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Characterization of a Novel Arachidonic Acid-Derived Neutrophil Chemoattractant

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The oxidation of arachidonic acid in a superoxide-generating environment results in the formation of a potent chemoattractant that appears to be identical to a chemotactic material generated by hepatocytes when they metabolize alcohol. The product was extracted, chromatographed and characterized by physical methods including GC/MS. The physical properties are consistant with the parent structure: 19-hydroperoxy, 20-hydroxyarachidic acid. This novel saturated 20 carbon product, derived from arachidonic acid by free radical-generating reactions, may play a role in the neutrophilic infiltration observed during the course of acute alcoholic hepatitis.

Acute alcoholic hepatitis is a disease characterized by the presence of neutrophils within the liver parenchyma (1). Previously, we reported that incubation of either rat or human hepatocytes with ethanol results in a time-dependent generation of a lipid that is chemotactic for human neutrophils (3, 4). The chemotactic lipid (CL) is generated by the interaction of acetaldehyde and components of the cell cytosol, in a process involving generation of oxygen-derived free radicals (5, 6). Although the biological activity of CL has been characterized (3, 5), structural identification has been hindered by the small quantities isolated from hepatocyte supernatants. Previous work (7) demonstrated that oxidation of arachidonic acid using a superoxide-generating system *in vitro* results in the formation of a novel product that is chemotactic for human neutrophils. This arachidonic acid-derived oxidation product appears to be identical to hepatocyte-generated CL (5). In the present report, we employed this *in vitro* system as a source of CL to determine its physical properties.

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Abbreviations used are: CL, chemotactic lipid; RP-HPLC, reverse-phase high performance liquid chromatography; GC/MS, mass chromatography/mass spectroscopy; Me_3Si (OTMS), trimethylsilyl; UV,ultraviolet.

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MATERIALS AND METHODS

Materials- Arachidonic acid was purchased from Nuchek Prep, Elysian, MN. Sep-Pak C18 cartridges were obtained from Waters Associates, Milford, MA. [^{3}H]-arachidonic acid and [1-^{14}C]-arachidonic acid were from New England Nuclear. bis-trimethylsilyl-trifluoroacetamide was from Pierce, Rockford, IL. Soybean lipoxygenase, sodium borohydride ($NaBH_4$), N-formyl-met-leu-phe (FMLP) and endotoxin-free water were purchased from Sigma. Monostearin was from Matreya, Inc., Pleasant Gap, PA. Bovine milk xanthine oxidase (E.C. 1.1.3.22) was from Calbiochem Corp. Organic solvents were HPLC grade.

Generation of CL- CL was prepared as in (7). For some experiments, labeled arachidonic acid was added to the incubation mixtures. In some cases, reactions were scaled up by adding up to 50 mg of arachidonic acid with proportionately increased amounts of acetaldehyde and xanthine oxidase. Generation of superoxide anion in these reaction conditions was verified by reduction of ferricytochrome *c* (7). Reactions were stopped by the addition of cold (dry ice/acetone) alcohol (1.5:1, vol/vol) (Fisher Chemicals) and extracted using Sep-Pak cartridges (5).

Characterization of CL- CL was extracted using C18 chromatography and materials eluted with methyl formate were subjected to RP-HPLC (5). Fractions (1.0 ml each) displaying chemotactic activity, exhibiting retention times from 8-10 min (see Fig. 1, A), were collected, concentrated under a stream of either argon or nitrogen and suspended in MeOH or hexane for further analysis. Gas chromatography/mass spectroscopy (GC/MS) was performed with a Hewlett-Packard 5988A MS equipped with a 5997A workstation and 5890 GC. A fused silica capillary SE-30 column (Supelco, Inc., Bellefonte, PA) (column 2-4004, 30 cm, 0.25 mm i.d., 0.25 mm df) was employed with a temperature program. A second series of experiments were carried out using a Hewlett-Packard 5890 GC Series II with a HP 5971A mass selective detector quadrupole equipped with a MS ChemStation (HPG1030A). The column was a HP-Ultra 2 (25 mm x 0.2 mm x 0.33 mm) and all injections were made in the splitless mode with hexane (ca. 2 ml) as solvent. The temperature program was initiated at 150°C, reached 250°C at 10 min and 325°C at 20 min. Diazomethane was prepared and materials converted to Me_3Si derivatives as in (8). Ultraviolet (UV) spectra were recorded using a HP8452 UV spectrophotometer.

Incubation of Hepatocytes with Ethanol- Hepatocytes were isolated from rats made selenium-deficient (3, 9). We have found that production of CL by hepatocyte cytosol is augmented by depletion of hepatic antioxidant defenses (9). Three hours after plating, hepatocytes were depleted of glutathione by addition of fresh medium containing 0.4 mM buthionine sulfoximine (10). Eighteen hours after plating, this medium change was repeated and the cells were then incubated with 10 mM ethanol for 6 hs (3). After incubation, the medium was decanted and processed using Sep-Pak and RP-HPLC (vide supra).

Chemotactic and Chemiluminescence Assays- Neutrophils were isolated from venous blood of healthy human volunteers. Cells suspensions contained >95-98% neutrophils, and chemotactic assays were performed as described (3). Lipid hydroperoxides eluted from RP-HPLC were detected in a reaction containing isoluminol and microperoxidase followed by fluorometric detection of chemiluminescence (11).

RESULTS

Arachidonic acid (50 mg) was incubated with xanthine oxidase and acetaldehyde (total vol. 56 ml) for 30 min in the dark. CL generated in this fashion was extracted using Sep-Pak C18 and chromatographed by RP-HPLC (Fig. 1, A). As expected, a wide range of compounds were generated in this system that exhibited a diversed array of structures when inspected by GC/MS. Biologically active material, however, eluted within 8-10 min (Fig. 1, B) and accounted for ≅ 0.03% of the initial material (based upon recovery of [^{3}H]-arachidonic acid tracer) (Fig. 1, C). It should be noted that virtually identical results were obtained with [1-^{14}C] arachidonic acid, indicating that CL carried radiolabel at carbon 1(not shown). As shown in Fig. 1, C, a wide array of products carried radiolabel. Biologically active fractions that also carried radioactivity were extracted and UV spectra were recorded in methanol. Scanning UV (220-400 nm) failed to reveal intense bands of absorbance indicative of conjugated double bonds (i.e., diene, triene, or tetraene).

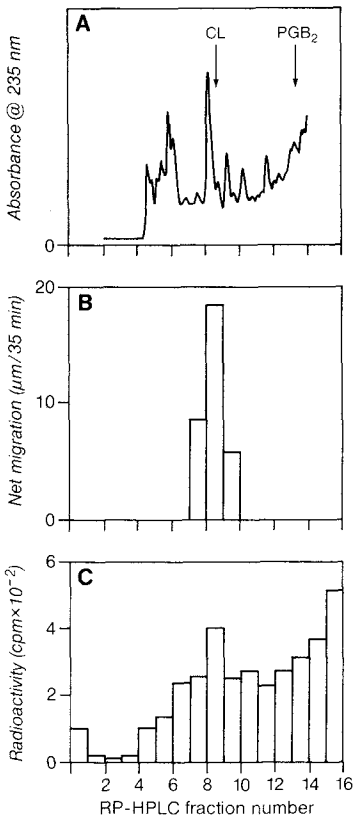

Fig. 1. **RP-HPLC profile and biological activity of material extracted following the incubation of arachidonic acid with xanthine oxidase and acetaldehyde.** [^{3}H]-arachidonic acid (1.0 mCi) was added as tracer and products extracted using a Sep-Pak cartridge. The methyl formate fraction was concentrated and subjected to RP-HPLC on an octadecylsilica column, at solvent flow of 1.0 ml/min. A. The UV detector was set at 235 nm. Arrows denote the elution positions of PGB_2 (synthetic standard) and of CL (determined by bioassay). The retention times for leukotriene B_4 (LTB_4), 20-OH-LTB_4 and 20-COOH-LTB_4 were 16.2 min, 6.1 min and 5.1 min, respectively. B. Chemotactic activity present in aliquots (1:100 dilution) of individual column fractions. Results are reported as net migration (total migration-random migration) (7) during the 35 min incubation (μm/35 min). C. Radioactivity of RP-HPLC fractions determined by scintillation counting of 50 μl aliquots of each fraction.

Aliquots of this material were treated with diazomethane and converted to OTMS derivatives (8). Further analysis by GC/MS showed that the major component of this material eluted as a relatively sharp peak (22.50-22.67 min) with an equivalent chain length corresponding to C24.4 (Ultra-2, HP). Its mass spectrum, (Fig. 2) gave prominent ions at m/z 399 (base peak; Me_3SiO^+ = CH-$(CH_2)_{17}$-$COOCH_3$), m/z 203, m/z 205 (Me_3SiO^+ = CH-CH_2-$OSiMe_3$), 341 (M-161, loss of SiO = $CHCO_2CH_3$), 315, 187 (205-18) and m/z 103 (CH_2=O^+SiMe_3). Less prominent ions were observed at m/z 502 (M), 487 (M-15, loss of CH_3), 443 (M-59, loss of CO_2CH_3), 412 (M-90), and 429 (M-73, loss of $(CH_3)_3Si^+$). Taken together, the UV, labelling and mass spectrometry data suggest that the major product obtained from fractions after RP-HPLC was a saturated methyl eicosatetraenoate carrying alcohol groups at carbons 19 and 20. The presence of a carboxylic acid

Table I. Effect of $NaBH_4$ reduction on the chemiluminescence and chemotactic activity of CL

Lower chamber	Chemiluminescence (relative fluorescence)	PMN migration (μm/35 min)	Ion at m/z 399 (relative abundance)
Buffer	---	86.5 ± 1.4	---
CL	10,172	112.3 ± 2.1	4.0×10^3
CL + $NaBH_4$	0	89.4 ± 1.2	4.0×10^5

Results represent mean values from one experiment performed in duplicate. Similar results were obtained in a separate experiment. For the chemotactic assays, CL was used at a dilution of 1:100.

at carbon 1 was supported by the radiolabel profile obtained with [1-^{14}C] arachidonic acid as precursor and the location of the hydroxyl groups was supported by high intensity of the ion at m/z 399 (base peak); an intensity suggestive of a vicinal diol. Furthermore, incubation of bioactive material obtained after RP-HPLC with soybean lipoxygenase (5.0 mg, for 30 min, pH 9.0) failed to generate chromaphores. This result supports the absence of cis-pentadienes in the parent structure and is consistent with the mass spectra data obtained with the methyl ester OTMS derivative which together indicate that the product is fully saturated.

Studies of the biological activity of CL indicated that it had a half-life of less than 24 h in aqueous solutions (not shown), suggesting that the chemotactic product contained a labile group (i.e., hydroperoxides). To assess this possibility, CL was generated as in Methods, extracted, resuspended in methanol and incubated in the presence or absence of $NaBH_4$ for 30 min. Reactions were then split, and equal aliquots were either stored in methanol (for GC/MS analysis) or evaporated with a stream of nitrogen, suspended in buffer and tested for the presence of chemotactic activity (Table I). Incubation of CL with $NaBH_4$ abolished its chemotactic activity. $NaBH_4$ had no effect on random motility, nor did it affect the chemotactic activity exhibited by FMLP (10^{-8} and 10^{-9}M) (not shown). Analysis of the reaction products by GC/MS revealed that the prominent ion at m/z 399 increased ≅ 100-fold in abundance after $NaBH_4$ treatment and biologically active fractions exhibited chemiluminescence that was also abolished by treatment with $NaBH_4$ (Table I).Injection of the derivatized material prior to treatment with $NaBH_4$ gave a weak ion at m/z 415 (≅ 5% of the base peak), consistent with a peroxy group at C19 that is labile and reduced to the alcohol. Although the present mass spectrum was obtained with derivatives prepared from incubations with arachidonic acid essentially identical GC retention time and mass spectrum were obtained with the bis(trimethylsilyl) derivative prepared from monostearin. For these experiments, monostearin (1.0 mg) was treated with bis-trimethylsilyl-trifluoroacetamide. The product eluted beneath a sharp peak with a retention time of 22.38 min. Its mass spectrum (Fig. 3)

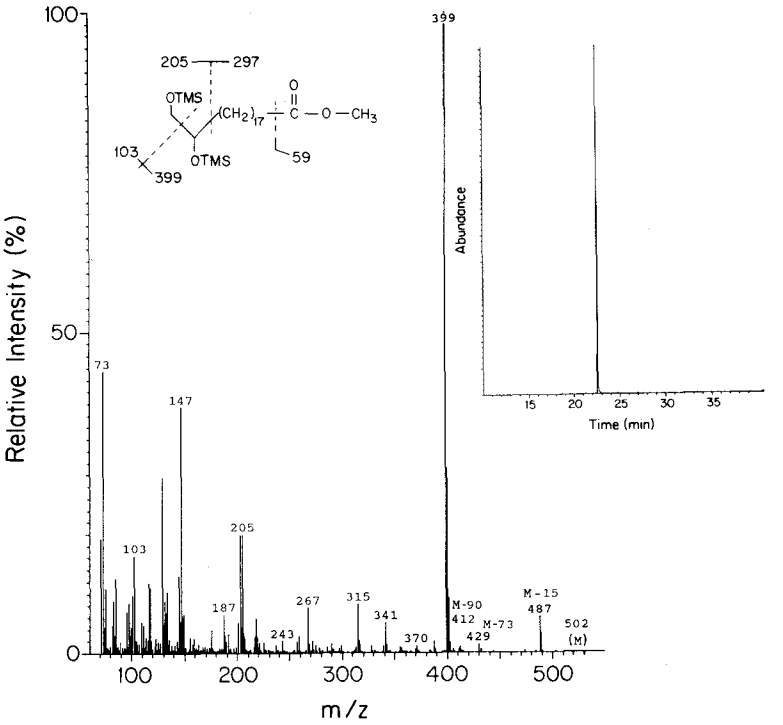

Fig. 2. **Mass spectrum of the methyl ester Me_3Si derivative of CL.** Left insert: assigment of prominent ions. The stereochemistry of the alcohol groups at carbons 19 and 20 remains to be determined. Right insert: gas chromatography-ion chromatogram (see Methods for temperature program). The column was a HP-Ultra 2 (25 mm x 0.2 mm x 0.33 mm) and the collected data was plotted as an ion (m/z 399.0) chromatograph.

gave prominent ions at m/z 399 (base peak; Me_3SiO^+ = CH-CH_2-OOC-$(CH_2)_{16}$-CH_3), 203, 205 (Me_3SiO^+ = CH-CH_2-$SiOMe_3$), 103 (M-399; loss of CH_2 = O^+Si $(CH_3)_3$), 341 (M-161; loss of SiO=CH-CO_2-CH_3) and 129 (CH_2=CH-CH^+OSi $(CH_3)_3$. Ions of lower intensity were observed at m/z 502 (M), 487 (M-15, loss of CH_3), 429 (M-73, loss of $(CH_3)_3Si^+$) and 412 (M-90, elimination of Me_3SiOH). The presence of these ions are consistent with the structure bis-(trimethylsilyl)monostearin which carries a stearic acid (n-octadecanoic) in the one position of a glycerol backbone (a monoacylglycerol). This mass spectrum was essentially identical to the spectra obtained with the methylester Me_3Si ether derivative of the product isolated from arachidonic acid incubations (Fig. 2). Thus, bis(trimethylsilyl)monostearin is a positional isomer of the methyl ester, Me_3Si ether derivative of 19,20-dihydroxyarachidic acid. The trimethylsilyl ether derivative of 2-monostearin (retention time 21.7 min), the position isomer of monostearin, gave prominent ions at m/z 129 (base peak), 191, 203, and 218 (approximately 80% of m/z 129), with less intense ions at m/z 267, 341, 399 (5% of base peak) 531 and 487. The dramatic difference between the spectra recorded for 2-monostearin trimethylsilyl ether and its isomer *bis* (trimethylsilyl) monostearin also suggests that a diol is present in CL product mass (Fig. 2). It should be noted that monosterin, at concentrations up to 0.1 μg/ml (concentrations at which CL is chemotactic) was not chemotactic for human neutrophils.

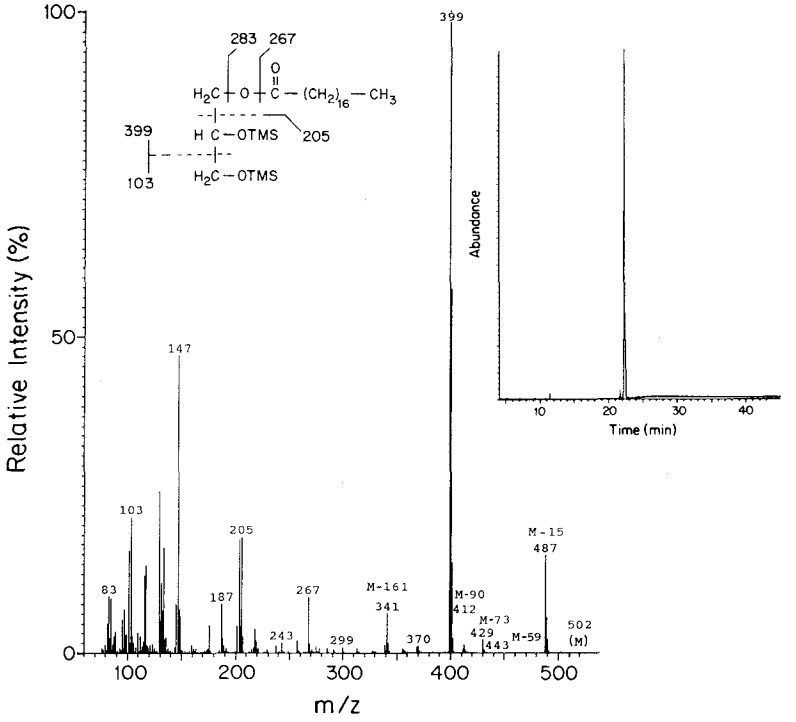

Fig.3. **Mass spectrum of the methyl ester bis Me_3Si derivative of monostearin.** Left insert: assignment of prominent ions. Right insert: total ion chromatogram.

Incubation of rat hepatocytes with ethanol (10 mM) results in the generation of CL (3). Biologically active supernatants obtained from ethanol-treated (10 mM, 6 h, 37°C) rat hepatocytes were processed as above and fractions exhibiting chemotactic activity (fractions #8-10 after HPLC) were treated with diazomethane, derivatized and analyzed by GC/MS. These combined fractions contained a product displaying a GC retention time and electron-impact fragmentation pattern that was essentially identical to that shown in Figs. 2 and 3, and gave prominent ions at m/z 399 (base peak, $Me_3SiO^+ = CH\text{-}(CH_2)_{17}\text{-}COOCH_3$), 203, 205 ($Me_3SiO^+ = CH\text{-}CH_2\text{-}OSiMe_3$) with lower intensity at m/z 341, 487 (M-15) and 502 (M). This product was not observed when ethanol was omitted from the hepatocyte incubations.

DISCUSSION

The results presented here provide evidence that the CL generated *in vitro* is a 19-hydroperoxy, 20-hydroxyarachidic acid. The proposed parent structure of CL is supported by its behavior in RP-HPLC, bioassays, chemiluminescence, absence of conjugated double bonds (UV spectroscopy, lack of chromaphores when incubated with lipoxygenase) and results from both radiolabeling and GC/MS analyses (Fig. 2, insert and Table I). Most biologically active eicosanoids are formed via specific enzymatic biosynthetic routes. Work by us (7) and others (12, 13) have provided

examples of free radical-mediated, non-enzymatic generation of biologically active products derived from arachidonic acid. Thus, the generation of bioactive lipids via a combination of both non-enzymatic and enzymatic routes working in concert may deserve further consideration in the pathophysiology of human diseases.

The mechanism involved in the generation of 19-hydroxperoxy, 20-hydroxyarachidic acid during exposure of arachidonic acid to free radical generating systems was not investigated in the present study. It is possible that the basic structure elucidated here contains a mixture of R and S isomers at carbons C19 and C20 since they were generated during non-enzymatic oxidation conditions. If present, these putative isomers did not separate in GC. The stereochemistry of the biologically active product will require total organic synthesis. Along these lines, several R and S configurations can be expected that can carry hydroperoxy groups at either 19 or 20 positions and may each carry biological activity to some degree.

The CL generated *in vitro* appears to be identical with respect to biological activity, migration on RP-HPLC and fragmentation pattern of its derivative on GC/MS (Results), to that obtained from ethanol-treated hepatocytes (3, 5). Recent evidence indicates a role for acetaldehyde, a cytosolic oxidase and free radicals in the generation of CL by ethanol-treated rat hepatocytes (5, 6, 9). Whether the CL is generated by the same or similar mechanism *in vitro* and *in vivo* remains to be determined. It appears that this material may be the first to carry biological activity in a human system with a fully saturated arachidic acid backbone structure. Whether other bioactive oxygenation products are derived from saturated fatty acids in human tissues remains to be determined. Interestingly, the chromatographic behavior of CL (Fig. 1, RP-HPLC) appears to correlate with the chromatographic characteristics exhibited by a recently reported (14) integrin modulating factor (IMF-1).

In summary, non-enzymatic oxidation of arachidonic acid generates a novel product which we identified as 19-hydroperoxy,20-hydroxyarachidic acid. This saturated hydroperoxy containing fatty acid product may be responsible, at least in part, for the neutrophilic infiltrate observed during the course of acute alcoholic hepatitis.

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