Assessment of 5-lipoxygenase involvement in human monocyte-mediated LDL oxidation[1]

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Abstract Lipoxygenase (LO) activity has been implicated in the process by which activated human monocytes oxidize normal human low density lipoprotein (LDL) and render it toxic to target cells. Here we examined the role of 5-LO in activated monocyte-mediated LDL modification. Five putative inhibitors of 5-LO (A63162, CGS8515, PF5901, RG6866, and MK886) were used to determine if they prevented activated monocytes from oxidizing LDL. Only RG6866, A63162, and CGS8515 inhibited monocyte-mediated LDL oxidation. Nonspecific effects of these drugs on LDL oxidation by activated monocytes were examined. RG6866 and A63162 were both found to be general antioxidants at their effective concentrations. CGS8515 was toxic at its effective concentration. A63162, CGS8515, and RG6866 also inhibited 15-LO activity in vitro. MK886 and PF5901 did not exhibit the nonspecific effects above and did not inhibit monocyte-mediated LDL oxidation, whereas both MK886 and PF5901 inhibited production of 5-LO metabolites by activated monocytes at concentrations that had no effect on LDL oxidation by the activated monocytes. Since neither of these agents inhibited LDL oxidation, we conclude that 5-LO is not involved in human monocyte oxidation of LDL. The possibility that a cellular 12- or 15-LO is involved in human monocyte-mediated LDL oxidation remains to be evaluated.—**Folcik, V. A., and M. K. Cathcart.** Assessment of 5-lipoxygenase involvement in human monocyte-mediated LDL oxidation. *J. Lipid Res.* 1993. **34:** 69–79.

Supplementary key words lipid peroxidation • human monocyte oxidation • oxidized lipoproteins • human monocyte activation • lipoprotein modification

Previous results from our laboratory have indicated that activated human monocytes oxidize LDL in a superoxide anion- and lipoxygenase-dependent manner, causing it to become toxic to proliferating fibroblasts (1, 2). Cell types other than human monocytes that are capable of oxidatively modifying LDL include neutrophils (3), aortic smooth muscle cells (4), endothelial cells (4), peripheral blood mononuclear cells (5), and mouse peritoneal macrophages (6, 7). In addition to our work with human monocytes, a role for the lipoxygenase enzyme in LDL oxidation has also been reported in LDL modification by rabbit aortic endothelial cells (8) and mouse peritoneal macrophages (9). Our laboratory has also shown that soybean lipoxygenase alone can cause LDL to become oxidized and cytotoxic (10).

Our laboratory has previously investigated the necessity for lipoxygenase activity in activated monocyte-mediated LDL oxidation and conversion to a form toxic to proliferating fibroblasts (2). The evidence that our laboratory has shown for LO involvement in monocyte-mediated LDL oxidation includes results from experiments using lipoxygenase inhibitors such as 5,8,11,14-eicosatetraynoic acid (ETYA) and reduced glutathione at concentrations that decreased LDL oxidation by activated monocytes and its subsequent toxicity while exhibiting minimal nonspecific properties such as general antioxidant capacity or toxicity to the monocytes. We have verified the effectiveness of ETYA for inhibiting cyclooxygenase and 5-LO activity using HPLC analysis of [^{14}C]arachidonic acid metabolites produced by activated monocytes in the presence and absence of ETYA (Folcik, V. A., and M. K. Cathcart, unpublished results). Our laboratory also found that cyclooxygenase inhibitors had no effect on LDL oxidation by the activated human monocytes (2). Similar results have been obtained by others regarding the lack of involvement of cyclooxygenase in modification of LDL by mouse peritoneal macrophages (7). Additional evidence for the participation of LO was provided by studies using copper II (3,5- diisopropylsalicylic acid)$_2$ (CuDIPS) an agent shown to enhance LO activity in vitro. CuDIPS increased monocyte-mediated LDL oxidation, an effect that may be explained by enhancement of intracellular lipoxygenase activity (2).

Abbreviations: LO, lipoxygenase; LDL, low density lipoprotein; CuDIPS, copper II (3, 5-diisopropylsalicylic acid)$_2$; PBS, phosphate-buffered saline; BCS, bovine calf serum; SLO, soybean lipoxygenase; HPLC, high performance liquid chromatography; ZOP, opsonized zymosan; TBARS, thiobarbituric acid reactive substances; ETYA, 5,8,11,14-eicosatetrayenoic acid.

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While our laboratory has developed a system using isolated human peripheral blood monocytes that requires activation of the monocytes for LDL modification and for conversion of LDL to a cytotoxin, others such as Parthasarathy et al. (6), Leake and Rankin (7), Rankin, Parthasarathy, and Steinberg (9), and Jessup et al. (11) have studied the ability of resident mouse peritoneal macrophages to modify LDL in transition metal-containing medium (Ham's F-10) where activation of the macrophages is not required. The goal of their studies was to determine how mouse peritoneal macrophages can oxidize human LDL and convert it into a form recognized and taken up via the mouse peritoneal macrophage scavenger receptor. Rankin et al. (9) have examined the role of lipoxygenase in their system and also reported that ETYA was capable of preventing LDL modification by mouse peritoneal macrophages.

Oxidized lipids are hypothesized to play a role in many diseases (12–15) and oxidatively modified LDL has properties that may render it atherogenic (reviewed in 16). Modified LDL is found in atherosclerotic plaques but not in normal vessels (17, 18). The ultimate goal of our studies is to determine how human monocytes modify LDL so that a means can be found to prevent such processes from occurring in vivo. Such knowledge would be valuable in providing a treatment that could help diminish the progression of tissue damage that occurs in atherosclerosis and chronic inflammatory diseases.

Monocytes are known to possess more than one type of lipoxygenase enzyme. 5-, 12- And 15-lipoxygenases are distinguished by the carbon of arachidonic acid to which they catalyze the addition of a hydroperoxyl group. 5-LO (19–22) and 15-LO (22) products of arachidonic acid metabolism have been found to be produced by activated human monocytes, and interleukin 4 has been shown to induce 15-LO activity in human monocytes (23). There are also reports of 12-LO activity in human monocytes; but such activity has been attributed by some to platelet contamination (20), as platelets are known to be a rich source of 12-LO. Stimulated porcine pulmonary intravascular and alveolar macrophages demonstrate both 5- and 12-LO activity in vitro (24) and mouse peritoneal macrophages have been shown to have 5-, 12-, and 15-LO activities (25–27).

Activated monocytes/macrophages produce potent inflammatory mediators such as leukotrienes and lipoxin (28, 29) via the 5-LO pathway. Products of the 5-LO pathway have also been shown to induce formation of reactive oxygen species by macrophages (30, 31). Because superoxide anion production is necessary for LDL oxidation by activated human monocytes (1), we sought to determine whether 5-LO activity was necessary for monocyte-mediated LDL oxidation and hypothesized that 5-LO products might regulate O_2^- production and affect the oxidation process. To address this issue, we used five chemically distinct and putatively specific 5-LO inhibitors and investigated whether they inhibited monocyte-mediated LDL oxidation. The drugs included MK886, PF5901, RG6866, CGS8515, and A63162. We have examined each of these drugs for specificity of action by evaluating their effects on 5- and 15-LO activity, their antioxidant properties and their effect on monocyte viability in addition to evaluating their effects on oxidation of LDL by activated human monocytes. Our results indicate that specific inhibition of 5-LO does not inhibit monocyte-mediated oxidation of LDL, suggesting that some other type of LO activity may be involved in monocyte-mediated LDL oxidation.

MATERIALS AND METHODS

Monocyte isolation

Human monocytes were isolated from freshly drawn, heparinized (LyphoMed Inc. Rosemont, IL) whole blood (2) or from a concentrated, citrated preparation of leukocytes obtained from apheresis performed on donors at the Cleveland Clinic Blood Bank. Whole blood or concentrated leukocytes were diluted 1:1 or 1:6 (respectively) with phosphate-buffered saline (PBS) without calcium or magnesium (Gibco Laboratories, Grand Island, NY or Whittaker Bioproducts Inc., Walkersville, MD) and centrifuged at 440 *g* over a cushion of Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ). The mononuclear cell layer was collected and washed twice with PBS, then resuspended and spun (150 *g*) 2–3 times through bovine calf serum (BCS; Hyclone Laboratories Inc., Logan UT) to remove platelets. The mononuclear cells were then resuspended in Dulbecco's Modified Eagle Medium (DMEM; Whittaker Bioproducts Inc.) with 10% BCS, antibiotic-antimycotic (100 units/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, and 0.25 μg/ml amphotericin C; Gibco Laboratories) and L-glutamine (2 mM; Whittaker Bioproducts Inc.) and allowed to adhere to BCS-coated 150-cm² flasks (Corning Glass Works, Corning, NY) for 1–3 h at 37°C in a humidified atmosphere with 10% CO_2 (32). After monocyte adherence, lymphocytes were removed by washing the flasks 4 times with 25 ml of prewarmed 10% BCS-DMEM. Greater than 98% monocytes were routinely obtained by this protocol as assessed by nonspecific esterase staining. The cells were cultured in 10% BCS-DMEM overnight or for one additional day before use in these experiments.

Monocyte-mediated LDL oxidation

Monocytes were removed from flasks with 20 ml of 5 mM EDTA-PBS (pH 7.4), and washed twice with RPMI-1640 (Whittaker Bioproducts Inc.), then resuspended, counted, and plated in 12-well (Costar, Cambridge, MA) tissue culture plates at 5×10^5 monocytes/

well in 1 ml of 10% BCS-DMEM. After adherence (1–2 h) the medium in the wells was aspirated and replaced with 1 ml of RPMI-1640 for the experiment. LO-inhibiting drugs were usually added at this point in 10 μl of dimethyl sulfoxide (DMSO; Sigma Chemical Co., St. Louis, MO). The drugs included MK886 (L-663,536; 3-[1-(4-chlorobenzyl)-3-t-butyl-thio-5-isopropyl-indol-2-yl]-2-2-dimethylpropanoic acid; Merck & Co. Inc., Rahway, NJ); CGS8515 (4-N-(2-amino-methylbenzoate)-1,2-o-naphthoquinone; Ciba-Geigy Corp., Summit, NJ); A63162 (N-hydroxy-N-[1-(4-phenylmethoxyphenyl) ethyl]acetamide; Abbott Laboratories, Abbott Park, IL); RG6866 (N-methyl-4-benzyloxyphenylacetohydroxamic acid; Rorer Central Research, Ft. Washington, PA); and PF5901 (alpha-pentyl-3-(2-quinolinylmethoxy)-benzene methanol; Purdue Frederick Company, Yonkers, NY). All of the 5-LO-inhibiting drugs were generous gifts of the aforementioned companies. ETYA was purchased from Cayman Chemical Company (Ann Arbor, MI). LDL was prepared by the Lipoprotein Core Laboratory (Department of Cell Biology, Cleveland Clinic Foundation) by modifications of the methods of Hatch and Lees (33) and Havel, Eder, and Bragdon (34). Prior to use in experiments, LDL was dialyzed in Spectra/Por 2 molecular porous membrane tubing (Spectrum Medical Industries, Inc., Los Angeles, CA) for 4–5 h against PBS at 4°C in the dark to remove EDTA (5 mM) used to inhibit oxidation during preparation. LDL was used in these experiments at 0.5 mg cholesterol/ml medium. After the cells had incubated with the 5-LO-inhibiting drugs for 15–30 min in serum-free RPMI, LDL was added to the cell culture plates. Opsonized zymosan (ZOP; 35) at 2 mg/ml medium was then added to activate the monocytes and the cell culture plates were incubated at 37°C, 10% CO_2 for 22–24 h. At the end of the incubation the plates were spun at 1000 g for 12 min and supernatants were collected in aliquots and stored frozen at −20°C for assay of LDL oxidation.

Assay for LDL oxidation

LDL oxidation was determined by detection of thiobarbituric acid reactive substances (TBARS), by the method of Schuh, Fairclough, and Haschemeyer (36) using malonaldehyde bis(dimethyl acetal) (MDA; Aldrich Chemical Co., Inc., Milwaukee, WI) as a standard. Briefly, 300-μl aliquots of monocyte supernatant were mixed with 750 μl of 25% trichloroacetic acid (Fisher Scientific, Fair Lawn, NJ) and 750 μl of 1% thiobarbituric acid (Sigma Chemical Co.). The samples were then incubated for 45 min at 95°C, after which they were centrifuged to pellet precipitated proteins. Fluorescence was detected on a Perkin-Elmer LS-3 Fluorescence Spectrometer at 515 nm excitation and 553 nm emission. Oxidation was expressed as TBARS in nanomoles of MDA-equivalents per ml of LDL-containing medium.

Assay for drug cytotoxicity

Drug toxicity to monocytes was determined by a modification of the method of Shirhatti and Krishna (37). Briefly, flasks of monocytes (approximately 1.5×10^7 cells/flask, 25 ml) were isolated as previously described and labeled overnight by incubation with 0.4 μCi [^{14}C]adenine (ICN Radiochemicals, Irvine, CA)/ml medium. Washed cells were plated in 12-well tissue culture plates as described above. After the medium was changed to RPMI-1640, LO inhibitors in DMSO or DMSO alone were added to the wells (final DMSO concentration in medium was 1.0% or less) followed by addition of ZOP (2 mg/ml). The plates were incubated for 24 h, centrifuged, and the supernatants were removed. Aliquots (200 μl) of the supernatants were mixed with scintillation fluid (CytoScint, ICN Biomedicals Inc., Irvine CA) and radioactivity (cpm) of [^{14}C]adenine metabolites released by the cells was detected with a Beckman LS-3801 beta-counter. The basic concept behind the assay is that damaged cells release the ^{14}C-labeled metabolic products (including ATP, ADP, and cAMP) while undamaged cells release relatively minor quantities. The data are expressed as:

$$\%\ \text{release} = \frac{\text{sample release} - \text{background}}{\text{SDS release} - \text{background}} \times 100$$

with background determined as the amount of [^{14}C]adenine metabolite release from a control with activated monocytes and 10 μl of the drug vehicle, DMSO. SDS was used to lyse the cells to determine the maximum possible release. Zero percent release (background) is interpreted as a lack of toxic effect and increased release is considered increased toxicity. Data were analyzed by the two-tailed, paired *t*-test where n = 3. Significant values were defined as having $P < 0.05$.

$CuSO_4$ and SLO-mediated LDL oxidation

$CuSO_4$ and soybean lipoxygenase (SLO)-mediated LDL oxidation assays were carried out in 12-well tissue culture plates in a 1 ml volume of RPMI-1640 and incubated for 24 h at 37°C in 10% CO_2 to imitate monocyte assay conditions, but without cells (2, 10). LO inhibitors were added as described for monocyte assays. $CuSO_4$ (Fisher Scientific Co.) was used at 2 μM to oxidize LDL at 0.5 mg cholesterol/ml, and affinity-purified SLO type V (Sigma Chemical Co.) was used at approximately 5000 U/ml. Supernatants were harvested and assayed for LDL oxidation as TBARS, as previously described.

HPLC separation of [^{14}C]arachidonic acid metabolites

Monocytes were isolated as described above, and plated in 12-well tissue culture plates in 10% BCS-DMEM at 1×10^6/well (1 ml). The cells were labeled with 1 μCi

(19 nmol) of [^{14}C]arachidonic acid (NEN Research Products, Dupont, Wilmington DE)/ml of medium, overnight. The medium was changed to RPMI-1640, LO inhibitors (or DMSO) were added, and ZOP was added to activate the cells. At the end of a 30-min incubation, 176.5 μl of ethanol (200 proof, Aaper Alcohol and Chemical Co., Shelbyville, KY) was added to the wells to yield 15% ETOH, and supernatants from three wells were pooled and centrifuged. Tritiated 12-HETE (2.5 nCi of 12(S)-hydroxy[5,6,8,9,11,12,14,15(n)]-[^{3}H]eicosatetraenoic acid, Amersham Corporation, Arlington Heights, IL) was added to the samples as an internal standard. This allowed calculation of extraction efficiency and quantitative comparison of [^{14}C]arachidonic acid metabolite formation. (No ^{14}C-radiolabeled 12-HETE was detected under the conditions described.) Samples were then acidified with 200 μl of 10% formic acid. Arachidonic acid metabolites were extracted by solid-phase extraction on C18 Sep-Pak cartridges (Waters Division of Millipore, Milford, MA) by the method of Powell (38). The supernatants were passed through the Sep-Pak cartridges, followed by 15 ml of water to wash the syringe containing the sample; then 20 ml of 15% acidified ETOH (pH 3) was added, followed by 20 ml of water to remove the ETOH, then 20 ml of petroleum ether (OmniSolv, E. M. Science, Gibbstown, NJ) to remove neutral lipids and water, and finally 5 ml of ethyl acetate (HPLC Grade, Aldrich Chemical Co., Inc.) was used to extract the arachidonic acid metabolites. The samples were then dried under nitrogen while shielded from light and stored at -20°C under N_2 until HPLC analysis. Reverse phase-HPLC was performed with a Rainin Microsorb C18 column (250 mm × 4.6 mm, Rainin Instrument Co., Inc., Woburn, MA) and a Rainin Dynamax Microsorb C18 column (250 mm × 4.6 mm), a Beckman Model 421A controller, Model 114M pumps and a Model 163 Variable Wavelength detector. Radioactivity was detected with an online Radiomatic Instruments Flow-one Beta Radioactive Flow Detector. Data were processed with the Flow-one Beta program on an IBM Personal Computer XT, Lotus 1-2-3 software (Lotus Development Corporation), and GraphPAD InPlot (GraphPAD Software, San Diego, CA). Packard Radiomatic Flo-Scint III (Meriden, CT) was used with solvent system A and Packard Radiomatic Flo-Scint II was used with solvent system B. Two different solvent systems were used due to slight differences in column chemistry of the two columns used. Solvent system A was used with the Rainin Microsorb column and consisted of a gradient of acetonitrile (HPLC grade, Fisher Scientific) and water adjusted to pH 4 with phosphoric acid, (J. T. Baker Chemical Co., Phillipsburg, NJ) beginning with 32% acetonitrile for the first 5 min, with an increase to 50% acetonitrile in the next 5 min, and an increase from 50% to 100% acetonitrile in the next 40 min. After 5 min at 100% acetonitrile, initial conditions were restored (39, 40). Solvent system B was used with the Rainin Dynamax Microsorb column and consisted of 80% methanol (HPLC grade, Fisher Scientific), 20% water, and 0.01% acetic acid (27) delivered isocratically for 21 min, then changed to 100% acetonitrile over the next 10 min, followed by 10 min at 100% acetonitrile; then the solvents were restored to initial conditions. The 100% acetonitrile was necessary to expedite arachidonic acid elution.

The samples were reconstituted in 250 μl of mobile phase, vortexed, sonicated, and filtered through 0.45-micron nylon-66 filters (Rainin Instrument Co., Inc.) before injection. Leukotriene and prostaglandin standards for determination of metabolite retention times were purchased from Cayman Chemical Co. and Biomol (Plymouth Meeting, PA).

RESULTS

To determine the necessity for 5-LO in activated human monocyte-mediated LDL oxidation, five putatively specific 5-LO inhibitors were employed. The drugs were included in 24-h incubations of activated monocytes with LDL and their effect on monocyte-mediated LDL oxidation was assayed as TBARS. The data are expressed as a percentage of LDL oxidation by activated monocytes in the presence of the drug vehicle, DMSO. DMSO had no effect on LDL oxidation induced by any of the means used. Spontaneous LDL oxidation (that which occurred in the absence of cells) and in the presence of drugs or vehicle (where appropriate) was determined and subtracted from all samples. None of the drugs promoted LDL oxidation and there was no monocyte-mediated LDL oxidation detected in the absence of monocyte activation.

Among the drugs tested, RG6866, A63162, and CGS8515 inhibited LDL oxidation by activated human monocytes (**Fig. 1**). The inhibitory effect was dose-dependent with RG6866. Fig. 1 also shows that MK886, the drug that has been reported to have the most specific mechanism of action for 5-LO, had no inhibitory effect on LDL oxidation by activated human monocytes when used at concentrations reported to inhibit leukotriene biosynthesis in human leukocytes (41, 42). In another experiment, MK886 at 0.1 and 0.3 μM was preincubated with monocytes for 1.5 h in serum-free RPMI medium prior to addition of LDL and ZOP. MK886 was without inhibitory effect under these conditions as well (data not shown). PF5901 also had no effect on LDL oxidation by activated human monocytes (Fig. 1) when used in a concentration range known to affect leukotriene production by human lung tissue (43) and canine PMN (44) in vitro.

The contradictory results among the drugs emphasized the necessity for determining their specificity. There were several possible means by which inhibitory effects could

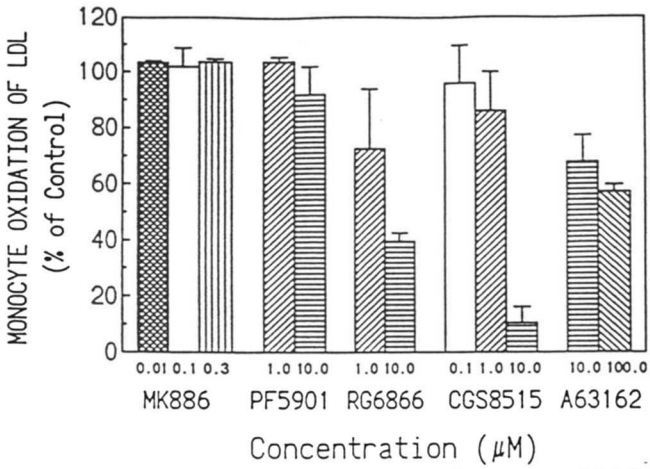


Fig. 1. Monocyte-mediated LDL oxidation in the presence of 5-LO inhibitory drugs. Monocytes (5×10^5) in a 1-ml volume were activated in the presence of LDL (0.5 mg cholesterol/ml) and the drugs indicated or DMSO (the drug vehicle) for 24 h and the supernatants were assayed for LDL oxidation as TBARS. The data are expressed as a percentage of the LDL oxidation mediated by activated monocytes in the presence of an appropriate quantity of the drug vehicle, DMSO. The error bars represent the spread of the mean of two experiments performed in duplicate with the exception of MK886 (0.1 μM) where they represent the standard error of the mean of four experiments performed in duplicate. The concentrations of drugs are indicated.

be exerted aside from inhibition of 5-LO activity in the activated monocytes. One possibility was that a lipoxygenase activity other than that of 5-LO could have been inhibited in the activated monocytes. Another possibility was that the inhibition could be via general antioxidant or metal ion chelating capacity of the drugs. A third was that the drugs might be toxic to monocytes. Each of the above was examined.

Our laboratory has previously shown that soybean lipoxygenase, a plant seed 15-LO, is alone capable of oxidatively modifying LDL, causing it to become toxic to proliferating target fibroblasts (10). Under conditions previously established (2, 10) the ability of all of the drugs to inhibit 15-LO-mediated LDL oxidation was determined. LDL oxidation was measured as TBARS and the data are expressed as a percentage of 15-LO-mediated LDL oxidation in the presence of drug vehicle alone. Spontaneous LDL oxidation in the absence of enzyme was subtracted from all samples with the appropriate concentrations of drug or drug vehicle included. Without exception the drugs that were capable of inhibiting LDL oxidation by activated human monocytes also inhibited 15-LO modification of LDL (**Fig. 2**), whereas, in contrast, MK886 and PF5901 did not inhibit 15-LO-mediated LDL oxidation.

The drugs were also evaluated for their general antioxidant capacity, a property that could account for apparent effectiveness in inhibiting both activated monocyte-mediated and 15-LO-mediated LDL oxidation. Our laboratory has previously established conditions for $CuSO_4$-mediated LDL oxidation that mimic the level of LDL oxidation by activated monocytes (2, 10). The ability of a compound to prevent $CuSO_4$ from oxidizing LDL is interpreted as general antioxidant capacity. The data in **Fig. 3** are expressed as a percentage of LDL oxidation measured as TBARS mediated by 2 μM $CuSO_4$ in the presence of the drug vehicle, minus the spontaneous LDL oxidation in the absence of $CuSO_4$ and with the appropriate concentration of drugs present.

The results presented in Fig. 3 demonstrate that all drugs exhibiting effective inhibition of LDL oxidation by activated monocytes and inhibition of SLO-mediated LDL oxidation were also effective at inhibiting $CuSO_4$-mediated LDL oxidation. Neither MK886 nor PF5901 were antioxidants under the conditions and concentrations tested. These results corroborate the specificity of these drugs and previous reports that they are not antioxidants (11, 45).

To evaluate cytotoxicity of the drugs, we used an assay of [^{14}C]adenine metabolite release from monocytes that had incorporated the [^{14}C]adenine into intracellular pools (37). The results presented in **Fig. 4** demonstrate that among the drugs that affected LDL oxidation by activated monocytes, only CGS8515 was significantly toxic at its effective concentration. A63162 was not toxic at 10 μM but had an unexpected effect on monocytes at 100 μM. The release of [^{14}C]adenine metabolites in the presence of 100 μM A63162 was significantly less than that found for vehicle-treated, activated monocytes. Such a phenomenon might be explained by the hydrophobic nature of the drug and a possible cell membrane interaction that

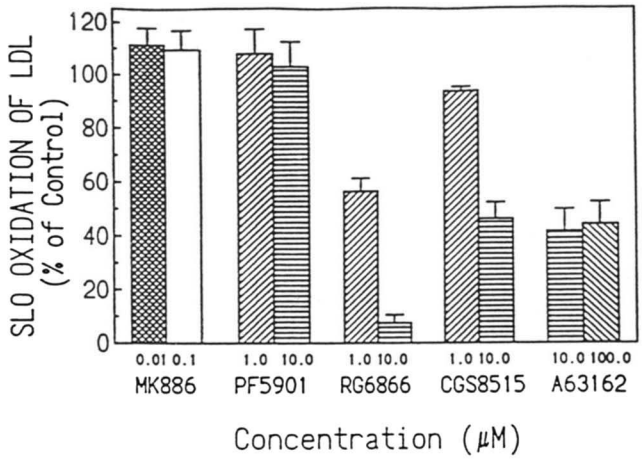


Fig. 2. SLO-mediated LDL oxidation in the presence of 5-LO-inhibiting drugs. LDL (0.5 mg cholesterol/ml) was incubated with SLO (5000 U/ml) in a 1-ml volume for 24 h in the presence of the drugs or the drug vehicle (DMSO). The data are expressed as a mean percentage of SLO-mediated LDL oxidation (measured as TBARS) in the presence of DMSO. The error bars represent the SEM of three experiments performed in duplicate. The concentrations of the drugs are indicated.

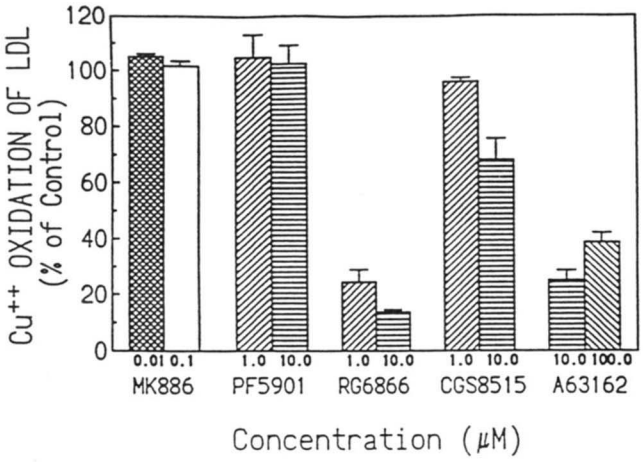


Fig. 3. $CuSO_4$-mediated LDL oxidation in the presence of 5-LO-inhibiting drugs. LDL (0.5 mg cholesterol/ml) was incubated for 24 h with $CuSO_4$ (2 μM) in a 1-ml volume, and LDL oxidation was assayed as TBARS. The data are expressed as a mean percentage of LDL oxidation mediated by $CuSO_4$ in the presence of the drug vehicle, DMSO. The error bars represent the SEM of three experiments performed in duplicate. The concentrations of the drugs are indicated.

markedly decreases the membrane fluidity. (Controls demonstrated that A63162 is not a quenching agent.) PF5901 was found to be toxic at 100 μM, but the decrease in release of [^{14}C]adenine metabolites was not significant at 10 μM. MK886 was not toxic at any concentration tested, a result consistent with previous reports (41).

The results just described suggest that 5-LO activity is not the lipoxygenase activity required for activated human monocyte-mediated LDL oxidation. To strengthen this conclusion it was necessary to determine that the 5-LO-specific drugs actually inhibited 5-LO activity within the monocytes. MK886 and PF5901 were both without effect on monocyte-mediated LDL oxidation and exhibited no nonspecific effects at the concentrations tested, so these were the drugs tested to determine whether they altered the profile of metabolites of [^{14}C]arachidonic acid produced by activated monocytes, as determined by HPLC analysis. These assays were performed in the absence of LDL to avoid any possible interference of the endogenous antioxidants within LDL in such a short incubation. The results shown in **Fig. 5** are from a representative experiment. Fig. 5A shows that in the absence of a stimulus, formation of [^{14}C]arachidonic acid metabolites was not detectable. Monocytes activated in the presence of drug vehicle produced cyclooxygenase and lipoxygenase metabolites from [^{14}C]arachidonic acid that had been incorporated into the cells during a 24-h preincubation with the radiolabeled substrate (Fig. 5B). Peak 1 shares the retention time of a prostaglandin standard and peaks 2 and 3 share the retention times of components of a standard mixture of several forms of leukotriene B_4. In Fig. 5C, MK886 at 0.1 μM decreased leukotriene formation by 40–50% and caused no decrease in prostaglandin production, confirming the specificity of MK886 and its effectiveness at the concentration used. It should be noted that in the assays of inhibition of LDL oxidation by activated monocytes three times this quantity of MK886 (0.3 μM) had no inhibitory effect (Fig. 1). PF5901 at 10 μM caused a 60–75% decrease in leukotriene formation and also a 66% decrease in prostaglandin production by activated monocytes (Fig. 5D). Thus MK886 and PF5901 inhibited 5-LO activity in activated human monocytes.

PF5901 apparently had an unknown nonspecific inhibitory effect on lipid metabolism by the activated monocytes at the concentration used as there was 40% less [^{14}C]arachidonic acid detected in the sample supernatants in the form of metabolites and free arachidonic acid compared to the control with activated monocytes and the drug vehicle DMSO. Drugs were not present during [^{14}C]arachidonic acid labeling of the monocytes so uptake was not affected. The overall decrease in [^{14}C]arachidonic acid and its metabolites was not due to a difference in extraction efficiency as the recovery of [^{3}H]12-HETE used to spike the samples was not decreased in the sample with PF5901. This effect of PF5901 was reproducible.

DISCUSSION

In light of the evidence that lipoxygenase activity may be involved in LDL oxidation by activated human mono-

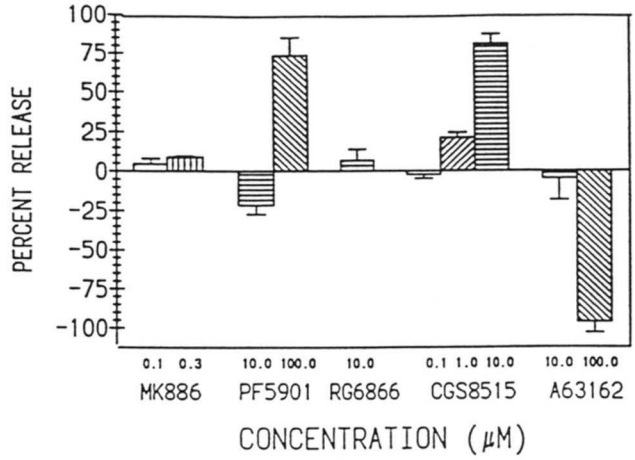
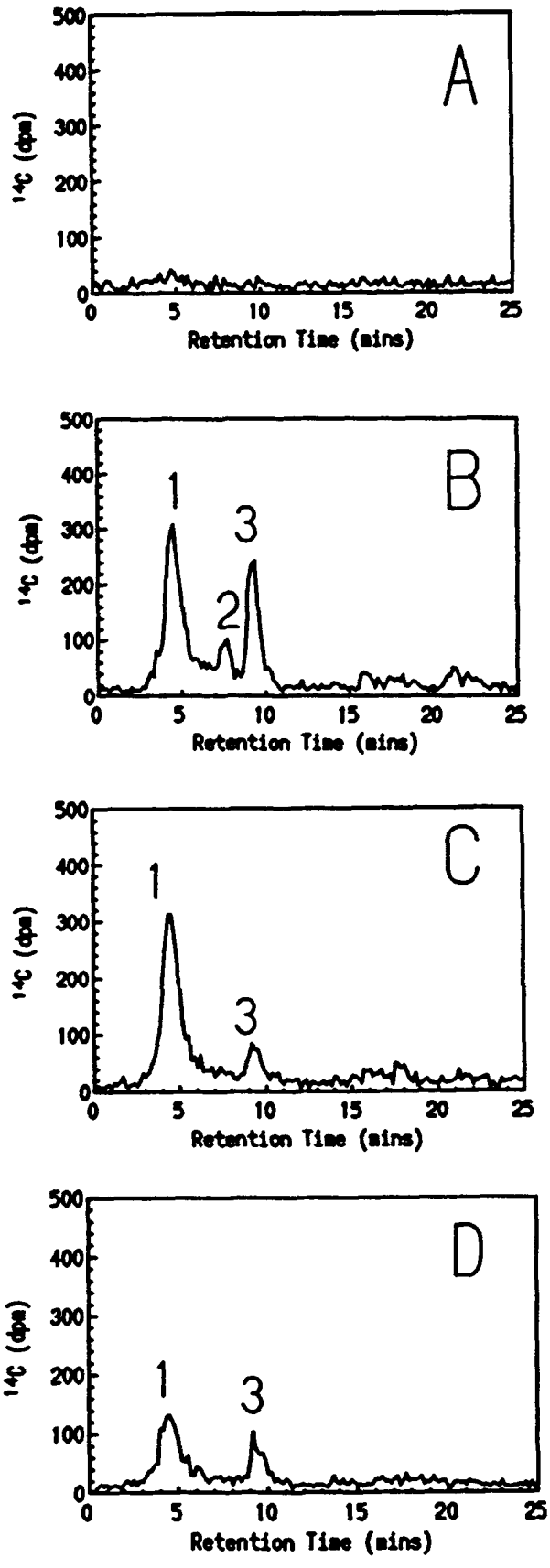


Fig. 4. Drug toxicity to monocytes. Monocyte ATP-pools were labeled by incubation of monocytes overnight with [^{14}C]adenine. The monocytes were washed and plated as described for LDL oxidation experiments and activated in the presence of drugs or the drug vehicle, DMSO. Release of [^{14}C]adenine metabolites was determined as cpm by scintillation counting of supernatants and calculated as described in Materials and Methods. The data are expressed as a mean percentage of the release found with complete lysis of the cells. The error bars represent the SEM of three experiments performed in duplicate, except for MK886 at 0.3 μM, PF5901 at 100 μM, and A63162 at 10 μM where the error bars represent the spread of the mean of two experiments performed in duplicate. The concentrations of the drugs are indicated.



cytes, we examined the role of 5-LO in our system. Monocytes/macrophages are known to produce 5-LO metabolites when stimulated with particulate activators such as ZOP (19, 21), the activator that we use in our system. We hypothesized that the 5-LO enzyme played a role in an activation pathway leading to the respiratory burst, as others have reported the ability of fatty acids and 5-LO products to elicit a respiratory burst in macrophages, and our laboratory has previously demonstrated the necessity for superoxide anion in monocyte-mediated LDL oxidation. In the results presented here we have investigated the necessity for 5-LO activity using five drugs reported to have specificity for inhibiting cellular 5-LO.

MK886 (also known as L-663, 536) has been shown to irreversibly block leukotriene synthesis in human leukocytes without affecting cyclooxygenase or 12-LO activity (41, 42). MK886 derives its high degree of specificity from its mechanism of action involving inhibition of a 5-LO-activating protein, or "FLAP", that is necessary for 5-LO activity in intact cells (46, 47). FLAP is a hydrophobic protein with structural similarity to integral membrane proteins and it has been suggested to be the membrane anchor for activated 5-LO (48). MK886 binds FLAP and blocks the membrane translocation of 5-LO and its subsequent activation (42). PF5901 (REV 5901) is a drug that was designed to specifically inhibit 5-LO by virtue of structural similarity to 15-HETE, a known inhibitor of 5-LO (49, 50). The heterocyclic nature of PF5901 is reported to render it specific for 5-LO and inactive against 12-LO and cyclooxygenase (49). PF5901 (REV 5901) has been shown to inhibit production of 5-LO metabolites as well as acting as a peptido-leukotriene receptor antagonist (44, 49). Others have found PF5901 not to have antioxidant properties (45). RG6866 is an inhibitor of isolated 5-LO and reported to be more potent than PF5901 in that regard. It has also been shown to prevent antigen-induced anaphylaxis in guinea pigs (51). The effects of RG6866 are mediated by preventing formation of 5-LO metabolites as it has been found to have no leukotriene antagonist capability as determined by bioassay (51, 52). A63162 is a 5-LO inhibitor found to be effective at inhibiting physiological phenomena that are presumed to be mediated by

Fig. 5. HPLC separation of [^{14}C]arachidonic acid metabolites. Shown is a representative experiment in which monocyte lipid pools were prelabeled by incubation overnight with [^{14}C]arachidonic acid. External arachidonic acid was removed and the cells were stimulated with opsonized zymosan in the presence or absence of the drugs. The supernatants were harvested 30 min after stimulation. The lipids were then extracted and separated on reversed phase-HPLC with solvent system B. A: Unstimulated monocytes. B: Monocytes stimulated in the presence of the drug vehicle, DMSO. C: Monocytes stimulated in the presence of 0.1 μM MK886. D: Monocytes stimulated in the presence of 10.0 μM PF5901. The number 1 indicates the retention time of prostaglandins and 2 and 3 indicate the retention times of leukotrienes.

leukotrienes (53). Both RG6866 and A63162 belong to a class of compounds that are arachidonic acid analogs designed to inhibit lipoxygenases by virtue of an N-substituted hydroxamate group that interacts with and reduces the non-heme ferric iron of lipoxygenase thereby inactivating the enzyme (54, 55). CGS8515 is a 5-LO inhibitor reported to prevent formation of 5-LO arachidonic acid metabolites in human and mammalian leukocytes (56). It is reported not to affect cyclooxygenase activity, 15- and 12-LO, and thromboxane synthetase at doses up to 100 μM (56). All of the drugs used were added in DMSO to yield a final concentration of 1.0%, and controls contained the same quantity of DMSO. DMSO is well known to possess hydroxyl-radical scavenging ability but had no effect on LDL oxidation in the systems that we analyzed.

When the putative 5-LO inhibiting drugs were tested to determine their effects on monocyte-mediated LDL oxidation, RG6866, A63162, and CGS8515 inhibited the LDL modification while MK886 and PF5901 did not. One of the inhibitors, CGS8515, was toxic to the activated monocytes at its only effective concentration, and also served as an antioxidant at the same concentration. It was further demonstrated that two of the drugs, RG6866 and A63162, decreased LDL oxidation mediated by 15-LO and $CuSO_4$, suggesting nonspecificity. A63162 demonstrated a lack of dose-dependence in this regard that may be attributable to low aqueous solubility.

The inhibition of all of three means of LDL oxidation tested by RG6866 and A63162 has several interesting implications. First, the hydroxamic acids are designed to reduce the metal ion center of lipoxygenases and inhibit their activity. As these agents inhibited SLO- and $CuSO_4$-mediated LDL oxidation it appears that they may form complexes with the metal ion center of SLO and/or $CuSO_4$ and interfere with oxidation. Furthermore, there is evidence that similar agents can scavenge lipid radicals in metal ion-free environments (11, 57) suggesting an alternative mechanism for inhibition in these three systems for LDL oxidation. While we may speculate on the possibility that RG6866 (having exhibited a dose-dependent inhibition without toxicity) may be inhibiting a cellular 15-LO, such interpretation must take into account that the mechanisms of such lipoxygenase inhibition are indistinguishable from inhibition of radical-mediated lipid peroxidation.

The two remaining 5-LO inhibitors, PF5901 and MK886, had no nonspecific effects on LDL oxidation at the concentrations tested. HPLC analysis of the 5-LO products of arachidonic acid metabolism produced by the activated monocytes in the presence and absence of MK886 and PF5901 verified that the drugs were inhibiting 5-LO activity within the monocytes, while allowing them to oxidize LDL. MK886 caused notable inhibition of leukotriene formation in the activated monocytes, without affecting prostaglandin production or [^{14}C]arachidonic acid release. Our results agree with those of Rouzer et al. (42), who found that MK886 demonstrated an IC_{50} of 102 nM for leukotriene biosynthesis in human leukocytes. PF5901 inhibited leukotriene production by the activated monocytes but also inhibited prostaglandin formation. Therefore, we conclude that specific inhibition of 5-LO activity in activated monocytes has no effect on their ability to oxidize LDL.

After the original submission of this manuscript, Sparrow and Olszewski (58) published a paper questioning the role of any lipoxygenases in LDL oxidation by mouse macrophages in Ham's F-10 medium. Their results indicated no contribution of 5-LO to this process and thereby confirm the findings of Jessup et al. (11). In additional studies using linoleic acid as LO substrate, Sparrow and Olszewski (58) reported that ETYA inhibits mouse macrophage 15-LO at ETYA concentrations 100-fold lower than those required to inhibit macrophage-mediated LDL oxidation. They concluded that 15-LO is not involved in LDL modification by these cells. We understand the rationale for drawing these conclusions; however other interpretations of these data are possible as well. First, the measurements of 15-LO activity were performed in the absence of LDL. We find that in the presence of LDL, less [^{14}C]linoleate is converted to product by 15-LO in human monocytes (Folcik, V. A., and M. K. Cathcart, unpublished observations) suggesting either that a large unlabeled substrate pool is available from LDL or that the labeled linoleic acid partitions into the LDL and therefore is less readily available to the cells. LDL could have a similar effect on ETYA, reducing its interaction with the lipoxygenase. An analogous effect has been shown to pertain to 15-LO from soybeans. Increasing quantities of linoleic acid competed for LO binding and prevented inhibition of LO activity by ETYA (59). Perhaps more importantly, 15-lipoxygenases have been shown to be able to oxidize fatty acids that are esterified either in phospholipids (60) or in cholesteryl esters (61 and Folcik, V. A., and M. K. Cathcart, unpublished observations). As the majority of fatty acids on LDL are esterified to cholesterol, triglyceride, or phospholipid, it is likely that the availability of esterified fatty acids far exceeds that of free fatty acids. One might therefore expect that an inhibitory analog of free fatty acid, such as ETYA, may not prove to be as effective or efficient an inhibitor of the oxidation of esterified fatty acids as it is for the free fatty acids which it resembles. Thus we question whether the need for larger quantities of ETYA to prevent LDL modification by macrophages, as presented by Sparrow and Olszewski (58), could be explained by the competitive effect of LDL in the assay of 15-LO activity and/or by the possible difference in substrate form provided by LDL. In order to minimize possible dilution of the hydrophobic 5-LO inhibitory

drugs by LDL, the compounds were allowed to incubate with the cells in RPMI medium for 15–30 min prior to addition of LDL or ZOP. The results of Fig. 5 demonstrate that this is sufficient time for the drugs to enter the cells and alter 5-LO activity.

Sparrow and Olszewski (58) also point out that ETYA is toxic to mouse peritoneal macrophages in Ham's F-10 medium at about twice the concentration required to inhibit cell-mediated LDL modification. Jessup et al. (11) also report toxicity of ETYA to mouse peritoneal macrophages in Ham's F-10 medium in the absence of serum. Jessup et al. (11) found it necessary to include lipoprotein-deficient serum in the incubations of mouse peritoneal macrophages in Ham's F-10 with ETYA and LDL to limit its toxic effect. In our studies, up to 30 μM ETYA is not toxic to ZOP-activated human monocytes in RPMI-1640 medium in the presence of human LDL at 0.5 mg cholesterol/ml for up to 24 h, as quantitated by the assay of [^{14}C]adenine metabolite release (less than 10% release in several determinations). We therefore believe that the inhibitory effect of ETYA in our system is not due to toxicity. In our view, the question of the role of lipoxygenases in monocyte/macrophage-mediated LDL oxidation remains open.

Studies of human atherosclerotic lesions have provided some circumstantial evidence that 15-LO may be involved in the modification of LDL found in atherosclerotic lesions. Ylä-Hertuala et al. (62) have used monoclonal antibody and cDNA probes to show that oxidized LDL, 15-LO mRNA, and 15-LO protein are found in areas of atherosclerotic lesions where macrophages are concentrated. The presence of esterified hydroxyoctadecadienoic acids, possible 15-LO products, was demonstrated in human atherosclerotic plaques more than 20 years ago (63). Evidence from studies such as these emphasizes the need for further investigation into the potential role of 15-LO in the atherosclerotic process.

We conclude from our investigation that 5-LO activity is not essential for human monocyte-mediated LDL oxidation. MK886 has proved in our studies to be a valuable tool for the study of 5-LO activity in monocyte-mediated LDL oxidation. Although our previous data showing ETYA- and glutathione-mediated inhibition of LDL oxidation by activated human monocytes do not definitively demonstrate a role for lipoxygenases in this process, neither of these agents is toxic when LDL is included in the incubation in RPMI-1640, and neither agent serves as a general antioxidant for LDL oxidation at its effective concentration (2). Additionally, we have shown that an agent that enhances 15-LO activity increases LDL oxidation by activated human monocytes (2) and large amounts of "15-LO like" products are found in monocyte modified LDL (Folcik, V. A., and M. K. Cathcart, unpublished observations). We believe that these data are not inconsistent with some role for a lipoxygenase in LDL modification by these cells. Other methods must be used to investigate whether LO activity actually has an essential role in LDL oxidation by activated monocytes and other cell types.

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