

Mouse Peritoneal Macrophages Contain Abundant ω -6 Lipoxygenase Activity That Is Independent of Interleukin-4

Joseph A. Cornicelli, Kathryn Welch, Bruce Auerbach, Steven J. Feinmark, Alan Daugherty

Abstract The action of an ω -6 lipoxygenase (LO) has been implicated in the development of atherosclerosis through a mechanism involving oxidation of LDL, and its regulation in macrophages may have important implications for the disease process. Human monocyte-derived macrophages (HMDMs) showed no demonstrable LO protein or activity unless they were incubated with interleukin-4 (IL-4). In contrast, mouse peritoneal macrophages (MPMs) possessed significant basal levels of LO activity and protein that were augmented by IL-4 treatment. Interferon gamma prevented the induction of LO in both HMDMs and MPMs. Whereas interferon gamma could completely block the IL-4 induction of LO in human cells, it did not suppress basal LO activity in MPMs. Both HMDMs and MPMs exhibited similar concentration-response relationships for stimulation of LO activity and protein, with maximal induction at 1 ng/mL IL-4. The time course of IL-4 induction of LO activity was markedly different in human and murine cells. IL-4 induced LO activity

and protein in human cells by 48 hours that were maximal by 72 hours; there was a decline to a new baseline by 96 hours. MPMs have a significant amount of LO activity at baseline, which declined with time by nearly 10-fold in the absence of IL-4. IL-4 blunted the decline of LO activity with time and restored activity to that found at baseline by 48 hours. IL-4 was not responsible for the LO activity present in freshly isolated MPMs since both activity and protein content were similar in cells harvested from IL-4^{+/+} and IL-4^{-/-} mice. Therefore, whereas IL-4 may be an important modulator of LO production in vitro, it is not essential for the in vivo expression of this protein. Further, these studies demonstrate that significant differences exist between monocyte-derived macrophages matured in vitro and tissue macrophages that have matured in vivo. (*Arterioscler Thromb Vasc Biol.* 1996;16:1488-1494.)

Key Words • interleukin-4 • lipoxygenase • macrophage • regulation

The LOs are a group of non-heme iron-containing enzymes that insert molecular oxygen into the 1,4-*cis, cis* pentadiene of polyunsaturated fatty acids. Mammalian enzymes identified to date are capable of performing this reaction at the 5, 12, or 15 carbon of arachidonic acid, and hence have been named 5-, 12-, and 15-LO, respectively. 5-LO has been extensively studied with regard to its role in generating the potent mediators of inflammation, leukotrienes.¹ The biological functions of 12- and 15-LO have not been as well characterized, although there is some evidence for their involvement in lipoxin synthesis.¹

Among these enzymes, 15-LO is unique in its ability to oxygenate polyenoic fatty acids esterified to various membrane lipids² and lipoproteins.^{3,4} The ability to oxidize esterified fatty acid has led to the speculation that the occurrence of 15-LO in reticulocytes is responsible for the degradation of mitochondrial membranes and subsequent red cell maturation.⁵⁻⁷ In addition to reticulocytes, 15-LO has been found in eosinophils,⁸ airway epithel-

ium,⁹⁻¹² pancreatic β -cells,¹³ and in intestinal cells derived from subjects with inflammatory bowel disease.¹⁴ The enzyme is absent from freshly isolated human monocytes and HMDMs.¹⁵ 15-LO mRNA and protein have been detected in atherosclerotic lesions in humans and rabbits in locations coincident with macrophages and epitopes of oxidized LDL.^{16,17} These observations may have particular significance for the pathogenesis of atherosclerosis in that it is believed that oxidation of LDL is a key feature in the generation of macrophage foam cells.¹⁸

A related 12-LO exists in porcine, bovine, and murine leukocytes. This LO, while distinct from the 12-LO of human platelets, is highly homologous to the 15-LO found in rabbit and human tissues at the molecular and immunologic level.^{19,20} Presentation of arachidonic acid to the leukocyte 12-LO gives the primary reaction product of 12-hydroperoxyeicosatetraenoic acid (12-HPETE) and small amounts of 15-HPETE. Conversely, human and rabbit 15-LO yield predominantly 15-HPETE from arachidonate and small amounts of the 12 positional isomer. Both leukocyte 12-LO and human and rabbit 15-LO produce 13-hydroperoxyoctadecadienoic acid when given linoleic acid as substrate.¹⁹ The molecular and immunologic characterization of the murine 12-LO have been described,²⁰ and the molecular basis for the differences in the positional specificities between the two groups of enzymes has been defined.²¹⁻²⁴ In view of the similarities of the human and homologous murine enzymes, these enzymes could be referred to collectively as ω -6 LOs.

The finding that 15-LO is not present in freshly isolated human monocytes or HMDMs led Conrad and coworkers¹⁵ to examine the regulation of expression of the en-

Received November 14, 1994; revision accepted April 24, 1996.

From the Department of Atherosclerosis Therapeutics (J.A.C., K.W., B.A.), Parke-Davis Pharmaceutical Research Division, Warner Lambert Co, Ann Arbor, MI; the Department of Pharmacology (S.J.F.), College of Physicians and Surgeons, Columbia University, New York, NY; and the Cardiovascular Division (A.D.), Department of Medicine, Washington University School of Medicine, St. Louis, Mo.

Correspondence to Joseph A. Cornicelli, PhD, Department of Vascular and Cardiac Disease, Parke-Davis Pharmaceutical Research, 2800 Plymouth Rd, Ann Arbor, MI 48105. E-mail cornicj@aa.wl.com.

© 1996 American Heart Association, Inc.

Selected Abbreviations and Acronyms

DMEM	= Dulbecco's modified Eagle's medium
FBS	= fetal bovine serum
HMDM	= human monocyte-derived macrophage
HODE	= hydroxyoctadecadienoate
IFN- γ	= interferon gamma
IL-4	= interleukin-4
LO	= lipoxygenase
MPM	= mouse peritoneal macrophage
PAGE	= polyacrylamide gel electrophoresis
PBS	= phosphate-buffered saline
rh	= recombinant human
rm	= recombinant murine
RP-HPLC	= reversed-phase high-performance liquid chromatography
SDS	= sodium dodecyl sulfate
SP-HPLC	= straight-phase high-performance liquid chromatography

zyme by various immune-response modulators. They have demonstrated that IL-4 and IFN- γ are potent regulators of 15-LO mRNA and activity in HMDMs. Of all the cytokines tested, IL-4 alone was capable of inducing 15-LO expression and activity. The effects of IL-4 were blocked by coinubation with IFN- γ . We compared the regulation of ω -6 LO expression in HMDMs and MPMs to determine the importance of IL-4 *in vitro*. Studies with cells obtained from IL-4-deficient mice²⁵ were performed to address the contribution of IL-4 to the regulation of LO expression *in vivo*.

Methods

Materials

DMEM, l-glutamine, penicillin, and streptomycin were purchased from GIBCO. Cytokines, along with their respective neutralizing antibodies, were obtained as rh and rm proteins from R&D Systems and were used with their respective cell types. rh- and rm-IFN- γ were purchased from Bio Source International. FBS and human AB serum were obtained from Hyclone Laboratories and Sigma Chemical Co, respectively. SDS gels (4% to 12%) and nitrocellulose blotting membranes were obtained from Schleicher and Schuell, radiolabeled and enhanced chemiluminescence reagents were from Amersham, and fresh elutriated human monocytes were obtained from Advanced Biotechnologies. IL-4 $-/-$ and $+/+$ mice were a generous gift from Dr O. Kanagawa, Washington University, St Louis, Mo, and Dr M. Kopf, Max-Planck-Institute for Immunobiology, Freiburg, Germany.

Cells

Resident MPMs were isolated from male Swiss Webster and IL-4-deficient mice by lavaging the peritoneal cavity with DMEM. The cells were centrifuged, resuspended in DMEM containing 10% FBS, and seeded into six-well tissue-culture plates at a concentration of 1×10^6 cells/well. MPMs were allowed to adhere to plastic overnight in a humidified incubator at 37°C with an atmosphere of 5% CO₂ and 95% air before the wells were extensively washed with DMEM, and fresh culture medium containing 10% FBS was added in the presence or absence of rm-IL-4 at a concentration of 10 ng/mL, unless stated otherwise. After this the cells were placed in the incubator at 37°C for an additional 96 hours; culture media and cytokines were replaced after 48 hours.

Freshly elutriated human monocytes were resuspended in human culture media consisting of high-glucose DMEM, 20% FBS, 10% human AB serum, antibiotics, and l-glutamine. More than 95% of the cells were determined to be monocytes as determined by differential cell counts and positive staining for CD14 and CD15. Cells were dispensed into polytetrafluoroethylene-coated

culture beakers at a density of 3 to 5×10^7 cells in a volume of 30 mL media and placed in a tissue-culture incubator for 5 days to allow the monocytes to mature to macrophages. Approximately 50% to 70% of the cells were recovered as viable. These cells were collected by centrifugation, resuspended in fresh human culture medium, and seeded into six-well tissue-culture plates at a density of 2×10^6 cells/well. Adherent HMDMs were incubated for an additional 72 hours, after which the medium was replaced with fresh human culture medium with or without rh-IL-4 at a concentration of 10 ng/mL, unless otherwise noted. HMDMs were cultured in the presence of IL-4 for 96 hours, with media and cytokines replaced after 48 hours, before the experiments were initiated.

Cell viability was monitored in all cultures throughout the experiment either by counting nuclei directly²⁶ or by photographing random fields of several culture wells, counting the number of cells per unit area, and then calculating the total number of cells per culture. These methods gave results that were internally consistent with one another. Human and murine macrophages cultured in the presence or absence of IL-4 differed in number only by $\approx 10\%$ at the end of the experiment and represented $\approx 90\%$ of the cells originally plated.

15-LO and Anti-15-LO

15-LO was isolated from rabbit reticulocyte lysates.⁷ Polyclonal antiserum directed against rabbit 15-LO was obtained by inoculating sheep with the purified protein and isolating the IgG fraction by affinity chromatography. This antiserum cross-reacts with both human and mouse ω -6 LO.

Cellular 15-LO Activity Assay

Macrophages (2×10^6) were scraped into PBS, placed in 15-mL conical tubes, and pelleted by gentle centrifugation. PBS was removed, and 450 μ L iced reaction buffer (PBS and 0.2% sodium cholate, pH 7.4) was added to the cell pellets. Cell pellets were sonicated on ice over 10 3-second cycles at a power setting of 1.5 using a Branson sonifier to liberate the cytosolic enzyme. 15-LO activity was determined under reaction conditions.²⁷ Briefly, 100- μ L aliquots of the sonicated cell suspension were incubated with 360 μ mol/L linoleic acid that was dissolved in a small volume of EtOH. The solution was incubated on ice for 10 minutes, and the reaction was stopped by adding an equal volume of mobile phase (acetonitrile/water/methanol/acetic acid, 350:250:150:1), followed by the addition of trimethylphosphite to reduce the hydroperoxides to the corresponding hydroxy fatty acids. Products were identified and quantified by using RP-HPLC against known standards of 13(S)-HODE. To verify that the RP-HPLC fraction accurately reflected the enzymatic oxidation of linoleate, the purified 13(S)-HODE fraction was rechromatographed by using SP-HPLC with a Nucleosil 100 Silica 5- μ column (250 \times 4.6 mm) eluted with hexane/isopropanol/acetic acid (98:2:0.1), and the HODE fractions were isolated. Over 90% of the hydroxy fatty acid was 13-HODE, with the remainder present as 9-HODE. The 13-HODE peak was collected, converted to its methyl ester by reaction with diazomethane, and fractionated on a Chiracel OB column (250 \times 4.6 mm) eluted with hexane/ethanol (100:2) at 0.8 mL/min at 40°C. Chromatography was performed by using authentic samples of 13(S)-HODE and a racemic mixture of 13-HODE.

Western Blot Analyses

Individual wells of cultured macrophages were scraped into SDS sample buffer, and lysates were subjected to SDS-PAGE on 4% to 12% gradient gels. Separated proteins were transferred to nitrocellulose paper by using the method of Towbin et al.²⁸ Blots were blocked with 5% dried milk, incubated with anti-15-LO, and then incubated with peroxidase-conjugated goat anti-sheep antibody. Immunopositive bands were visualized by reaction with diaminobenzidine to produce a colored product or by using enhanced chemiluminescence. Authentic 15-LO was included as

a positive control; nonimmune sheep IgG was used as a negative control.

Results

The oxidation of linoleate by ω -6 LO results in the formation of a chiral product, 13-(S)-hydroperoxyoctadecadienoic acid, as opposed to a racemic mixture that would result from nonenzymatic oxidation. Chiral analysis of the 13-HODE fraction after RP- and SP-HPLC chromatography (Fig 1A) demonstrated that the 13-HODE derived from the cell lysate reaction and measured with the RP-HPLC column was 13(S)-HODE and therefore accurately reflects ω -6 LO activity. Treatment of HMDMs or MPMs with IL-4 resulted in upregulation of ω -6 LO activity (Fig 1B and 1C). As reported by Conrad et al,¹⁵ 15-LO activity in nonstimulated HMDMs was undetectable. Treatment of these cells with IL-4 resulted in a dramatic increase in activity that was completely blocked by preincubating rh-IL-4 with its neutralizing antibody. In contrast, MPMs cultured for 96 hours in the absence of IL-4 displayed considerable ω -6 LO activity. LO activity was augmented in MPMs by incubation with rm-IL-4 and blocked by preincubation of the cytokine with a neutralizing antibody. Incubation of HMDMs and MPMs with the appropriate neutralizing antibody alone had no effect on enzyme activity (data not shown). Increased enzyme activity was accompanied by an increase in cellular LO protein. Fig 2A shows the absence of detectable LO protein in untreated HMDMs (lane 2) and in cells incubated with neutralizing antibody alone (lane 3). Extracts prepared from HMDMs incubated with IL-4 revealed a band of immunoreactivity in lane 4 that comigrated with authentic 15-LO (lane 1). The presence of immunodetectable LO was prevented by treating cells with IL-4 that had been preincubated with neutralizing IL-4 antibody (lane 5). Similar results were obtained when MPMs were used (Fig 2B and 2C). When increasing amounts of cell extract from control (lanes 2 through 5) or IL-4-treated (lanes 6 through 9) cells were subjected to Western blot analyses, an enhanced signal was observed from the IL-4-incubated cells. This observation held true whether the blots were prepared from extracts containing identical amounts of protein (Fig 2B) or from extracts of equal cell numbers (Fig 2C). Densitometric scans of these blots revealed that the signal intensity from IL-4-incubated preparations was about twice that of control extracts (bottom panels).

Treatment of HMDMs or MPMs with IL-4 in the presence of IFN- γ did not result in any induction of LO activity or protein (Fig 3), and the suppression of induction of LO activity was concentration dependent. Whereas the effects in human and murine cells were qualitatively similar, they were quantitatively different. Human cells responded to IFN- γ over a concentration range of 1 to 50 U/mL. LO activity was not detectable in HMDMs exposed to high concentrations of rh-IFN- γ , as reflected by the absence of immunoreactive protein on Western blots (data not shown). Murine cells were much more sensitive (about two orders of magnitude) to the effects of rm-IFN- γ . The maximal suppression achieved in these cells resulted in LO activity that was identical to the level of activity observed in cells not treated with IL-4.

The experiments described above were performed with IL-4 at a concentration of 10 ng/mL. Investigations were conducted to determine the concentration-response relationship for IL-4 and the induction of LO activity (Fig 4).

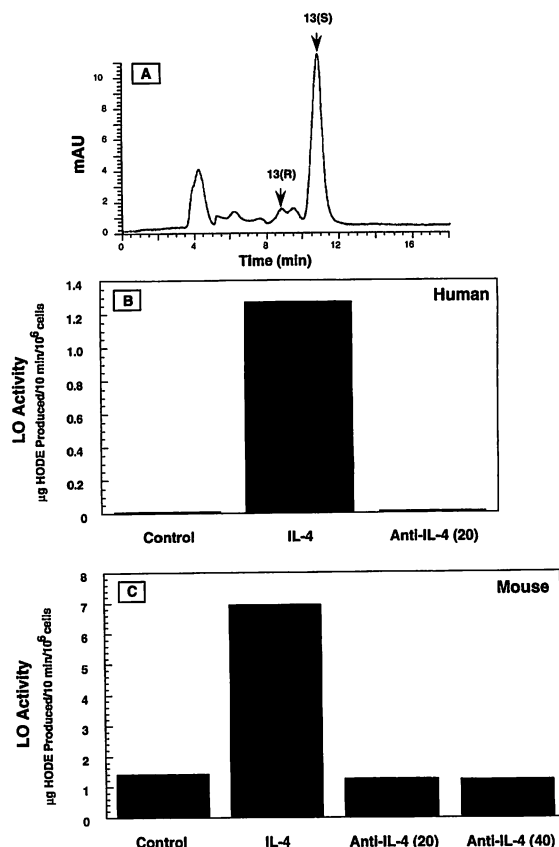
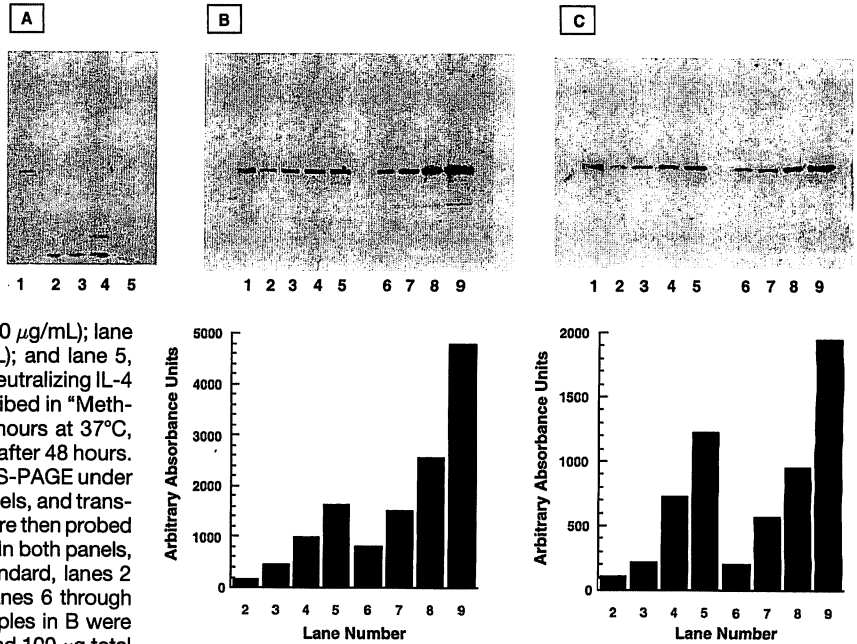


Fig 1. A, Macrophage ω -6 LO activity was assayed from cells treated with IL-4 as described in "Methods." The hydroxy fatty acid peak obtained from RP-HPLC was fractionated by SP-HPLC, where >90% of the eluted product was 13-HODE. This peak was methylated with diazomethane and analyzed by chiral-phase HPLC on a Chiracel OB column. The retention times of authentic (R) and (S) isomers are shown. This tracing reflects activity in material obtained from HMDMs; essentially identical results were obtained from MPMs. B and C, Bar graphs. IL-4 was preincubated in the presence or absence of specific neutralizing antibodies for 1 hour at 37°C, added at a final concentration of 10 ng/mL to HMDMs (B) or MPMs (C) and incubated for 48 hours at 37°C. The medium was changed, preincubated IL-4 was added, and cells were incubated for an additional 48 hours at 37°C. Cells were harvested and lysed in assay buffer, and LO activity was determined. Values are the mean of duplicate determinations and are representative of three separate experiments. Concentrations of anti-IL-4 used were 20 μ g/mL for experiments using human cytokine, and 20 or 40 μ g/mL for incubations with murine reagents.

Human and murine cells responded in a similar manner to the range of concentrations of IL-4 tested. Substantial changes in enzyme activity could be detected with concentrations of IL-4 as low as 0.3 ng/mL, with maximal activity occurring at \approx 1 ng/mL.

The time course of induction of LO activity was determined in HMDMs and MPMs. LO activity in human cells was not detectable until after 48 hours of exposure to IL-4 (Fig 5A); it rose significantly at 72 hours and declined at 96 hours to a level that was still easily detectable and well above baseline. The time course of enzyme induction in MPMs was somewhat different (Fig 5B). At the time of isolation (0 hour), MPMs produced 12 to 14 μ g HODE/10⁶ cells in a 10-minute incubation. In our experiments, this activity declined with time in the absence of IL-4 such

Fig. 2. A, IL-4 was preincubated in the presence or absence of neutralizing antibody (20 $\mu\text{g}/\text{mL}$) for 1 hour at 37°C before addition to HMDMs at a final concentration of 10 ng/mL. Cells were then treated as described in Fig 1. After a 96-hour incubation, cells were harvested, lysed, subjected to SDS-PAGE under reducing conditions on 4% to 12% gradient gels, and transferred to nitrocellulose membranes. Filters were then probed with antibody to rabbit reticulocyte 15-LO. Lane 1, Rabbit reticulocyte 15-LO; lane 2, control cells; lane 3, cells incubated with anti-IL-4 alone (20 $\mu\text{g}/\text{mL}$); lane 4, cells incubated with IL-4 alone (10 ng/mL); and lane 5, cells incubated with IL-4 preincubated with neutralizing IL-4 antibody. B and C, MPMs prepared as described in "Methods" were exposed to IL-4 or buffer for 96 hours at 37°C, with a change to fresh medium and cytokine after 48 hours. Cells were harvested, lysed, subjected to SDS-PAGE under reducing conditions on 4% to 12% gradient gels, and transferred to nitrocellulose membranes. Filters were then probed with an antibody to rabbit reticulocyte 15-LO. In both panels, lane 1 contains rabbit reticulocyte 15-LO standard, lanes 2 through 5 lysates from control MPMs, and lanes 6 through 9 samples from cells cultured with IL-4. Samples in B were normalized for protein, loading 12.5, 25, 50, and 100 μg total cell protein in lanes 2 through 5 and 6 through 9, respectively. The data in C reflect results obtained when lysates from equal cell numbers were applied. Lysate from 0.125, 0.25, 0.5, and 1.0 $\times 10^5$ cells were loaded in lanes 2 through 5 and 6 through 9, respectively. Bar graphs show densitometric quantification of blots B and C.



that by 72 to 96 hours, the time at which the previous experiments were begun, there was nearly a 10-fold reduction in LO activity. The inclusion of rm-IL-4 in the culture medium restored LO activity to a level somewhat greater than that measured at 0 hour, and although it seemed to decline with time throughout the course of the experiment, it remained significantly elevated relative to the level of LO activity in cells cultured in the absence of IL-4. At 96 hours the differences in LO activity between IL-4-treated and control cultures were essentially the same as those observed in Fig 1.

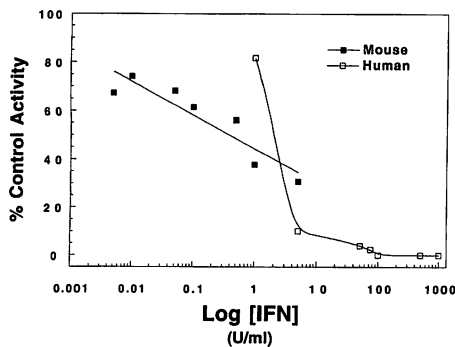


Fig 3. Plot shows IFN- γ prevention of stimulation of LO activity by IL-4 in HMDMs and MPMs, which were prepared as described in Fig 1. Cell preparations were treated with rh- or rm-IL-4 (10 ng/mL) in the presence of the indicated concentrations of recombinant IFN- γ from the appropriate species. Cultures were incubated at 37°C for 48 hours, after which the medium was removed, replaced with fresh medium and cytokines, and incubated for an additional 48 hours. Cells were harvested and lysed in assay buffer to determine LO activity. Values are presented as means of duplicate determinations from three experiments and are given as the percentage of LO activity observed in cells treated with IL-4 in the absence of IFN- γ .

Our observations that IL-4 dramatically induces LO protein and activity in vitro and that MPMs possess substantial LO protein and activity while HMDMs do not led us to hypothesize that IL-4 regulates the synthesis of LO in vivo. We reasoned that since mast cells are a rich source of IL-4,²⁹ and since these cells are numerous in the peritoneal cavity, the peritoneal macrophages expressed LO in response to local concentrations of IL-4. To test this hypothesis, we harvested macrophages from the peritoneal cavities of IL-4 $-/-$ and $+/+$ mice.²⁵ Freshly isolated MPMs from animals that were homozygous for IL-4 deficiency actually exhibited enhanced LO activity (Fig 6). When extracts prepared from identical numbers of cells from IL-4 $-/-$ and $+/+$ mice were subjected to Western blot analyses, a slight increase in the amount of LO protein expressed in the cells from the deficient animals was also observed.

Discussion

As reported by Conrad et al,¹⁵ human IL-4 selectively regulates expression of LO in HMDMs in culture. LO enzyme is absent from cultured HMDMs when assayed by a variety of techniques. Treatment of the cells with IL-4 resulted in time- and concentration-dependent expression of both protein and enzymatic activity. MPMs, on the other hand, possess considerable LO activity and protein in the absence of exogenous IL-4, both of which quickly diminished with time in culture. The addition of exogenous IL-4 to these cultures permitted the cells to maintain LO protein and activity at levels comparable to those in freshly isolated cells. Therefore, both HMDMs and MPMs are capable of responding to IL-4 with respect to LO in vitro, albeit starting from significantly different baseline values.

Interactions between signaling molecules of the immune system provide a convenient method for homeostatic control. IL-4 modifies some of the effects of IL-2 and IFN- γ ,

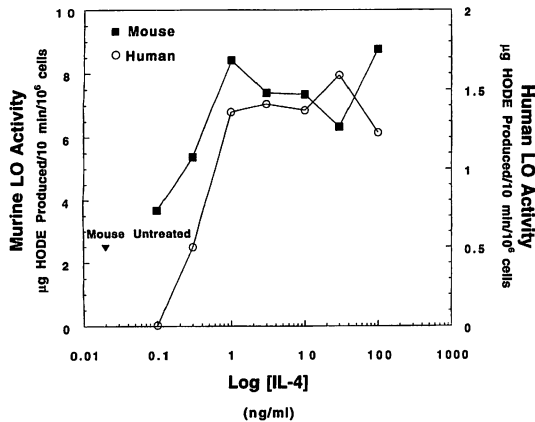


FIG 4. Graph shows concentration-response relationships between IL-4 and LO activity in HMDMs and MPMs, which were prepared as described in the text. Each cell type was cultured in the presence or absence of IL-4 at the indicated concentrations for 48 hours at 37°C. Medium was changed, fresh IL-4 was added, and cells were incubated for an additional 48 hours at 37°C. Each cell type was assayed for LO activity. LO activity was undetectable in untreated HMDMs; that observed in untreated MPMs is shown.

while IFN- γ modulates many of the actions of IL-4.³⁰ In both MPMs and HMDMs, IFN- γ suppresses the IL-4 induction of LO. In human cells, <10 U/mL IFN- γ can block induction by >90%. At this concentration, LO activity in murine cells, while significantly reduced, is still comparable to that observed in non-IL-4-treated cells in the absence of IFN- γ , although it is possible that it might be diminished at higher concentrations. These observations demonstrate that both MPMs and HMDMs can respond similarly to the actions of IFN- γ on LO in vitro, ie, the level of LO achieved in the presence of the cytokine is essentially that observed at baseline.

Preincubation of human and murine IL-4 with the appropriate neutralizing antibody was performed to ensure that the observed effects were not related to contaminants of the recombinant proteins. In each case, the neutralizing antibody was quite effective at preventing the induction of LO. In the case of HMDMs, the antibody totally prevented the induction of the enzyme. In the murine system, the antibody prevented the IL-4 induction of LO above baseline. It did not, however, suppress the preexisting enzyme, even when the amount of neutralizing IL-4 antibody was doubled. This suggests that the preexisting LO in MPMs was derived from a signal generated in vivo.

An investigation into the regulation of LO expression in vivo was performed with mice made deficient for IL-4 by targeted gene disruption. A potential source for IL-4 that may affect MPMs in vivo is mast cells, which are widely distributed throughout the peritoneal cavity and contain IL-4.²⁹ If the induction of LO activity in MPMs was absolutely dependent on IL-4 from mast cells or other IL-4-producing cells of the peritoneal cavity, this enzyme would not be found in freshly isolated MPMs from IL-4-deficient mice. Our results demonstrated that the presence of LO and its activity were not compromised by a deficiency of IL-4. One must be careful, however, not to overinterpret these negative results. IL-4 is known to affect the development of various lymphoid cells in vitro,³¹ yet in IL-4-deficient mice T and B cell development is normal.³² "Normal" lymphoid development and "normal" levels of

LO in these animals do not necessarily imply that IL-4 does not play a role in the generation of T and B cells or in the regulation of LO expression. Rather, it means that whereas IL-4 is an important determinant of LO induction in vitro, its role in vivo may be fulfilled by other signal-transducing molecules. It is quite possible that the related cytokines IL-10 and/or IL-13^{33,34} may substitute for IL-4 in the deficient mice. In fact, Nassar et al³⁵ have shown that IL-13 is capable of inducing LO activity in HMDMs in vitro. Our work provides evidence that this observation may be physiologically relevant in vivo.

The present study demonstrated a significant difference between freshly harvested MPMs and HMDMs. Considerable LO activity resides in freshly isolated MPMs, whereas HMDMs are devoid of the protein and its activity. IL-4 is able to induce LO in both cell types with virtually identical dose-response relationships, which suggests that the differences in LO activity are due not to differences in responsiveness to the cytokine, but rather to signal(s) generated in vivo. Macrophages derived from human peripheral blood are matured in culture from monocytes, whereas tissue macrophages have emigrated from the circulation into the various organs and spaces. The manifold differentiation signals to which the cells are exposed in the latter instance are likely to be quite different from those in the former. This could account for the observed quantitative differences. In support of this hypothesis, Levy and co-workers³⁶ have demonstrated basal LO protein and activity in human alveolar macrophages that were enhanced by

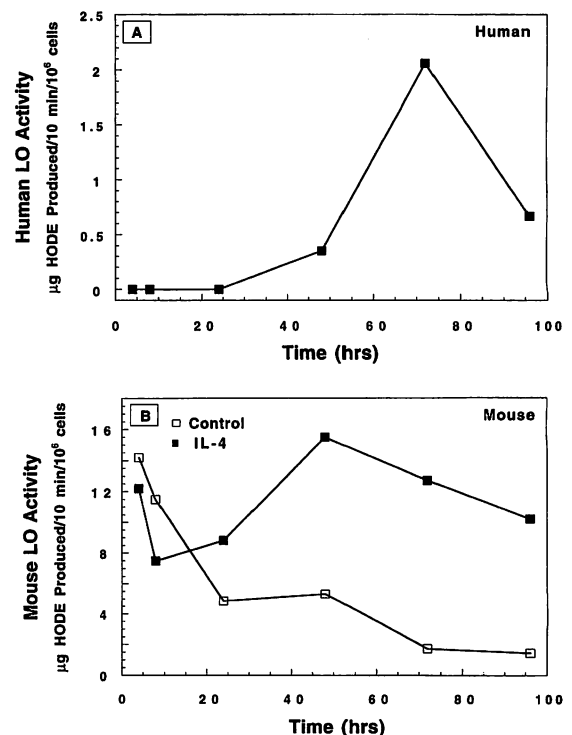


FIG 5. Line graphs show time course of IL-4 induction of LO activity in HMDMs (A) and MPMs (B), which were prepared as described in the text. One set of cultures was treated with recombinant IL-4 (10 ng/mL) from the appropriate species, and cultures were taken at the indicated times for the measurement of LO activity. A second set of cultures that was not treated with IL-4 was also tested. No detectable LO activity was found in the HMDMs that were not exposed to IL-4.

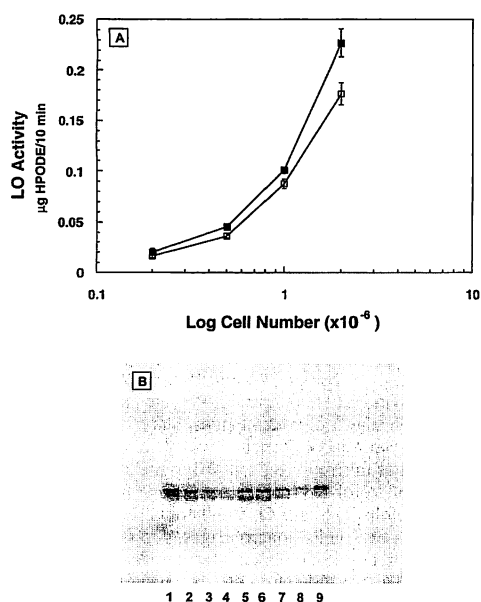


FIG 6. A, Graph. MPMs were obtained from IL-4 $-/-$ (■) or $+/-$ mice as described in the text. The cells were counted, pelleted, divided into aliquots containing the indicated numbers of cells, and lysed in assay buffer, and LO activity was determined as indicated. B, An identical number of cells were prepared for SDS-PAGE, electroblotted onto nitrocellulose, and subjected to Western blot analysis by using an antibody to rabbit reticulocyte LO. Lanes 1 through 4, cell extracts from control MPMs; lanes 5 through 8, cell extracts from IL-4-deficient mice; lane 9, rabbit LO standard. Cell extracts are from 2×10^6 , 1×10^6 , 5×10^5 , and 2×10^5 cells for each series.

IL-4. Furthermore, the quantitative aspects of their observations are in reasonable agreement with those we obtained in MPMs.

A good deal of discussion has centered on the proposition that LO may play a pivotal role in atherosclerosis (for a review, see Reference 37). The control of 15-LO expression could be important in determining the pathogenesis of the disease. This takes on particular significance in view of the growing appreciation of the participation of lymphocytes in atherogenesis.^{18,38-43} Lymphocytes are important sources of the cytokines IL-4 and IFN- γ , both of which can modulate LO expression. In addition to its putative role in the oxidative modification of LDL, LO may be important in regulating the expression of many activities in the arterial wall related to atherogenesis. For example, Marui et al⁴⁴ have shown that the transcription and expression of vascular cell adhesion molecule-1 are regulated in endothelial cells by an antioxidant-sensitive mechanism. Given the ability of LO to "seed" nonenzymatic lipid peroxidation in biological systems,⁴⁵ it is conceivable that this enzyme might participate in nuclear signaling. That this might occur in vivo is strongly supported by results in a mouse model of atherosclerosis in which atherosclerosis-susceptible mice have a greater oxidative stress index than mice resistant to the disease process.⁴⁶ The hydroperoxide products of 15-LO can stimulate many molecular events associated with smooth muscle cell proliferation, such as stimulation of *c-fos*, *c-myc*, and *c-jun* mRNA expression and mitogen-activated protein kinase activity.⁴⁷ Taken together, these observations show that within the arterial wall there exist the appropriate cell types, molecular signals, and mechanisms for modulating

atherosclerosis vis-à-vis LO. Definitive proof for these speculations awaits the discovery of specific LO inhibitors that lack significant antioxidant activity.

Acknowledgments

Alan Daugherty is an Established Investigator of the American Heart Association. The authors thank Dr O. Kanagawa, Department of Pathology, Washington University, and Dr M. Kopf, Max-Planck-Institute for Immunobiology, Freiburg, Germany, for supplying IL-4 $+/-$ and $-/-$ mice. The authors are deeply indebted to Beth Engeszer for her expert assistance in the editing of this manuscript.

References

- Samuelsson B, Dahlen S-E, Lindgren JA, Rouzer CA, Serhan CN. Leukotrienes and lipoxins: structures, biosynthesis, and biological effects. *Science*. 1987;237:1171-1176.
- Kuhn H, Brash AR. Occurrence of lipoxygenase products in membranes of rabbit reticulocytes: evidence for a role of the reticulocyte lipoxygenase in the maturation of red cells. *J Biol Chem*. 1990;265:1454-1458.
- Sparrow CP, Parthasarathy S, Steinberg D. Enzymatic modification of low density lipoprotein by purified lipoxygenase plus phospholipase A2 mimics cell-mediated oxidative modification. *J Lipid Res*. 1988;29:745-753.
- Cathcart MK, McNally AK, Chisolm GM. Lipoxygenase-mediated transformation of human low density lipoprotein to an oxidized and cytotoxic complex. *J Lipid Res*. 1991;32:63-70.
- Schewe T, Rapoport SM, Kuhn H. Enzymology and physiology of reticulocyte lipoxygenase: comparison with other lipoxygenases. *Adv Enzymol Relat Areas Mol Biol*. 1986;58:192-271.
- Rapoport SM, Schewe T, Wiesner R, Halangk W, Ludwig P, Janicke-Hohne M, Tannert C, Hiebsch C, Klatt D. The lipoxygenase of reticulocytes: purification, characterization, and biological dynamics of the lipoxygenase: its identity with the respiratory inhibitors of the reticulocyte. *Eur J Biochem*. 1979;96:545-561.
- Schewe T, Wiesner R, Rapoport SM. Lipoxygenase from rabbit reticulocytes. *Methods Enzymol*. 1981;71:430-441.
- Nadel JA, Conrad DJ, Ueki IF, Schuster A, Sigal E. Immunocytochemical localization of arachidonate 15-lipoxygenase in erythrocytes, leukocytes, and airway cells. *J Clin Invest*. 1991;87:1139-1145.
- Sigal E, Dicharry S, Highland E, Finkbeiner WE. Cloning of human airway 15-lipoxygenase: identity to the reticulocyte enzyme and expression in epithelium. *Am J Physiol Lung Cell Mol Physiol*. 1992;262:L392-L398.
- Shannon VR, Chanez P, Bousquet J, Holtzman MJ. Histochemical evidence for induction of arachidonate 15-lipoxygenase in airway disease. *Am Rev Respir Dis*. 1993;147:1024-1028.
- Hunter JA, Finkbeiner WE, Nadel JA, Goetzl EJ, Holtzman MJ. Predominant generation of 15-lipoxygenase metabolites of arachidonic acid by epithelial cells from human trachea. *Proc Natl Acad Sci U S A*. 1985;82:4633-4637.
- Shannon VR, Crouch EC, Takahashi Y, Ueda N, Yamamoto S, Holtzman MJ. Related expression of arachidonate 12- and 15-lipoxygenases in animal and human lung tissue. *Am J Physiol Lung Cell Mol Physiol*. 1991;261:L399-L405.
- Shannon VR, Ramanadham S, Turk J, Holtzman MJ. Selective expression of an arachidonate 12-lipoxygenase by pancreatic islet β -cells. *Am J Physiol Endocrinol Metab*. 1992;263:E828-E836.
- Shannon VR, Stenson WF, Holtzman MJ. Induction of epithelial arachidonate 12-lipoxygenase at active sites of inflammatory bowel disease. *Am J Physiol Gastrointest Liver Physiol*. 1993;264:G104-G111.
- Conrad DJ, Kuhn H, Mulkins M, Highland E, Sigal E. Specific inflammatory cytokines regulate the expression of human monocyte 15-lipoxygenase. *Proc Natl Acad Sci U S A*. 1992;89:217-221.
- Yla-Herttuala S, Rosenfeld ME, Parthasarathy S, Sigal E, Sarkioja T, Witztum JL, Steinberg D. Gene expression in macrophage-rich human atherosclerotic lesions: 15-lipoxygenase and acetyl low density lipoprotein receptor messenger RNA colocalize with oxidation specific lipid protein adducts. *J Clin Invest*. 1991;87:1146-1152.
- Yla-Herttuala S, Rosenfeld ME, Parthasarathy S, Glass CK, Sigal E, Witztum JL, Steinberg D. Colocalization of 15-lipoxygenase mRNA and protein with epitopes of oxidized low density lipoprotein in macrophage-rich areas of atherosclerotic lesions. *Proc Natl Acad Sci U S A*. 1990;87:6959-6963.

18. Daugherty A, Roselaar SE. Lipoprotein oxidation as a mediator of atherogenesis: insights from pharmacological studies. *Cardiovasc Res.* 1995;29:297-311.
19. Yamamoto S. Mammalian lipoxygenases: molecular structures and functions. *Biochim Biophys Acta.* 1992;1128:117-131.
20. Chen XS, Kurre U, Jenkins NA, Copeland NG, Funk CD. cDNA cloning, expression, mutagenesis of C-terminal isoleucine, genomic structure, and chromosomal localizations of murine 12-lipoxygenases. *J Biol Chem.* 1994;269:13979-13987.
21. Sloane DL, Leung R, Craik CS, Sigal E. A primary determinant for lipoxygenase positional specificity. *Nature.* 1991;354:149-152.
22. Sigal E, Sloane DL, Conrad DJ. Human 15-lipoxygenase: induction by interleukin-4 and insights into positional specificity. *J Lipid Mediat.* 1993;6:75-88.
23. Gan Q-F, Witkop GL, Sloane DL, Straub KM, Sigal E. Identification of a specific methionine in mammalian 15-lipoxygenase that is oxygenated by the enzyme product 13-HPODE: dissociation of sulfoxide formation from self-inactivation. *Biochemistry.* 1995;34:7069-7079.
24. Sloane DL, Leung R, Barnett J, Craik CS, Sigal E. Conversion of human 15-lipoxygenase to an efficient 12-lipoxygenase: the side-chain geometry of amino acids 417 and 418 determine positional specificity. *Protein Eng.* 1995;8:275-282.
25. Kopf M, Le Gros G, Bachmann M, Lamers MC, Bluethmann H, Köhler G. Disruption of the murine IL-4 gene blocks Th2 cytokine responses. *Nature.* 1993;362:245-248.
26. Nakagawara A, Nathan CF. A simple method for counting adherent cells: application to cultured human monocytes, macrophages, and multinucleated giant cells. *J Immunol.* 1983;56:261-268.
27. Auerbach BJ, Kiely JS, Cornicelli JA. A spectrophotometric microtiter-based assay for the detection of hydroperoxy derivatives of linoleic acid. *Anal Biochem.* 1992;201:375-380.
28. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A.* 1979;76:4350-4355.
29. Bradding P, Feather IH, Howarth PH, Mueller R, Roberts JA, Britten K, Bews JPA, Hunt TC, Okayama Y, Heusser CH, Bullock GR, Church MK, Holgate ST. Interleukin 4 is localized to and released by human mast cells. *J Exp Med.* 1992;176:1381-1386.
30. Banachereau J, Brière F, Galizzi JP, Miossec P, Rousset F. Human interleukin 4. *J Lipid Mediat.* 1994;9:43-53.
31. Paul WE, Ohara J. B-cell stimulatory factor/interleukin 4. *Annu Rev Immunol.* 1987;5:429-459.
32. Kühn R, Rajewsky K, Müller W. Generation and analysis of interleukin-4 deficient mice. *Science.* 1991;254:707-710.
33. Doherty TM, Kastelein R, Menon S, Andrade S, Coffman RL. Modulation of murine macrophage function by IL-13. *J Immunol.* 1993;151:7151-7160.
34. Moore KW, O'Garra A, De Waal Malefyt R, Vieira P, Mosmann TR. Interleukin-10. *Annu Rev Immunol.* 1993;11:165-190.
35. Nassar GM, Morrow JD, Roberts LJ II, Lakkis FG, Badr KF. Induction of 15-lipoxygenase by interleukin-13 in human blood monocytes. *J Biol Chem.* 1994;269:27631-27634.
36. Levy BD, Romano M, Chapman HA, Reilly JJ, Drazen J, Serhan CN. Human alveolar macrophages have 15-lipoxygenase and generate 15(S)-hydroxy-5,8,11-cis-13-trans-eicosatetraenoic acid and lipoxins. *J Clin Invest.* 1993;92:1572-1579.
37. Yla-Herttuala S. Gene expression in atherosclerotic lesions. *Herz.* 1992;17:270-276.
38. Stemme S, Holm J, Hansson GK. T lymphocytes in human atherosclerotic plaques are memory cells expressing CD45RO and the integrin VLA-1. *Arterioscler Thromb.* 1992;12:206-211.
39. Stemme S, Rymo L, Hansson GK. Polyclonal origin of T lymphocytes in human atherosclerotic plaques. *Lab Invest.* 1991;65:654-660.
40. Hansson GK, Seifert PS, Olsson G, Bondjers G. Immunohistochemical detection of macrophages and T lymphocytes in atherosclerotic lesions of cholesterol-fed rabbits. *Arteriosclerosis.* 1991;11:745-750.
41. Jonasson L, Holm J, Skalli O, Bondjers G, Hansson GK. Regional accumulations of T cells, macrophages, and smooth muscle cells in the human atherosclerotic plaque. *Arteriosclerosis.* 1986;6:131-138.
42. Stemme S, Hansson GK. Immune mechanisms in atherosclerosis. *Coron Artery Dis.* 1994;5:216-222.
43. Swanson SJ, Rosenzweig A, Seidman JG, Libby P. Diversity of T-cell antigen receptor Vbeta gene utilization in advanced human atherosclerosis. *Arterioscler Thromb.* 1994;14:1210-1214.
44. Marui N, Offermann MK, Swerlick R, Kunsch C, Rosen CA, Ahmad M, Alexander RW, Medford RM. Vascular cell adhesion molecule-1 (VCAM-1) gene transcription and expression are regulated through an antioxidant-sensitive mechanism in human vascular endothelial cells. *J Clin Invest.* 1993;92:1866-1874.
45. Kuhn H, Belkner J, Wiesner R, Brash AR. Oxygenation of biological membranes by the pure reticulocyte lipoxygenase. *J Biol Chem.* 1990;265:18351-18361.
46. Liao F, Andalibi A, Qiao JH, Allayee H, Fogelman AM, Lusis AJ. Genetic evidence for a common pathway mediating oxidative stress, inflammatory gene induction, and aortic fatty streak formation in mice. *J Clin Invest.* 1994;94:877-884.
47. Rao GN, Alexander RW, Runge MS. Linoleic acid and its metabolites, hydroperoxyoctadecadienoic acids, stimulate c-fos, c-jun, and c-myc mRNA expression, mitogen-activated protein kinase activation, and growth in rat aortic smooth muscle cells. *J Clin Invest.* 1995;96:842-847.