part was dissolved in 95% ethanol to quantify non-esterified 13-HODE. The other part was dissolved in 0.28 N potassium hydroxide in 95% ethanol. In this solution hydrolysis of lipids was carried out for 45 min at 55° under argon [8]. Subsequently, distilled water was added to both ethanolic solutions till a final concentration of 15% ethanol was attained [14]. The solutions were then acidified (HCl, pH 3–4) for solid phase extraction with 1 mL octadecylsilyl (C_{18}) silica cartridges (Bond Elut, Analytichem International, Harbor City, CA, U.S.A.). The latter had been conditioned with 2 × 1 mL ethanol, followed by 2 × 1 mL water. After successive washes with 1 mL 15% ethanol, 1 mL water and 2 × 1 mL petroleum ether, the monohydroxy fatty acids were eluted with 1 mL petroleum ether:chloroform (1:1). Thereafter they were quantified by reversed phase HPLC as described later. Peak areas were integrated and the amount of 13-HODE and 15-HETE determined using internal standard calculation. Similar results were obtained when peak height was measured.

Preparation of standards. 13-HODE, 15-HETE and the internal standard (15-HEDE) were prepared by incubation of, respectively, linoleic acid, arachidonic acid, and 11,14-*cis*-eicosadienoic acid (10 mg in 100 mL Tris buffer 0.2 M; pH 9 at 4°) with soybean lipoxydase type I (linoleate: oxygen oxidoreductase; EC 1.13.11.12) (126,000 units; 4°; 30 min), followed by reduction with 100 mg gluthatione (20°; 1 hr). The metabolites were extracted and purified by thin layer chromatography as described [15]. Next, preparative reversed-phase HPLC was performed with a Varian 5000 liquid chromatograph. A Nucleosil C_{18} column (4.6 × 250 mm, 5 μm particles, Alltech Europe, Eke, Belgium) was used and elution (1 mL/min) was carried out with tetrahydrofuran–acetonitrile–water–acetic acid (22:40:38:0.05, by vol.) [16]. 13-HODE, 15-HETE and 15-HEDE were monitored at 235 nm in a Varian UV50 variable wavelength detector. The standards were kept in ethanol at −20°.

Enzymatic assay of total cholesterol. The extracts of aorta in which 13-HODE and 15-HETE had been measured could not be used to determine the cholesterol content of the vessel, because the BHT present in these extracts interfered with the enzymatic assay of cholesterol. Therefore, the total cholesterol content of an adjacent segment was measured. The tissue was homogenized with a Tissue Tearer™ and extracted three times with chloroform:methanol (2:1). This organic phase was consecutively washed with 0.88% KCl and methanol:water (1:1). Then, the solvent was evaporated, the residue dissolved in isopropanol and the cholesterol measured enzymatically [17].

Materials. The four diets were obtained from Altromin, Lage, F.R.G. Arachidonic acid, linoleic acid, 11,14-*cis*-eicosadienoic acid, butylated hydroxytoluene and soybean lipoxygenase were from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Acetonitrile for HPLC, chloroform p.a., ethanol p.a., hexane p.a., hydrochloric acid p.a., isopropyl alcohol p.a., methanol p.a. and petroleum ether p.a. (boiling range 100–140°) were purchased from Merck (Darmstadt, F.R.G.); tetrahydrofuran for HPLC was from LabScan (Dublin, Ireland); potassium hydroxide highest purity from UCB (Brussels, Belgium) and sodium pentobarbital from Psyphac (Brussels, Belgium). The physiological salt solution contained (mM) NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, Ca-EDTA 0.025 and glucose 11.1.

Data analysis. All data are given as the mean ± SEM. The number of aortic segments reported (N) equals the number of rabbits used. A two-way analysis of variance (ANOVA) was used to analyse the data [18]. Regression lines were computed by means of the method of the least squares [18]. The SPSS/PC$^+$ package (SPSS, Chicago, IL, U.S.A.) was applied for these purposes. A 5% level of significance (2-tailed) was selected.

RESULTS

Plasma cholesterol levels, plasma triglyceride levels, total cholesterol content and macroscopic evaluation of fatty streaks

In most rabbits receiving a high cholesterol diet the total plasma cholesterol levels and the total cholesterol content of the aorta were clearly increased as compared to the control values. The plasma triglyceride concentrations were not significantly affected by the hypercholesterolaemia. Addition of a NO-donor to the diet neither altered the plasma cholesterol, nor the triglyceride levels, nor the total cholesterol content of the aorta (Table 1).

Fatty streaks were not observed in aortae which had been repeatedly exposed to an exogenous source of NO (group 3). This was also the case for the control group, except for one rabbit whose aorta contained a few small fatty streaks. On the other hand, in those rabbits in which cholesterol feeding led to hypercholesterolaemia fatty streaks were clearly visible. The macroscopic score attributed to the lesions correlated significantly with the level of plasma cholesterol (correlation coefficient = 0.76, $P < 0.001$, $N = 19$) and with the total cholesterol content of the vessel (correlation coefficient = 0.63, $P = 0.004$, $N = 19$).

Amounts of 13-HODE and 15-HETE

In the control group the amount of non-esterified

Table 1. Plasma cholesterol levels, plasma triglyceride levels, total cholesterol content and macroscopic evaluation of fatty streaks in the rabbit thoracic aorta

Group/diet	Plasma cholesterol (mg/dL)	Plasma triglycerides (mg/dL)	Total cholesterol (μmol/g tissue)	Visual score of fatty streaks (scale: 0–5)*
1. Control	26 ± 3	27 ± 11	5.5 ± 0.4	0.1 ± 0.1
2. Cholesterol	706 ± 177	159 ± 134	23.7 ± 8.5	2.4 ± 0.6
3. NO-donor	22 ± 4	16 ± 5	6.0 ± 0.8	0.0
4. Cholesterol + NO-donor	970 ± 192	86 ± 42	51.9 ± 13.6	2.8 ± 0.5
Significance of factors in analysis of variance:				
Cholesterol	$P < 0.001$	NS	$P < 0.001$	$P < 0.001$
NO-donor	NS	NS	NS	NS
Interaction	NS	NS	NS	NS

Values shown as mean ± SEM for at least nine rabbits in each group.
NS, not significant.
* Scale: 0–5 [2]:
0: no visible fatty streaks; 1: only a few, focal points of small fatty streaks; 2: a few fatty streaks spread over the intimal surface; 3: a lot of small fatty streaks which are not in contact with each other; 4: fatty streaks separated by little spots of "intact" intimal surface; 5: complete surface is covered by fatty streaks.

Table 2. Amount of 13-HODE and 15-HETE in rabbit aorta

	13-HODE (nmol/g tissue)		15-HETE (nmol/g tissue)	
Group/diet	Free	Esterified	Free	Esterified
1. Control	0.4 ± 0.1	6.2 ± 0.9	0.4 ± 0.1	6.6 ± 2.1
2. Cholesterol	0.6 ± 0.2	2.2 ± 0.4	0.8 ± 0.4	3.9 ± 1.2
3. NO-donor	0.5 ± 0.1	22.1 ± 5.5	0.7 ± 0.2	15.0 ± 3.5
4. Cholesterol + NO-donor	0.4 ± 0.1	12.2 ± 4.2	0.2 ± 0.1	4.6 ± 1.1
Significance of factors in analysis of variance:				
Cholesterol	NS	$P = 0.002$	NS	$P = 0.005$
NO-donor	NS	$P < 0.001$	NS	$P = 0.044$
Interaction	NS	NS	NS	NS

Values are given as mean ± SEM for at least eight rabbits in each group.
NS, not significant.

13-HODE and 15-HETE was 0.4 ± 0.1 nmol/g tissue. Comparable quantities were present in aortae of groups 2, 3 and 4 (Table 2).

The majority of 13-HODE and 15-HETE was, however, present in esterified form. The content of these monohydroxy fatty acids was significantly decreased in aortae of hypercholesterolaemic rabbits (group 2; Fig. 1; Table 2). The amount of esterified 13-HODE displayed a negative correlation with the degree of fatty streak formation (correlation coefficient = −0.76, $P = 0.018$) and the total cholesterol content of the vessel (correlation coefficient = −0.83, $P = 0.006$) (Fig. 2). On the other hand, the quantity of esterified 13-HODE and 15-HETE augmented markedly after repeated exposure to exogenous NO (group 3). The aortae of rabbits treated with the combination (group 4) contained intermediate levels of esterified 13-HODE and 15-HETE (Fig. 1; Table 2).

DISCUSSION

Feeding a cholesterol-rich diet to rabbits results in hypercholesterolaemia together with the formation of experimental atherosclerotic lesions, which bear a superficial resemblance to the fatty streaks observed in certain human arteries [1, 2]. The present study quantified 13-HODE and 15-HETE derived from endogenous substrates, both in normal and atherosclerotic rabbit aortae. The major part of these hydroxy fatty acids was present in esterified form. The amount of 13-HODE in the control aortae was comparable to that reported earlier [8]. Surprisingly, the esterified 13-HODE and 15-HETE content of atherosclerotic aortae was significantly decreased. Moreover, the amount of esterified 13-HODE in these vessels correlated negatively with the total cholesterol content and the degree of fatty streaks. Hence, these results do not support the hypothesis

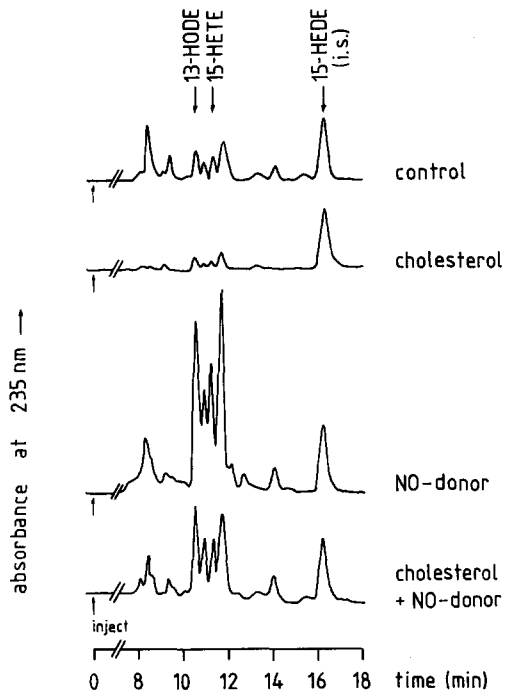

Fig. 1. Representative reversed phase chromatograms of thoracic aorta extracts. The aorta segments were taken from rabbits fed for 16 weeks with control diet, a cholesterol-rich diet, a diet with a NO-donor and a diet containing cholesterol plus a NO-donor. 13-HODE and 15-HETE (esterified + non-esterified) were monitored at 235 nm. 15-Hydroxy-11,13-eicosadienoic acid (15-HEDE) was used as internal standard (i.s.) to correct for losses during the extraction procedure.

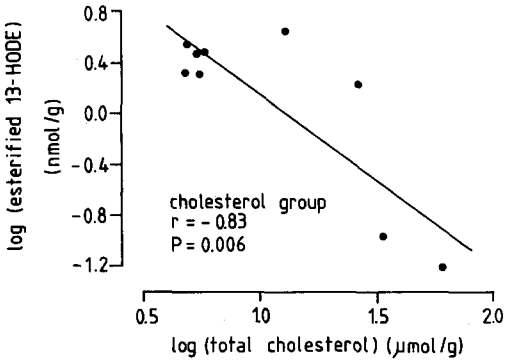

Fig. 2. Correlation between the amount of esterified 13-HODE (nmol/g tissue) and the total cholesterol content (μmol/g tissue) in the thoracic aorta obtained from 16-week hypercholesterolaemic rabbits.

that fatty acid hydroperoxides accumulate in atherosclerotic vessels, but suggest that a diminished 13-HODE content may be an index of vascular injury. Several explanations are conceivable for the decreased amounts of esterified 13-HODE and 15-HETE in vessels from hypercholesterolaemic rabbits. It is possible that the availability of linoleic and arachidonic acid is diminished. However, in aortae of rabbits fed an atherogenic diet a positive correlation between the amount of these fatty acids and atheromatous lesions has been reported [19]. A decreased activity of the 15-lipoxygenase or a diminished non-enzymatic oxidation *in vivo* forms another explanation. Arterial endothelium appears to have a role in fatty acid oxidation as well, because it has been demonstrated that vascular endothelial cell denudation by forceps or cotton swab caused a decrease in oxidation of both oleic and arachidonic acid [20]. Previously, the conversion of radiolabeled fatty acids into radiolabeled unesterified metabolites has been investigated [5–7]. In these studies the synthesis of 13-HODE from linoleic acid and 15-HETE from arachidonic acid was observed in aortae from hypercholesterolaemic rabbits, but not in normal rabbit aortae. However, the major difference with the present study is that in the former investigations the rate of conversion of *exogenous* fatty acids was measured. Possibly, the availability of endogenous linoleic acid and arachidonic acid is diminished in aortae from hypercholesterolaemic rabbits. Furthermore, exogenous added arachidonic acid or linoleic acid may be more available or susceptible to the activity of a 15-lipoxygenase or non-enzymatic oxidation. In addition, in the present study the *esterified* 13-HODE and 15-HETE content of atherosclerotic aortae was decreased, whereas in the referenced studies unesterified metabolites were measured.

Since the formation of NO is suppressed in atherosclerotic vessels, the second purpose of the present investigation was to study the effect of supplementation with a donor of NO on the 13-HODE and 15-HETE content in aorta. Molsidomine was selected for this purpose. Its liver metabolite SIN-1 releases NO spontaneously in the presence of oxygen. The latter is thereby converted to superoxide anion ($O_2^{\cdot-}$) [21]. Hence, SIN-1 is not only a donor of NO, but also of superoxide anions. The formation of these radicals ($O_2^{\cdot-}$, $NO^{\cdot}$) may explain the increased amounts of esterified 13-HODE and 15-HETE in aortae or rabbits exposed to the high dose of molsidomine. Since free hydroxy fatty acids are not well incorporated into either vascular endothelial and smooth muscle cells [8], it is most likely that esterified linoleic or arachidonic acid is oxygenated by these highly active species to form, respectively, esterified 13-HODE and 15-HETE via their hydroperoxy precursor. The effects of cholesterol and the NO-donor on the levels of esterified 13-HODE and 15-HETE were additive, as indicated by the absence of interaction.

The amount of free 13-HODE and 15-HETE seemed not to be altered by any of the diets. This is possibly due to the fact that only a very small part of linoleic and arachidonic acid is present in the non-esterified form [8] and consequently changes in the 13-HODE and 15-HETE content are difficult to detect. Moreover, since there was limited availability of tissue (about 2 × 50 mg per segment) the amount of free 13-HODE and 15-HETE was near the detection limit of the assay (0.01 nmol).

In summary, in this study we have demonstrated that cholesterol-induced fatty streak formation was accompanied by a decrease, rather than an increase, of the amounts of endogenous esterified 13-HODE and 15-HETE in the rabbit thoracic aorta. The esterified 13-HODE content displayed a negative correlation with the severity of the lesions. Since measurement of 13-HODE and 15-HETE may provide a good indication of the level of their respective hydroperoxyprecursors, our results do not support the hypothesis that fatty acid hydroperoxides accumulate in the arterial wall during early atherosclerosis. On the other hand, repeated exposure to a high dose of molsidomine (an exogenous donor of NO, but also of superoxide anions) could have initiated radical reactions thereby leading to an increase in the amount of hydroperoxy fatty acids, as reflected by the raise of the 13-HODE and 15-HETE content of the vessel.

Acknowledgements—We wish to thank Mrs Rita Van den Bossche and Mrs Hermine Fret for the determination of cholesterol and triglyceride levels. We are also grateful to Mrs Liliane Van den Eynde and Mrs Lydie Van Laerhoven for typing the manuscript.

G.R.Y. De Meyer is a Research Assistant of the National Fund for Scientific Research, Belgium. This study was supported by FGWO Grant No. 3.0015.89.

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