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Gas chromatography–mass spectrometry of *cis*-9,10-epoxyoctadecanoic acid (*cis*-EODA) I. Direct evidence for *cis*-EODA formation from oleic acid oxidation by liver microsomes and isolated hepatocytes

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Abstract

Oleic acid, *cis*-9-octadecenoic acid, is the major fatty acid in mammals. Its oxide, *cis*-9,10-epoxyoctadecanoic acid (*cis*-EODA), has been identified in blood and urine of humans, its origin is, however, still unknown. Lipid peroxidation and enzyme-catalyzed epoxidation of oleic acid are two possible sources. In the present article, we investigated by HPLC and GC–MS whether *cis*-EODA is formed enzymatically from oleic acid by the cytochrome P450 (CYP) system. Oleic acid, *cis*-EODA and its hydratation product *threo*-9,10-dihydroxyoctadecanoic acid (*threo*-DiHODA) were quantitated by HPLC as their *p*-bromophenacyl esters. For structure elucidation by GC–MS, the pentafluorobenzyl (PFB) esters of these compounds were isolated by HPLC and converted to their trimethylsilyl ether derivatives. Liver microsomes of rats, rabbits and humans oxidized oleic acid into *cis*-EODA. This is the first direct evidence for the enzymatic formation of *cis*-EODA from oleic acid. The epoxidation of oleic acid was found to depend on CYP, $NADPH+H^+$, and O_2. *cis*-EODA was measurable in incubates of liver microsomes for up to 30 min of incubation. Maximum *cis*-EODA concentrations were reached after 5–7 min of incubation and found to depend upon oleic acid concentration. Isolated rat hepatocytes hydratated *cis*-EODA into *threo*-DiHODA which was further converted to unknown metabolites. However, from incubation of oleic acid with these cells we could not detect *threo*-DiHODA or *cis*-EODA. Our study suggests that circulating and excretory *cis*-EODA may originate, at least in part, from CYP-catalyzed epoxidation of oleic acid. GC–MS of intact *cis*-EODA as its PFB ester in the negative-ion chemical ionization mode should be useful in investigating the physiological role of *cis*-EODA in man.

Keywords: *cis*-9,10-Epoxyoctadecanoic acid; Oleic acid

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1. Introduction

Fatty acids, prostaglandins and leukotrienes are endogenous substrates for monooxygenases [1,2]. Microsomal cytochrome P450 (CYP) monooxygenases catalyze the ω- and (ω−1)-oxidation of fatty acids in the presence of O_2 and $NADPH+H^+$ [3]. In addition to ω- and (ω−1)-oxidation products, unsaturated fatty acids are converted to epoxides by CYP [3]. Commonly, epoxides appear in oxidation reactions as intermediates, which are further rapidly metabolized. One major route is the hydratation of the epoxide to the corresponding dihydroxy compound by microsomal and/or cytosolic epoxide hydratases [4]. The second metabolic pathway of epoxides is their enzymatic conversion to glutathione (GSH) conjugates by GSH *S*-transferases [5].

The polyunsaturated fatty acid arachidonic acid is the best investigated fatty acid, because its metabolites, collectively named eicosanoids, which include prostaglandins, thromboxane and leukotrienes, are biologically highly active compounds. The arachidonic acid oxide leukotriene A_4, 5,6-epoxyeicosatetraenoic acid, is formed from the catalytical action of 5-lipoxygenase on arachidonic acid [6,7]. Enzymatic hydratation of leukotriene A_4 leads to the formation of leukotriene B_4, while its conjugation with GSH results in the formation of leukotriene C_4, the first member of the cysteinyl leukotriene family [6,7].

The major monounsaturated fatty acid in mammals is oleic acid (*cis*-9-octadecenoic acid). Until now only little attention has been paid to the metabolism of oleic acid. A series of studies suggests, however, potential roles for oleic acid, such as in lung injury [8–10]. The mode of action of oleic acid is incompletely understood. It is still unknown whether the effects induced by oleic acid are attributed to oleic acid itself or to unknown metabolites of oleic acid. Analogous to arachidonic acid, one can assume that oleic acid may undergo epoxidation to its oxide *cis*-9,10-epoxyoctadecanoic acid (*cis*-EODA), which may be further metabolized by epoxide hydratases and GSH *S*-transferases. Indeed, the oleic acid oxide *cis*-EODA has been identified by Ulsaker and Teien in human blood [11,12]. More recently, the same group has identified *cis*-EODA in human urine and estimated its concentration to be 2.1 n*M* by GC–MS [13]. This concentration is of the same order of magnitude as that of epoxyeicosatrienoic acids in human urine [14].

The identification of *cis*-EODA in vivo elucidates the metabolic pathway of oleic acid [11–13]. The origin of *cis*-EODA in urine is unknown. Ulsaker and Teien have assumed that urinary EODA is a real metabolic product of oleic acid excreted by the kidney, and not an artefact formed from urinary oleic acid during sample treatment [13]. In principle, there are two possible pathways for the formation of *cis*-EODA from oleic acid: (1) epoxidation of oleic acid by microsomal CYP systems; and (2) non-enzymatic epoxidation, i.e. lipid peroxidation, of oleic acid in lipids and subsequent hydrolysis of the epoxidized lipids to produce the free acid of *cis*-EODA.

Unlike other fatty acid epoxides, *cis*-EODA is stable enough [13] in order to be isolated from biological fluids prior to GC–MS analysis [11–13]. However, the presence of *cis*-EODA in human blood and urine has been shown only indirectly, i.e. after reduction of the epoxy and carboxy groups with $LiAlH_4$ to generate equal amounts of 1,9-dihydroxyoctadecane and 1,10-dihydroxyoctadecane [11–13].

We found that the oxirane ring of *cis*-EODA is sufficiently thermally stable for GC–MS analysis of intact *cis*-EODA as its methyl or pentafluorobenzyl (PFB) ester. In the present study, we investigated the enzymatic formation of *cis*-EODA from oleic acid by the catalytical action of microsomal CYP and by isolated hepatocytes. Since liver cells and microsomes possess epoxide hydratases, we also investigated the metabolism of *cis*-EODA to *threo*-9,10-dihydroxyoctadecanoic acid (*threo*-DiHODA) by microsomes and isolated hepatocytes. In the present communication, we report the first direct evidence by GC–MS for the CYP-dependent formation of *cis*-EODA from oleic acid. *cis*-EODA and its product of hydratation *threo*-DiHODA were quantitated in incubates of microsomes and isolated hepatocytes by HPLC after conversion of the free acids into their *p*-bromophenacyl esters. The same HPLC system was used to separate the PFB esters of *cis*-EODA and *threo*-DiHODA prior to structure elucidation by GC–MS.

2. Experimental

2.1. Chemicals

Collagenase (type IV), oleic acid, *cis*-EODA, *trans*-9,10-epoxyoctadecanoic acid (*trans*-EODA), *threo*-DiHODA, *erythro*-9,10-dihydroxyoctadecanoic acid (*erythro*-DiHODA), undecanoic acid for use as the internal standard in HPLC, dodecanoic acid, 2-methyl-1,2-di-3-pyridyl-1-propanone (metyrapone), 1,2-epoxy-3,3,3-trichloropropane (TCPO) and *N,N*-diisopropylethylamine (99.5%) were obtained from Sigma (Munich, Germany). These chemicals were of the highest purity available (≥98%). *cis*-EODA was also prepared from oleic acid using *m*-chloroperbenzoic acid (97%; Merck, Darmstadt, Germany) as described by Swern et al. [15]. From this product the corresponding diol was prepared by acid-catalyzed hydrolysis. The structure of the newly synthesized epoxide and its diol were identified by ^{1}H NMR and GC–MS. Acetonitrile and methanol of gradient grade were obtained from Merck. Reduced nicotinamide (NADPH+H^+, 98%) and isocitrate dehydrogenase were purchased from Boehringer-Mannheim (Mannheim, Germany). *p*-Bromophenacyl bromide (98%) was bought from Serva (Heidelberg, Germany). Triethylamine was purchased from Fluka (Neu Ulm, Germany). 2,3,4,5,6-Pentafluorobenzyl bromide (99%) and *N,O-bis*(trimethylsilyl) trifluoroacetamide (BSTFA, 99%) were obtained from Pierce (Rockford, IL, USA).

2.2. Enzyme preparation, isolation of hepatocytes and incubation conditions

Microsomes were prepared by standard methods from liver of male Wistar rats, male bastard rabbits and humans who had died of liver diseases [16]. The CYP content of the three species was determined spectrophotometrically by the method of Omura and Sato [17] to be 1.12, 1.84 and 0.84 nmol/mg protein, respectively. Epoxide hydratase activity in the rat, rabbit and human liver microsomes was 660, 234 and 215 pmol *threo*-DiHODA/min mg protein, respectively, towards the substrate *cis*-EODA, as analysed and estimated by HPLC. In all experiments freshly prepared microsomes were used. The incubation mixtures (5 ml) consisted of 0.5 ml $MgCl_2$ (50 m*M*), 0.5 ml nicotinamide (50 m*M*), 0.5 ml sodium isocitrate (50 m*M*), 0.5 ml NADPH+H^+ (10 m*M*), 10 μl isocitrate dehydrogenase (24.7 U/ml), 1.9 ml of 0.1 *M* potassium phosphate buffer, pH 7.4, and microsomes suspension of varying amounts. Sodium isocitrate and isocitrate dehydrogenase served as the NADPH+H^+ regenerating system. The volume of the mixture was adjusted to 5 ml using distilled water. Incubations were carried out in glass tubes (10×100 mm) which were shaken for 5 min at 37 °C. Reaction was started by the addition of varying amounts of oleic acid from a stock solution in ethanol, the final concentration of ethanol in the reaction mixture was less than 1 vol%. The mixture was incubated aerobically at 37 °C in a shaking water bath for various times as specified in the respective experiments. The reaction was stopped by adding under vortexing of 1 ml of a stock solution of the internal standard undecanoic acid (100 μ*M*) in 0.1 *M* phosphate buffer, pH 6.5, and of 4 ml of peroxide-free diethylether. By this procedure unoxidized fatty acids used as substrates, such as oleic acid and dodecanoic acid, are rapidly extracted from the aqueous reaction mixture into the organic solvent, terminating this enzymatic reaction. Addition of the internal standard undecanoic acid to the reaction mixture prior to solvent extraction ensures very similar conditions for reaction products and the internal standard. Because of the higher water-solubility of the oxidised fatty acids, tubes were then shaken for 90 min overhead to maximise extraction recovery. After centrifugation (500 *g*, 5 min) the ether phase was transferred into clean glass tubes and the solvent was evaporated in vacuo.

Isolated rat hepatocytes were freshly prepared by the method of Seglen [18] as described by Mito et al. [19]. Viability of freshly isolated hepatocytes was determined to be 80–95% by the Trypan Blue exclusion test. Experiments with isolated hepatocytes, typically 3×10^5 cells/ml of incubation medium, were carried out immediately after their isolation under the same experimental conditions for incubation medium, termination of the reaction and solvent extraction as described above for microsomes, except for the volumes, which were half of those used in experiments with microsomes (total

incubation volume, 2.5 ml). Incubation time was specified in the respective experiments.

2.3. Analytical procedures

2.3.1. Derivatization procedures

*p*BrPh esters and PFB esters of fatty acids were prepared by standard derivatization procedures [20,21]. Briefly, the residue of the ether evaporation was reconstituted in a 200-μl aliquot of water-free acetonitrile. To prepare the *p*BrPh esters, 10 μl of triethylamine and 20 μl of a *p*BrPh bromide solution in acetonitrile (30%, w/v) was added to this solution, and the mixture was heated at 60 °C for 2 h. For preparation of the PFB esters, 20 μl of *N,N*-diisopropylethylamine and 20 μl of a solution of PFB bromide in acetonitrile (30%, v/v) were added, and the mixture was heated at 30 °C for 60 min. For structure elucidation by GC–MS, the PFB esters were converted subsequently to their trimethylsilyl (TMS) ether derivatives as follows. Solvent and reagent excess were removed under a nitrogen stream, the residue was treated with 20 μl of BSTFA, and the reaction mixture was heated at 60 °C for 60 min. Sample were stored in BSTFA at 4 °C until analysis.

2.3.2. High-performance liquid chromatography

A Perkin-Elmer HPLC apparatus model Series 3B was employed for the analysis of *p*-bromophenacyl (*p*BrPh) esters. Samples (20-μl aliquots) were injected by means of a Perkin-Elmer autosampler model ISS 100. The effluent was monitored by a UV detector (Spectromonitor III, Milton Roy) set at 254 nm. Analysis of PFB esters was carried out on an LKB apparatus consisting of a pump model 2150, a controller model 2152, a solvent conditioner model 2156, an ultragrade mixer driver model 11300, a Rheodyne injection system fitted with a 200-μl sample loop, and a variable UV–Vis LKB detector model 2151 set at 254 nm. Chromatogram recordings and all calculations were carried out on Shimadzu integrators models C-R1A and C-R3A. For quantitation of the oxidation products, the residue of the ether evaporation was dissolved in acetonitrile, derivatized with *p*-bromophenacyl bromide to the corresponding esters and analyzed by HPLC. *p*BrPh esters as well as PFB esters of oleic acid, its oxidation products and of the internal standard were separated by HPLC on analytical columns (250 mm×4.6 mm I.D.) packed with Nucleosil 100-5C_{18}, 5-μm particle size, from Macherey-Nagel (Düren, Germany). The flow-rate was 1 ml/min. For GC–MS analysis, HPLC fractions corresponding to the PFB esters of *cis*-EODA and *threo*-DiHODA were collected separately, and methanol was removed under a stream of nitrogen. The PFB esters were then extracted by vortexing from the remaining aqueous phase with heptane (2 ml). The heptane phase was dried over anhydrous sodium sulphate, the solvent was removed under nitrogen, and the residue was treated with BSTFA.

2.3.3. Gas chromatography–mass spectrometry

GC–MS analyses were carried out on a Finnigan MAT TSQ model 45 (Finnigan, San Jose, CA, USA) coupled with a Finnigan 9611 gas chromatograph. Some GC–MS analyses were also carried out on a Finnigan MAT TSQ model 7000 coupled with a gas chromatograph model Trace Series 2000. Analyses were carried out in the negative-ion chemical ionization (NICI) or electron impact ionization (EI) mode. Ionization energy was 120 eV in NICI and 70 eV in EI. Methane was used as a reagent gas at a pressure of 65 Pa. Chromatographic separation was carried out on fused-silica capillary columns DB1 (15 m× 0.25 mm I.D., 0.25 μm film thickness) from J & W Scientific (Rancho Cordova, CA, USA) and Optima 17 (30 m×0.25 mm I.D., 0.25 μm film thickness) from Macherey-Nagel (Düren, Germany). Helium was used as a carrier gas at a pressures of 55 and 90 kPa, respectively. One-microliter aliquots were injected in the splitless mode.

3. Results

3.1. High-performance liquid chromatography

Optimal separation of the fatty acid esters was achieved using a linear gradient elution from 70% solvent A (methanol–water, 50:50, v/v) to 100% solvent B (methanol) within 25 min followed by an isocratic elution for 5 min at 100% solvent B. The PFB esters of the reaction products and oleic acid

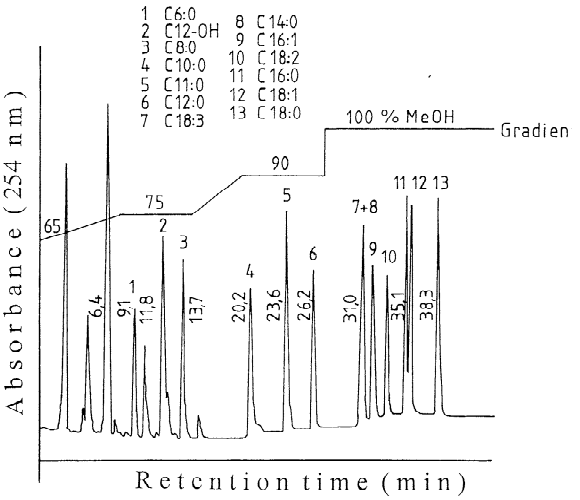

Fig. 1. HPLC chromatogram of a mixture of fatty acids as their *p*-bromophenacyl esters. Fatty acids were added to phosphate buffer at 100 μ*M* each, extracted by solvent extraction with diethyl ether and converted to their *p*-bromophenacyl esters using *p*-bromophenacyl bromide. Gradient elution was carried out as indicated; MeOH, methanol. Peak identification: Numbers on the peaks give the retention time (in min) of the respective fatty acid *p*-bromophenacyl ester. Other conditions were as described in Section 2.

had nearly the same retention times on HPLC as the corresponding *p*BrPh esters.

Fig. 1 shows a chromatogram from the HPLC analysis of a mixture of fatty acids as their *p*BrPh esters after their extraction from phosphate buffer. Unlabelled peaks in the chromatogram derive in part from remaining or degraded *p*BrPh bromide and in part from impurities present in the buffer. The same elution order was obtained for the PFB esters of these fatty acids (not shown). Obviously, the retention time of a fatty acid ester in this HPLC system mainly depends on the number of carbon atoms and on the number of the double bonds in the fatty acid molecule in such a way that unsaturated fatty acids elute in front of the corresponding saturated fatty acid. The result of this dependence is that the esters of tetradecanoic acid ($C_{14:0}$) coelute with those of the longer linolenic acid ($C_{18:3}$), and that the ester of hexadecanoic acid ($C_{16:0}$) cannot be baseline-separated from that of *cis*-9-octadecenoic acid ($C_{18:1}$) by a gradient elution within 40 min of analysis. The HPLC chromatogram of Fig. 1 further indicates that the elution of *p*BrPh esters also depends on the oxidative status of the fatty acid, with hydroxylated fatty acids eluting far in front of the corresponding non-hydroxylated fatty acids. Thus, the *p*BrPh ester of 12-hydroxydodecanoic acid (abbreviated as C12-OH in Fig. 1) elutes approximately 13 min earlier than the *p*BrPh ester of the parent molecule dodecanoic acid ($C_{12:0}$). HPLC analysis of fatty acids as their *p*BrPh esters has been reported previously [20,22–24].

These findings suggest that determination of oleic acid and its expected oxidative metabolites, i.e. of *cis*-EODA and *threo*-DiHODA, should be possible within a single run by this HPLC system. For rapid analysis and in order to be able to use undecanoic acid ($C_{11:0}$) as an internal standard, the gradient elution form was changed appropriately and simplified. The following gradient elution was found to be satisfactory in quantitative determinations of oleic acid metabolites in microsomal and hepatocyte suspensions: Linear increase from 70% solvent A to 100% solvent B within 25 min, followed by an isocratic elution for 5 min at 100% solvent B at a constant flow-rate of 1.0 ml/min. In this HPLC system, the *p*BrPh esters of oleic acid, *cis*-EODA, undecanoic acid and *threo*-DiHODA eluted at (mean±SD, $n=4$) 27.7±0.31, 19.9±0.23, 16.1±0.14, and 13.1±0.18 min, respectively.

As a rule, calibration curves were prepared for quantitation with concentrations of *cis*-EODA and *threo*-DiHODA ranging between 0 and 20 μ*M*. The concentration of the internal standard undecanoic acid in the incubation mixtures was usually 20 μ*M*. In some experiments with conditions differing from those described in Section 2, quantitation was carried out using calibration curves prepared under the changed conditions. Analyses were carried out in duplicate. Linear relationships were observed from linear regression analysis of the ratio of peak area of *cis*-EODA or *threo*-DiHODA to the peak area of undecanoic acid and the concentration of *cis*-EODA or *threo*-DiHODA with regression coefficients (r)= 0.985. Intra- and inter-day precision (RSD) of the method were determined to be below 8% for both analytes added at concentrations of 20 μ*M* each. The lowest concentration of *cis*-EODA and *threo*-DiHODA added to buffer, i.e. 0.2 μ*M* each, was determined with an imprecision (RSD) of 12 and

8%, respectively, and with a recovery (accuracy) of 85 and 92%, respectively.

Using isocratic elution (acetonitrile–water, 70:30, v/v; flow-rate 1.5 ml/min), the PFB esters of *cis*-EODA (retention time 18.7 min) and *trans*-EODA (retention time 19.6 min) were incompletely separated on a column (125×3.0 mm I.D.) packed with Nucleosil 120-3C_{18}, 3-μm particle size, from Macherey-Nagel.

3.2. Gas chromatography–mass spectrometry

The major goal of the present study was to show formation of *cis*-EODA from oleic acid metabolism with microsomes or isolated hepatocytes by GC–MS analysis of a derivative of this compound still carrying the oxirane group. In addition, we analysed by GC–MS a series of other compounds, which could, at least theoretically, be formed from *cis*-EODA (Table 1).

Under the derivatization conditions for the preparation of the *p*BrPh esters (heating 2 h at 60 °C) and the PFB esters (heating 1 h at 30 °C) the oxirane group of *cis*-EODA remained intact (see above). After trimethylsilylation (heating 1 h at 60 °C with BSTFA) of the PFB ester of authentic *cis*-EODA each one major peak at 18.6 min (OV17 column) was obtained both in the NICI mode and in the EI mode. The most abundant ion in the EI mass spectrum of the PFB ester of *cis*-EODA was observed at *m/z* 181 (100%) corresponding to the PFB cation. Other mass fragments were found at *m/z* 365 (10%) (cleavage between C_{10} and C_{11}) and *m/z* 155 (55%) (cleavage between C_8 and C_9). These ions strongly suggest that the PFB ester of EODA carries an intact oxirane group at C_9–C_{10}. The largest ion observed in the EI mass spectrum of this derivative was *m/z* 478 (1%), most likely the molecular cation of the *cis*-EODA-PFB ester. The most abundant ion in the NICI mass spectrum of the PFB ester of *cis*-EODA was observed at *m/z* 297 which corresponds to the carboxylate anion formed from loss of the PFB radical, i.e. $[M\text{-}PFB]^-$. The second mass fragment found in the NICI mass spectrum of the *cis*-EODA-PFB ester was *m/z* 279 (10%), which is most likely produced by loss of H_2O from *m/z* 297, i.e. $[M\text{-}PFB\text{-}H_2O]^-$.

The GC–MS chromatogram of *cis*-EODA-PFB ester showed two minor peaks which eluted with the retention times of the PFB esters of authentic 9-hydroxyoctadecanoic acid and 10-hydroxyoctadecanoic acid, and had identical NICI mass spectra with these derivatives (Table 1). Whether these compounds were initially present in the *cis*-EODA reference compound or were formed during derivatization and/or GC–MS analysis of the derivatives, was not investigated. In addition, the mass fragment at *m/z* 297 $[M\text{-}PFB]^-$ was subjected to collision-induced dissociation (CID) with argon. This ion was relatively stable to CID even at the very

Table 1
GC–MS and GC-tandem MS spectra of the pentafluorobenzyl (PFB) ester trimethylsilyl ether derivatives of the fatty acids investigated

Compound	Retention time (min)	*m/z* of $[M\text{-}PFB]^-$	*m/z* of the product ions derived from $[M\text{-}PFB]^-$
cis-9-Octadecenoic acid	14.77	281	None
9-Hydroxyoctadecanoic acid	16.52	371	281, 89
10-Hydroxyoctadecanoic acid	16.59	371	281, 89
12-Hydroxyoctadecanoic acid	16.75	371	281, 89
threo-9,10-Dihydroxyoctadecanoic acid	17.60	459	369, 353
erythro-9,10-Dihydroxyoctadecanoic acid	17.79	459	369, 353
trans-9,10-Epoxyoctadecanoic acid	18.55	297	279, 171, 155
cis-9,10-Epoxyoctadecanoic acid	19.10	297	279, 171, 155

Instrument: TSQ 7000; GC–MS and GC-tandem MS conditions: column: Optima 17; oven temperature program: 2 min at 80 °C, to 280 °C at 25 °C min^{-1}, to 320 °C at 4 °C min^{-1}; carrier gas: helium: 90 kPa, constant pressure. Reactand gas: methane: 0.5 kPa; collision gas: argon: 0.27 Pa; collision energy: 25 eV; ion source: 180 °C; emission current: 300 μA; electron energy: 200 eV; injector and interface temperature: 280 and 300 °C, respectively.

high collision energy of 30 eV. A few product ions were observed, the most intense of which were at *m/z* 279 (45%) ([M-PFB-H_2O]$^-$), *m/z* 171 (20%) (cleavage between C_9 and C_{10}) and *m/z* 155 (5%)(cleavage between C_8 and C_9). Similar mass spectra were also obtained from the *trans*-EODA-PFB which, however, emerged from the capillary column considerably earlier (Table 1). These findings strongly suggest that *cis*-EODA as well as *trans*-EODA are thermally stable in order to be analysed by gas chromatography as PFB esters without loss of the oxirane group to a considerable extent. The PFB ester TMS ether derivatives of *threo*-DiHODA and *erythro*-DiHODA showed virtually identical EI and NICI mass spectra, but they were separated by gas chromatography (Table 1).

3.3. Direct evidence for epoxidation of oleic acid by microsomes

Fig. 2 shows HPLC chromatograms from the analysis of the diethylether extract of an incubation mixture of oleic acid with microsomal CYP in the presence of NADPH + H^+ and O_2 after an incubation time of 10 min (A) and 30 min (B), respectively. After 10 min of incubation, two reaction products with the retention times of 12.6 and 19.8 min were observed, which were not present in the absence of CYP. In the sample taken 30 min after incubation, the peak (labelled on the chromatograms as *Epoxide*) eluting behind the internal standard disappeared almost completely, while the peak (labelled on the chromatograms as *Diol*) eluting in front of the internal standard increased further. The PFB esters of these products had the same retention times as those of authentic *threo*-DiHODA and *cis*-EODA, respectively.

For structure elucidation, HPLC fractions of sample A were collected, the reaction products were extracted, derivatized with BSTFA and analysed by GC–MS. The reaction product with the retention time of 19.8 min on HPLC (Fig. 2A) emerged from the GC column at the same time as the PFB ester of synthetic *cis*-EODA. The NICI mass spectrum of this compound (not shown) contained practically a single abundant ion at *m/z* 297. Fig. 3A shows a partial EI mass spectrum of the PFB ester of this metabolite, which is virtually identical to that of the PFB ester of authentic *cis*-EODA (oleic acid epoxide). The mass fragments at *m/z* 365 and *m/z* 155 strongly suggest the presence of one oxirane group at C_9–C_{10}. The absence of a mass fragment with *m/z* of 73 (TMS^+) in Fig. 3A indicates the absence of a hydroxy group in this metabolite. The molecular peak at *m/z* 478 and all other data observed from HPLC and GC–MS analyses unequivocally identify the compound eluted

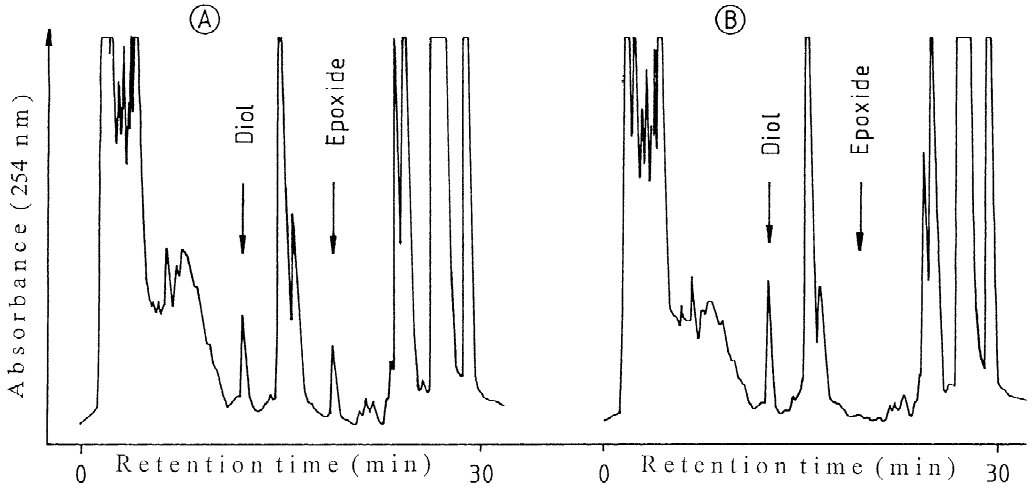

Fig. 2. HPLC chromatograms of the PFB esters of the reaction products from the incubation of oleic acid (2 m*M*) with rat liver microsomes (53.5 nmol CYP) for 10 min (A) and 30 min (B) at 37 °C. The peak with the retention time of 15.6 min corresponds to the internal standard (undecanoic acid, 20 μ*M*). The peak eluted at 27.6 min corresponds to the PFB ester of oleic acid.

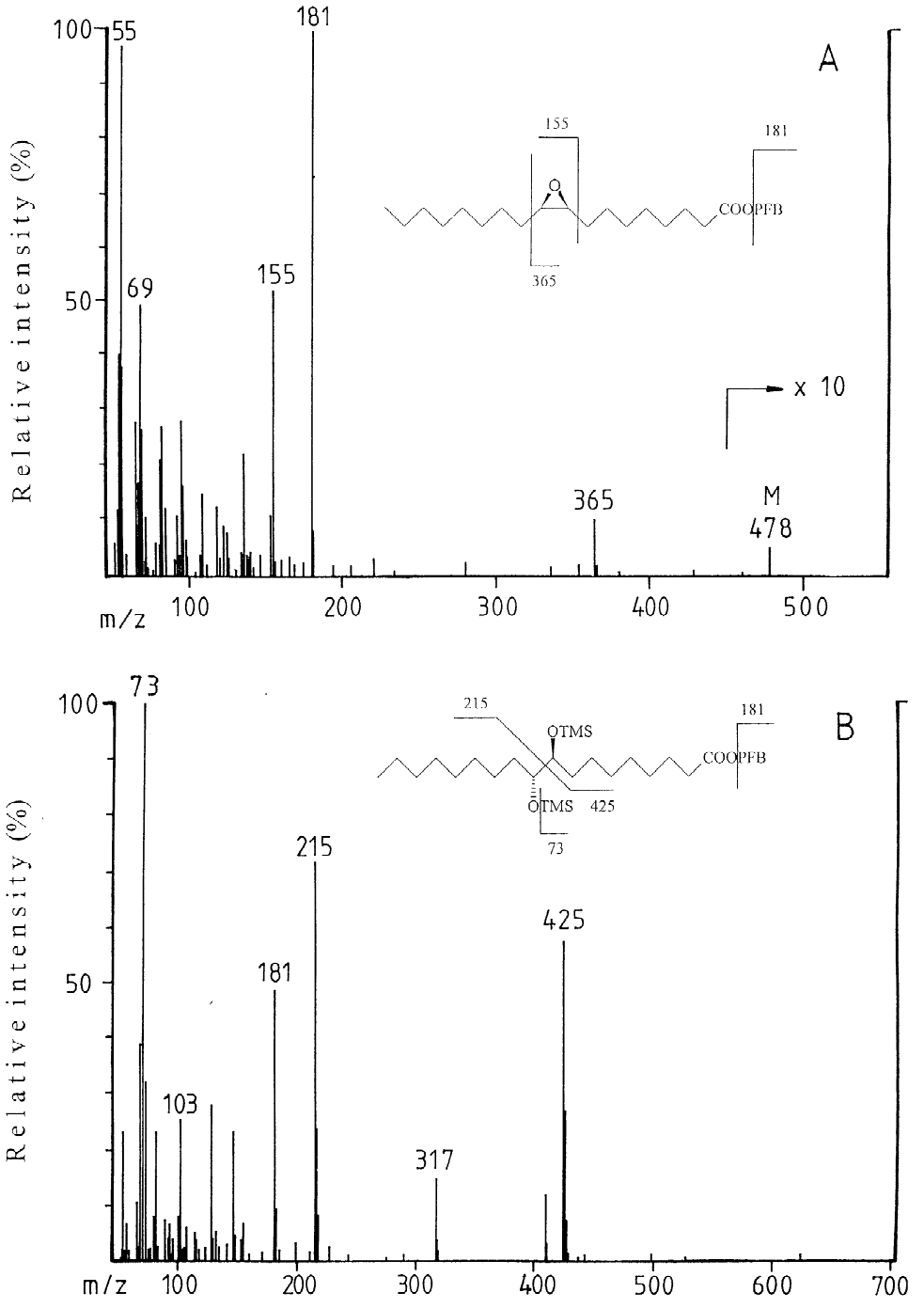

Fig. 3. EI mass spectra generated from the reaction products of oleic acid formed 10 min after its incubation with rat liver microsomes as described in the legend to Fig. 2. After solvent extraction of the reaction mixture, PFB esters were prepared and separated by HPLC (Fig. 2A). The HPLC fractions of the compounds eluted at 19.8 and 12.3 min were extracted separately, the residues were derivatized with BSTFA, and analysed by GC–MS. (A) Mass spectrum of the compound eluted in the HPLC fraction labelled as *Epoxide* in Fig. 2A. (B) Mass spectrum of the compound eluted in the HPLC fraction labelled as *Diol* in Fig. 2A.

at 19.8 min on HPLC (Fig. 2A, *Epoxide*) as the PFB ester of *cis*-EODA. EI mass spectra of the methyl esters of intact epoxides of arachidonic acid have been reported [25].

Fig. 3B shows the EI mass spectrum of the PFB ester TMS ether derivative of the more polar reaction product of oleic acid with the retention time of 12.6 min on HPLC (Fig. 2A, labelled on the chromatograms as *Diol*). The mass fragments at m/z 215 and m/z 425 strongly suggest that this compound is *threo*-DiHODA. Similar EI mass spectra of methyl esters of vicinal dihydroxy fatty acid esters derived from arachidonic acid have been described in the literature [25,26]. In the NICI mass spectrum of this compound, the most intensive ion was m/z 459 $[M\text{-}PFB]^-$. Retention times on GC–MS, EI and NICI mass spectra of the PFB ester TMS ether derivative of this reaction product were virtually identical with that of authentic *threo*-DiHODA. These observations strongly suggest that the compound eluted at 12.6 min on HPLC (Fig. 2A, *Diol*) is the PFB ester of *threo*-9,10-epoxyoctadecanoic acid (*threo*-DiHODA). All these findings together give unequivocal evidence for the epoxidation of oleic acid to *cis*-EODA, which is further metabolized to *threo*-DiHODA via a microsomal epoxide hydratase (activity: 660 pmol *threo*-DiHODA/min×mg protein, respectively, towards *cis*-EODA). Under the experimental conditions used in the present study, no oleic acid epoxides were formed during ether extraction, and EODA was not hydrolyzed chemically into *threo*-9,10-dihydroxystearic acid during the extraction and derivatization procedures.

3.4. Kinetics of the epoxidation of oleic acid by liver microsomes

The time course of the concentrations of *cis*-EODA and *threo*-DiHODA in microsomal incubates of oleic acid is shown in Fig. 4. Oleic acid was rapidly oxidized to *cis*-EODA, which was subsequently further converted to *threo*-DiHODA. *cis*-EODA formation reached a maximum (7.5 μ*M*) after 5 min of incubation. The concentration of its hydratation product increased constantly up to 30 min. After 30 min of incubation, hardly detectable amounts of *cis*-EODA were found. Watabe et al. [27] have also investigated microsomal oleic acid oxidation, but they took a sample only 30 min after incubation and could not detect any *cis*-EODA. Apparently, epoxidation of oleic acid in our experiment was complete 10 min after incubation. The total extent of the conversion of oleic acid into *cis*-EODA and *threo*-DiHODA was about 1.5% (Fig. 4).

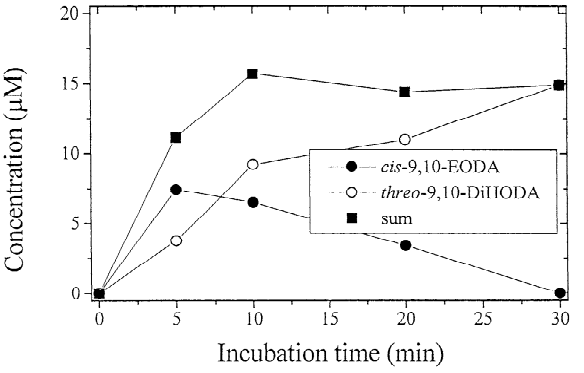

Fig. 4. Time course of the concentrations of *cis*-EODA, *threo*-DiHODA (DiHODA) and the sum of *cis*-EODA plus *threo*-DiHODA during incubation of oleic acid (2 m*M*) with rat liver microsomes (53.5 nmol CYP).

Fig. 5 shows the concentration of *cis*-EODA and *threo*-DiHODA after 10 min of incubation of different amounts of oleic acid with a constant amount of CYP from rat liver microsomes. This figure indicates that *cis*-EODA is measurable even at low oleic acid concentrations, but the highest amount is formed at 1000 μ*M* of oleic acid. At oleic acid concentrations

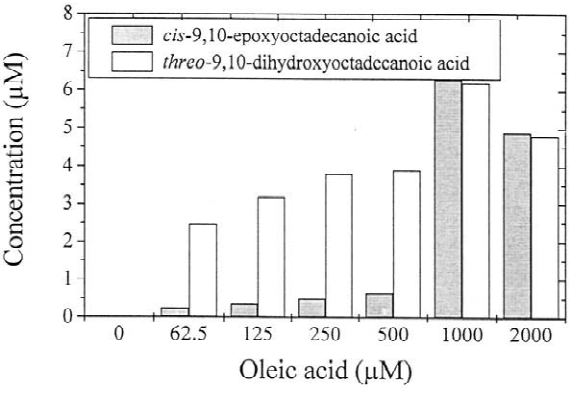

Fig. 5. Concentrations of *cis*-EODA and *threo*-DiHODA measured 10 min after incubation of the indicated oleic acid concentrations with 53.5 nmol CYP each from rat liver microsomes.

of 62.5, 125, 250 and 500 μ*M*, the major oxidative metabolite of oleic acid was *threo*-DiHODA, while at oleic acid concentrations of 1000 and 2000 μ*M*, similar concentrations of the epoxide and its diol were measured. At these concentrations, conversion of oleic acid to *cis*-EODA plus *threo*-DiHODA was of the order of 1.0%.

The results obtained from incubations of oleic acid with liver microsomes of rabbit (see below) and man (not shown) were qualitatively the same as with microsomes from rat liver. Fig. 6 shows the formation of *cis*-EODA and *threo*-DiHODA was dependent upon the CYP content from rabbit liver microsomes. The concentrations of *cis*-EODA and *threo*-DiHODA were found to depend upon the enzyme concentration. In this experiment, the concentrations of *threo*-DiHODA were considerably higher than those of *cis*-EODA. Total conversion of oleic acid to *cis*-EODA plus *threo*-DiHODA ranged between 0.6% (41.7 nmol of CYP) and 0.7% (83.4 nmol of CYP). Epoxide hydratase activity in rabbit liver microsomes was determined as 234 pmol *threo*-DiHODA/min×mg protein towards *cis*-EODA as the substrate.

3.5. Inhibition studies

The epoxidation of oleic acid was found to depend upon the presence of $NADPH+H^+$, O_2 and CYP. Inhibitors of CYP, such as carbon monoxide (CO) and metyrapone, were found to inhibit the epoxidation of oleic acid (Fig. 7). CO inhibited completely the epoxidation of oleic acid. Metyrapone was rather a weak inhibitor of CYP.

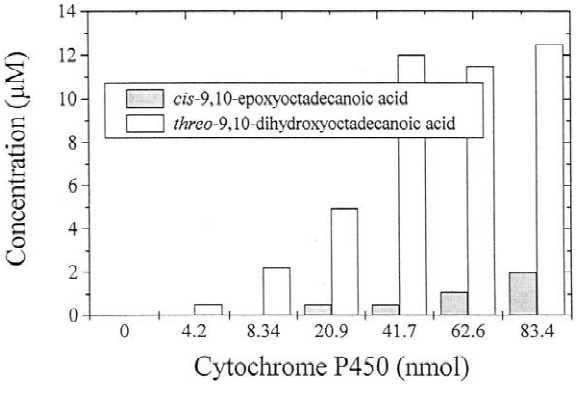

Fig. 6. Concentrations of *cis*-EODA and *threo*-DiHODA formed after 10 min of incubation of oleic acid (2 m*M*) with the indicated amounts of CYP from rabbit liver microsomes.

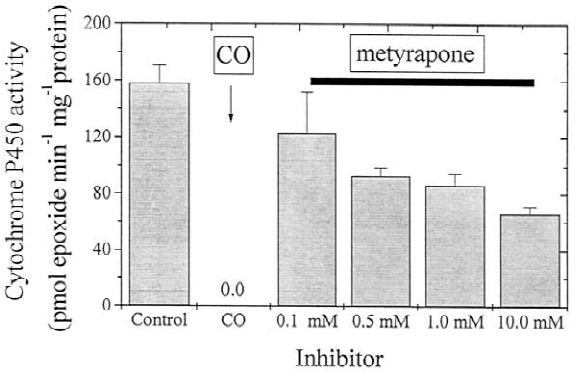

Fig. 7. Effect of carbon monoxide (CO) and metyrapone on the epoxidation of oleic acid (2 m*M*) catalyzed by liver microsomes from clofibrate/phenobarbital-treated rats (53.5 nmol CYP). Incubation time was 5 min. Data are shown as (mean±SD) from triplicate incubations.

Inhibition studies were also carried out with 1,2-epoxy-3,3,3-trichloropropane (TCPO), an inhibitor of epoxide hydratases [28,29]. TCPO inhibited the epoxidation of oleic acid as well the hydratation of oleic epoxide to its diol (Table 2). At 10 m*M* TCPO, epoxide hydratase activity was inhibited almost completely, whereas CYP activity was inhibited by 40%. Importantly, even 30 min after incubation of oleic acid with rat liver microsomes, *cis*-EODA was present in the incubation mixture at a concentration of 7 μ*M* in the presence of 10 m*M* of TCPO. These data suggest that under some circumstances, e.g. inhibition of epoxide hydratases, *cis*-EODA may exist for a relatively long period of time in microsomal suspensions.

Table 2
Effect of TCPO on the oxidative metabolism of oleic acid (2 m*M*) by rat liver microsomes (53.5 nmol CYP) to *cis*-EODA and *threo*-DiHODA after an incubation time of 30 min

TCPO (m*M*)	*threo*-DiHODA (μ*M*)	*cis*-EODA (μ*M*)
0	22	Not detectable
1	5.0	0.8
10	0.9	7.0

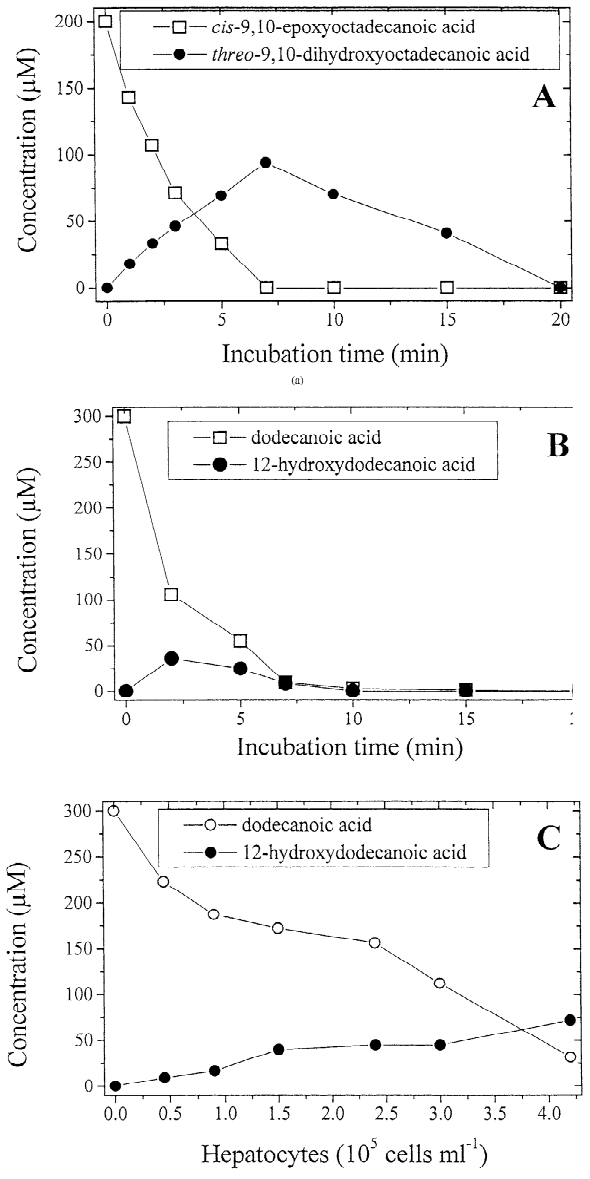

Fig. 8. Metabolism of (A) *cis*-EODA (initial concentration, 200 μ*M*) and (B) dodecanoic acid (initial concentration, 300 μ*M*) with isolated hepatocytes (3×10^5 cells/ml) from rats treated with clofibrate and phenobarbital. (C) Concentrations of dodecanoic acid and 12-hydroxydodecanoic acid after 3 min of incubation of dodecanoic acid (300 μ*M*) with various amounts of isolated hepatocytes. Substrates and products were determined by HPLC as *p*BrPh esters using undecanoic acid as the internal standard at a concentration of 50 μ*M* each. Other conditions were as described in Section 2.

3.6. Experiments with isolated rat hepatocytes

Incubation of oleic acid (0–2 m*M*) with isolated rat hepatocytes did not result in formation of *cis*-EODA and *threo*-DiHODA in detectable amounts. We therefore investigated the ability of hepatocytes to metabolise *cis*-EODA. Fig. 8A shows that isolated rat hepatocytes metabolized *cis*-EODA rapidly and completely, obviously via intermediate hydratation to *threo*-DiHODA, which must have been subsequently further metabolized. Neither *cis*-EODA nor *threo*-DiHODA were detectable in the incubation mixture after 20 min of incubation. Fig. 8B shows that hepatocytes possess monooxygenase activity, because dodecanoic acid was rapidly and quantitatively metabolized, at least in part, via ω-hydroxylation to 12-hydroxydodecanoic acid, which is subsequently metabolized most likely to dodecane-1,12-dioic acid. Incubation of 12-hydroxydodecanoic acid (350 μ*M*) with isolated rat hepatocytes also resulted in complete metabolism of this hydroxylated fatty acid (not shown). Hydroxylation of dodecanoic acid to 12-hydroxydodecanoic acid was dependent upon the hepatocyte concentration in the incubation mixture (Fig. 8C). All these findings suggest that *threo*-DiHODA could be further metabolized to its dicarboxylic acid via ω-hydroxylation. Therefore, absence of *cis*-EODA and *threo*-DiHODA in the hepatocyte incubates of oleic acid does not exclude intermediate formation of these oleic acid metabolites.

4. Discussion

4.1. Analysis of fatty acids and their oxidative metabolites by HPLC and GC–MS

HPLC analysis of fatty acids as *p*BrPh esters [20,22–24] or PFB esters on reversed-phase materials is a useful approach to quantify fatty acids in various matrices. Due to their higher molar absorptivity, *p*BrPh esters can be quantified more sensitively by UV detection than PFB esters. On the other hand, PFB esters capture considerably stronger electrons than *p*BrPh esters so that PFB fatty acid esters can be detected with much higher sensitivity by GC–MS operating in the NICI mode than *p*BrPh

esters. Our study shows that assessment of a wide spectrum of saturated and unsaturated fatty acids, including their hydroxylated and epoxidized metabolites, requires separation by gradient elution in HPLC and oven temperature programmed conditions in GC–MS. The HPLC method allows separation and simultaneous detection of oleic acid and its metabolites as *p*BrPh or PFB esters. The GC–MS method applies to directly detect intact *cis*-EODA. The PFB esters of *cis*-EODA and *trans*-EODA show virtually identical mass spectra both in the NICI and in the EI mode, but they emerge at different retention times from the GC capillary column. The PFB esters of *threo*-DiHODA and *erythro*-DiHODA show similar behavior.

4.2. Direct evidence for enzyme-catalysed formation of cis-EODA from oleic acid

Among the unsaturated fatty acids, little attention has been paid to oleic acid and its metabolism in the past. However, today it is generally accepted that oleic acid may play much more physiological roles than originally expected [8–13,29–34]. Perhaps with a single exception [34], it is still unknown whether the effects induced by oleic acid are attributed to oleic acid itself or to metabolites of oleic acid. Oleic acid, linoleic acid and other polyunsaturated fatty acids, but not saturated fatty acids, seem to have a direct regulatory effect on the stimulation of CD36 gene expression in human macrophages [34]. However, since metabolites of polyunsaturated fatty acids, notably of arachidonic acid, such as prostaglandins, thromboxane and leukotrienes, are the actual biologically active compounds, much more so than arachidonic acid itself, it is likely that the apparently direct effects of unsaturated fatty acids are indeed induced by their oxidative metabolites.

Early studies on oleic acid metabolism have shown that this monounsaturated fatty acid undergoes extensive oxidative metabolism to CO_2, acetate, and keto bodies [35,36]. Unesterified oleic acid, in particular, however, oleic acid esterified to lipids also undergoes non-enzymatic oxidative metabolism, which leads to formation of the free and the esterified *cis*-EODA, respectively [37–39]. *cis*-EODA has been identified indirectly both in human plasma [11,12] and in human urine [13], strongly suggesting that epoxidation of oleic is a physiological metabolic pathway of oleic acid.

The origin of circulating and excretory *cis*-EODA is still unknown. Analogous to other unsaturated fatty acids, free radical-catalysed epoxidation, i.e. lipid peroxidation, of oleic acid, as well as enzyme-catalysed epoxidation may be the two sources for *cis*-EODA. Indirect evidence for the microsomal $NADPH + H^+$- and O_2-dependent epoxidation of oleic acid has been reported three decades ago [26]. This evidence was supported basically on the measurement of *threo*-DiHODA in microsomal incubations of oleic acid, the epoxide hydratase product of the intermediate *cis*-EODA [27]. A third source for *cis*-EODA in human body may be nutrition, as plants are also capable in synthesizing enzymatically this epoxide from oleic acid [40]. Knoche has demonstrated by GC–MS that ^{18}O from O_2 but not from $H_2^{18}O$ is incorporated into the oxirane ring of *cis*-EODA [40]. Hamberg and Hamberg have shown that oleic acid was epoxidized hydroperoxide-dependently to *cis*-EODA in the broad bean, whereas the epoxide oxygen was derived from hydroperoxide and not from O_2 [41]. Like oleic acid, *cis*-EODA is rapidly absorbed, distributed and extensively metabolized in vitro and in vivo in the rat [42]. Racemic *cis*-EODA has been isolated from total lipids of human leukocytes [43]. Its identification was based mainly on GC–MS and on its chemical conversion into *threo*-DiHODA. More than 90% of *cis*-EODA occurred in its esterified form in leukocyte lipids [43].

In consideration of the potential significance of the free radical- as well as enzyme-catalysed epoxidation of oleic acid, the aim of the present study was to directly identify *cis*-EODA in incubates of oleic acid in liver microsomes and isolated hepatocytes. For this purpose we developed and applied HPLC and GC–MS methods. These unique chromatographic and mass spectrometric possibilities enabled studies on the metabolism of oleic acid by CYP present in microsomes and isolated hepatocytes. The present article presents direct and unequivocal evidence for the CYP-dependent formation of *cis*-EODA from oleic acid in microsomes. Our study shows that *cis*-EODA may not be solely an obligatory intermediate in the oxidative metabolism of oleic acid, as had been postulated previously, but it may accumu-

late under some circumstances, such as in an imbalanced situation between synthesizing and metabolizing enzymes of *cis*-EODA. In the case of a high CYP epoxide activity and lower epoxide hydratase activity, this epoxide may exist long enough to reach the circulation and subsequently the kidney.

4.3. Proposal of the oleic acid cascade

cis-EODA has been shown to be a specific substrate for various GSH *S*-transferases [32,33]. GSH conjugates of *cis*-EODA and metabolites of them have not yet been detected in vivo. However, the appearance of *cis*-EODA in human blood [11,12] and urine [13], and the existence of GSH conjugates of endogenous fatty acid epoxides suggest that in humans an oleic acid cascade may exist (Fig. 9). *cis*-EODA and *threo*-DiHODA has been shown to be substrates for microsomal CYP systems from *Vicia sativa*, which converted these compounds into 18-hydroxy-9,10-epoxyoctadecanoic acid and 9,10,18-trihydrocyoctadecanoic acid, respectively, via ω-hydroxylation [44]. On the basis of our present results and of the current knowledge about oleic acid metabolism, we suppose that *cis*-EODA is an endogenous compound, which is formed from oleic acid esterified to lipids by nonenzymatic lipid peroxidation followed by enzymatic hydrolysis, and from oleic acid by the catalytical action of CYP systems. *cis*-EODA is the common substrate for microsomal

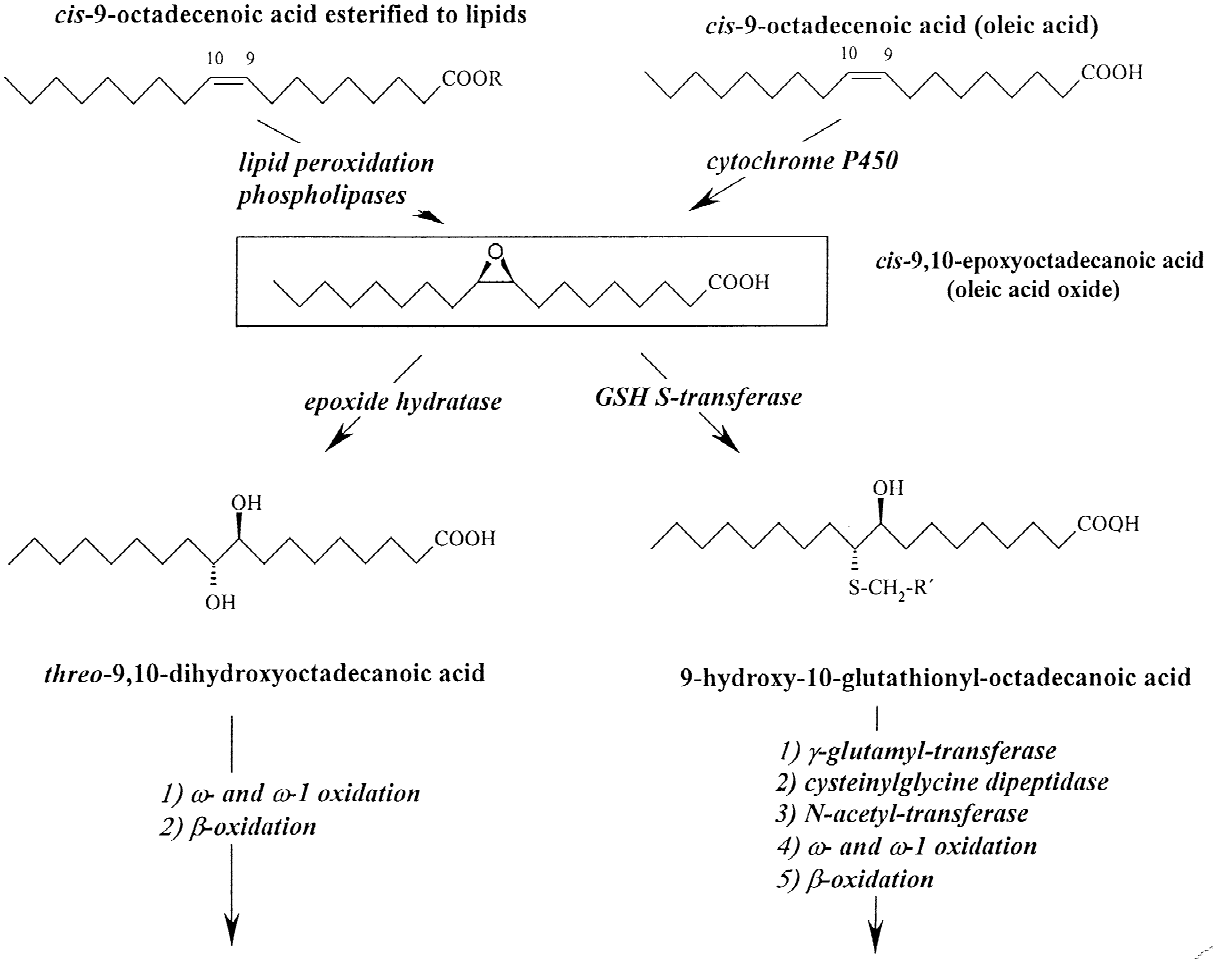

Fig. 9. Schematic of the proposed oleic acid cascade. S-CH_2-R′, glutathionyl. See the text for further details.

CYP, microsomal and cytosolic epoxide hydratases and GSH *S*-transferases, which form *threo*-DiHODA and 9-hydroxy-10-glutathionyl-octadecanoic acid, respectively. To the best of our knowledge, these compounds have not yet been identified in human blood or urine. We suppose that in humans *cis*-EODA, *threo*-DiHODA and 9-hydroxy-10-glutathionyl-octadecanoic acid are substrates for specific enzymes, the reaction products of which are eliminated by the lung, liver and kidney (Fig. 9). In addition to the metabolic fate of oleic acid and its metabolites illustrated in Fig. 9, 9-hydroxy-10-glutathionyl-octadecanoic acid and *threo*-DiHODA may exert biological activity in vivo. This is supported by the finding that the GSH conjugate of *cis*-EODA has been shown to stimulate a human erythrocyte membrane ATPase [45].

4.4. Possible role of cis-EODA as a biomarker of oxidative-stress in vivo

Oleic acid, both unesterified and esterified to lipids, undergoes non-enzymatic oxidative metabolism, which leads to formation of the free and the esterified *cis*-EODA, respectively [37–39]. The detection of racemic *cis*-EODA in lipids of human leukocytes [43] suggests a free radical-catalysed epoxidation of esterified oleic acid in lipids. In human atherosclerotic plaque non-enzymatic oxidative metabolites of arachidonic acid (e.g. F_2-isoprostanes) and linoleic acid (e.g. 9-oxo-octadecadienoic acid and 13-oxo-octadecadienoic acid) have been detected [46,47], suggesting a possible role of these substances in the pathogenesis of plaque. The occurrence of *cis*-EODA, *trans*-EODA and other possible oxidative metabolites of oleic acid in human atherosclerotic plaque remains to be investigated. The HPLC and GC–MS methods described in this article should be useful to investigate oleic acid metabolism, origin and occurrence of *cis*-EODA and its metabolites in vivo in humans, and their physiological and pathophysiological significance. Currently, we are developing a GC–MS method for the quantitative determination of free and esterified *cis*-EODA in human plasma and urine. By means of this method the reliability of *cis*-EODA as a biomarker of oxidative stress in vivo will be investigated in forthcoming studies.

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