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Differential low density lipoprotein receptor-dependent formation of eicosanoids in human blood-derived monocytes

(arachidonic acid/prostaglandins/leukotrienes/inflammation)

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ABSTRACT **We studied the ability of low density lipoproteins (LDLs) to provide arachidonic acid (AA) for eicosanoid biosynthesis in human blood-derived monocytes. When incubated in the presence of reconstituted LDL that contained cholesteryl [1-^{14}C]arachidonate (recLDL-[^{14}C]AA-CE), resting monocytes formed three labeled products of the prostaglandin (PG) H synthase pathway: 6-keto-PGF$_{1\alpha}$, thromboxane B$_2$, and PGE$_2$. The amounts of these eicosanoids in response to recLDL-[^{14}C]AA-CE were comparable to or exceeded those that were produced in response to the addition of 10 μM unesterified [1-^{14}C]AA. By contrast, resting monocytes formed only small amounts of products of the 5-lipoxygenase pathway, leukotriene (LT) B$_4$ and LTC$_4$ from either recLDL-[^{14}C]AA-CE or [^{14}C]AA, indicating preferential utilization of AA in the PGH synthase reaction. However, they converted LDL-derived [^{14}C]AA efficiently into LTB$_4$ and LTC$_4$, when they were first incubated with recLDL-[^{14}C]AA-CE and subsequently stimulated with the chemotactic peptide *N*-formylmethionylleucylphenylalanine or the Ca^{2+} ionophore A23187. The classical LDL receptor pathway mediated the synthesis of all of the above eicosanoids from LDL but not from unesterified AA. These results demonstrate that the LDL receptor pathway preferentially promotes the synthesis of PGH synthase products in resting human blood-derived monocytes and that an additional mechanism is required to promote effective synthesis of 5-lipoxygenase pathway products from AA that originates in LDL cholesteryl esters.**

We and others have observed stimulatory effects of native plasma lipoproteins on the formation of prostacyclin and prostaglandin (PG) E$_2$ in cultured endothelial cells, smooth muscle cells, and fibroblasts (1–3). Furthermore, we have shown (4) that the stimulatory effect of low density lipoprotein (LDL) on prostacyclin and PGE$_2$ production by platelet-derived growth factor-stimulated fibroblasts was a consequence of the LDL receptor-dependent delivery of arachidonic acid (AA) to the PGH synthase (EC 1.14.99.1) reaction and that it was followed by a profound inhibition of PGH synthase. This raised the possibility that the LDL pathway might have a role in the regulation of eicosanoid synthesis in fibroblasts in addition to its known role in the maintenance of cellular cholesterol homeostasis. Furthermore, since all animal cells can express the LDL receptor and also can convert AA into one or more eicosanoids (of which more than 40 with very different biological activities have been identified to date; for review, see refs. 5 and 6), it seemed possible that the LDL pathway might play a similar role in other cell types. To investigate the latter possibility, we turned our attention to human blood-derived monocytes. Unlike most mammalian cells, monocytes express both the PGH synthase pathway that leads to the formation of prostacyclin, thromboxane (TX) A$_2$, and PGE$_2$ (7) and the 5-lipoxygenase (EC 1.13.11.12) pathway that leads to the formation of leukotriene (LT) B$_4$ and LTC$_4$ (8). Furthermore, the products of the two pathways play distinct, and in some instances even opposing, biological roles (5). Our results demonstrate that human monocytes use the classical LDL receptor pathway (for review, see refs. 9 and 10) to deliver AA for the production of prostacyclin, TXA$_2$, and PGE$_2$, and that *N*-formylmethionylleucylphenylalanine (fMet-Leu-Phe) induces formation of LTB$_4$ and LTC$_4$ from AA that originates in LDL cholesteryl esters (CEs).

MATERIALS AND METHODS

Materials. fMet-Leu-Phe, AA, Ca^{2+} ionophore A 23187, and all other materials were from Sigma; [^{14}C]AA and [^{14}C]AA-CE (specific activity, 54.9 mCi/mmol; 1 Ci = 37 GBq) were from NEN.

Cell Preparation and Cell Culture. Human blood-derived mononuclear cells were isolated by leukapheresis from fasting volunteers, and monocytes were purified as described (11, 12). Cells were maintained in minimum essential medium supplemented with undialyzed 20% (vol/vol) autologous blood serum, penicillin (100 units/liter), and streptomycin (100 μg/ml). Before addition of reconstituted (rec) LDL-[^{14}C]AA-CE or unesterified [^{14}C]AA (7) to cells, the medium was removed and replaced with fresh medium supplemented with 4% (vol/vol) plasma-derived lipoprotein-deficient serum for 4 h (3, 4, 13). Ca^{2+} ionophore- and fMet-Leu-Phe-dependent LT syntheses were determined as described (7) except that AA or recLDL was used as described in the figure legends.

Reconstitution of LDL. Native human plasma LDL (density, 1.020–1.060 g/ml) was isolated and reconstituted as described by Krieger (14) with minor modifications. Briefly, [^{14}C]AA-CE (specific activity, 54.9 mCi/mmol; NEN, lot 2664-056) was repurified under argon on thin layer chromatography H plates (250 μm; Merck) using hexane/ether/acetic acid, 108/24/1.2 (vol/vol), as the solvent system. Purified [^{14}C]AA-CE was mixed with unlabeled AA-CE (Nu Chek Prep, Elysian, MN) to specific activities of 2.1 × 10^5 dpm/μg of LDL protein and 43 mCi/mmol of CE (see Table 1, experiment 1; Table 2; and Fig. 4) and 1.4 × 10^5 dpm/μg of LDL protein and 31 mCi/mmol of CE (see Table 1, experiment 2; Table 2; and Figs. 1–3). Reconstitution and

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Abbreviations: AA, arachidonic acid; CE, cholesteryl ester; LT, leukotriene; PG, prostaglandin; LDL, low density lipoprotein; recLDL, reconstituted LDL; TX, thromboxane.

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storage were performed under argon in the dark at 4°C to avoid oxidation.

Anti-LDL Receptor Antibodies. Polyclonal anti-LDL receptor antibodies were generated in rabbits against bovine adrenal LDL receptor as described (15, 16). The antibody was purified by DEAE-cellulose chromatography and was shown to block by 95% the binding of ^{125}I-labeled LDL to human fibroblasts at an IgG concentration of 75 μg/ml. Anti-LDL receptor IgG or an unrelated control antibody (150 μg/ml) was added 30 min before addition of recLDL-[^{14}C]AA-CE.

Eicosanoid Analyses. Radiolabeled tracer eicosanoids were added to parallel samples to determine recovery of the biological samples as described (17–19). PGH synthase or 5-lipoxygenase products, respectively, were extracted and separated by reversed-phase high pressure liquid chromatography systems as reported (7, 17).

RESULTS

The LDL Pathway Delivers AA to Human Monocytes for the Production of Prostacyclin, TXA_2, and PGE_2. To investigate whether LDL delivers AA to monocytes for eicosanoid production, we incubated monocytes with recLDL-[^{14}C]AA-CE protein (20 μg/ml). This led to formation of three labeled products of the PGH synthase pathway, 6-keto-$PGF_{1\alpha}$ (the stable derivative of prostacyclin), TXB_2 (the stable derivative of TXA_2), and PGE_2 (Fig. 1*A*). The relative amounts of these eicosanoids varied in different experiments, but PGE_2 was the major product followed by 6-keto-$PGF_{1\alpha}$ and TXB_2 (Fig. 1 and Table 1).

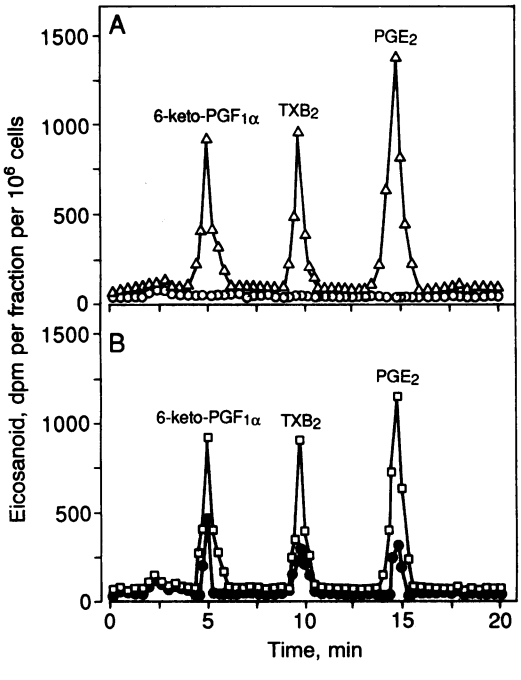

FIG. 1. Formation of 6-keto-$PGF_{1\alpha}$, TXB_2, and PGE_2 in response to recLDL-[^{14}C]AA-CE depends on the LDL receptor pathway. Approximately 6 × 10^6 monocytes per 60-mm dish were maintained in 3 ml of culture medium supplemented with 20% undialyzed human blood serum; 15 h later, the medium was removed and replaced with medium supplemented with 4% calf plasma-derived lipoprotein-deficient serum for 4 h. recLDL-[^{14}C]AA-CE at 20 μg of protein per ml was added for 4 h (Δ). Before addition of recLDL-[^{14}C]AA-CE at 20 μg/ml, parallel cultures were incubated for 30 min with polyclonal anti-LDL receptor antibody IgG (○) or unrelated rabbit IgG (□) (each at 150 μg/ml), or 60 μM chloroquine (●). Eicosanoids were analyzed and chromatograms represent single samples of duplicate dishes. Data in *A* and *B* are separated for easier reading.

Table 1. Formation of 6-keto-$PGF_{1\alpha}$, TXB_2, and PGE_2 in response to recLDL-[^{14}C]AA-CE or unesterified [^{14}C]AA

Exp.	Eicosanoid	Radiolabeled eicosanoid, ng per 10^6 cells: recLDL-[^{14}C]AA-CE	[^{14}C]AA
1	6-keto-$PGF_{1\alpha}$	20.5 ± 0.7	17.2 ± 0.1
	TXB_2	4.8 ± 0.6	4.2 ± 0.3
	PGE_2	20.0 ± 0.7	16.0 ± 0.3
2	6-keto-$PGF_{1\alpha}$	16.0 ± 0.2	8.4 ± 0.1
	TXB_2	15.7 ± 0.5	8.4 ± 3.2
	PGE_2	20.5 ± 0.9	9.0 ± 0.2

Cells (2 × 10^6 cells per 35-mm dish, experiment 1; 6 × 10^6 cells per 60-mm dish, experiment 2) were cultured as described in Fig. 1. On day 1, monocytes were exposed to recLDL-[^{14}C]AA-CE protein at 10 μg/ml or 10 μM [^{14}C]AA for 4 h. Radioactivity in eicosanoids was determined and expressed as the mean ±SD of duplicate dishes.

In parallel cultures we determined whether the synthesis of eicosanoids in response to recLDL-[^{14}C]AA-CE depended on the LDL receptor pathway. Preincubation of monocytes with a polyclonal rabbit anti-LDL receptor antibody completely prevented formation of all products of the PGH synthase pathway, whereas control rabbit IgG did not (Fig. 1*B*). Furthermore, chloroquine, an inhibitor of lysosomal hydrolase activity (1, 4), greatly inhibited the response from recLDL-[^{14}C]AA-CE. Other experiments showed no effect of chloroquine on the formation of PGH synthase products in response to unesterified [^{14}C]AA indicating that chloroquine did not interfere with the PGH synthase reaction (data not shown).

Significant eicosanoid formation was observed with concentrations of recLDL-[^{14}C]AA-CE as low as 0.5 μg/ml and the rate of synthesis increased up to concentrations of recLDL-[^{14}C]AA-CE protein of 5 μg/ml (Fig. 2*A*). Significant rates of eicosanoid synthesis were observed as early as 15 min and a further increase was observed thereafter (Fig. 2*B*). In most experiments, additional but smaller increases in eicosanoid formation were observed between 2 and 8 h of incubation. No experiments were performed for time periods that exceeded 8 h.

The magnitude of the response to recLDL-[^{14}C]AA-CE protein at 10 μg/ml during a 4-h period was similar to, and in several experiments significantly exceeded, that of the response to 10 μM unesterified [^{14}C]AA (Table 1). Thus, recLDL-[^{14}C]AA-CE could deliver high substrate concentrations to the PGH synthase reaction (7, 17, 18) by an LDL-receptor-dependent mechanism.

When we studied the response of monocytes to recLDL-[^{14}C]AA-CE as a function of time that the cells were maintained in culture, we noticed that the cells were able to produce significant amounts of eicosanoids within 4 h after isolation (the earliest time period studied). Eicosanoid synthesis rates increased within the first 24 h by ≈25%, remained constant for an additional 24–36 h, and then declined between days 3 and 12 reaching ≈50% of the maximal rate on day 12. The decline of LDL-dependent eicosanoid formation between days 3 and 12 could have been the result of downregulation of LDL receptor expression (12). To clarify this issue, we studied LDL-receptor-independent eicosanoid synthesis rates by adding 10 μM unesterified [^{14}C]AA at various times after isolation of the cells. However, these synthesis rates initially increased and then declined similarly indicating that the changes in the rate of eicosanoid synthesis from LDL were due to more than simply LDL receptor regulation (data not shown). The mechanism and functional significance of this phenomenon, therefore, remain unclear at present but may be related to the limited life span of terminally differentiated blood-derived monocytes in culture. It is noteworthy that treatment of the monocytes with granulocyte–macrophage colony-stimulating

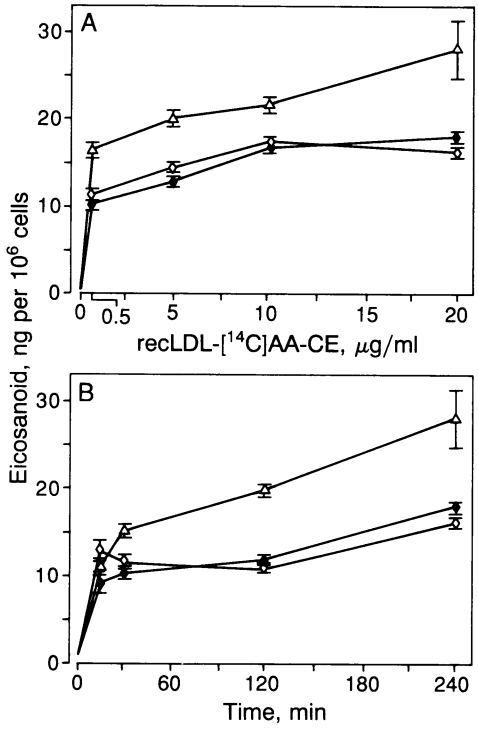

FIG. 2. Concentration dependence and kinetics of formation of labeled 6-keto-$PGF_{1\alpha}$, TXB_2, and PGE_2 from recLDL-[^{14}C]AA-CE. Monocytes were cultured as described in Fig. 1. recLDL-[^{14}C]AA-CE was added at increasing concentrations and incubated for 4 h (*A*) or was added at 10 μg/ml and incubated for increasing time periods (*B*), and radioactivity in eicosanoids (△, PGE_2; ◇, 6-keto-$PGF_{1\alpha}$; ◆, TXB_2) was determined as described in Fig. 1. Data represent the mean ± SD of duplicate dishes.

factor for the first 12 h after cell isolation increased the subsequent eicosanoid response toward LDL or AA by ≈50% (data not shown).

Other experiments revealed that LDL-dependent and cell-type-specific eicosanoid responses occur in human umbilical vein endothelial cells, $HepG_2$ cells (a differentiated human hepatoma cell line), and $p388D_1$ cells (a differentiated mouse macrophage cell line) (P.B.S., J.A.G., and A.J.R.H., unpublished observations).

Thus these results showed that recLDL-[^{14}C]AA-CE delivers high substrate concentrations to the PGH synthase reaction in resting human monocytes, that the LDL-derived AA is utilized for the constitutive formation of three major eicosanoids of this pathway, and that delivery of AA to the PGH synthase reaction completely depends on LDL-receptor-mediated uptake and lysosomal hydrolysis of the lipoproteins.

Inflammatory Stimuli Induce the Formation of LTB_4 and LTC_4 from LDL-Derived [^{14}C]AA. When we analyzed the culture medium from recLDL-[^{14}C]AA-CE-treated cells for the presence of 5-lipoxygenase products, we found no or little constitutive production of LTB_4 or LTC_4 (Table 2). Moreover, similar results were obtained upon analyzing the culture medium from cells that were treated with 10 μM unesterified [^{14}C]AA. However, monocytes showed high LT synthesis rates when unesterified AA was added with Ca^{2+} ionophore (data not shown). This data suggested that the AA delivered by the LDL pathway or taken up directly from the culture medium was being differentially utilized by the PGH synthase and 5-lipoxygenase reactions in resting cells. To study this possibility in greater detail, we determined whether the chemotactic peptide fMet-Leu-Phe or a Ca^{2+} ionophore could induce the monocytes to form larger amounts of LTs

Table 2. Constitutive and fMet-Leu-Phe- and Ca^{2+} ionophore-dependent formation of LTs

Incubation condition	Radiolabeled eicosanoid, ng per 10^6 cells	
	LTC_4	LTB_4
Constitutive		
recLDL-[^{14}C]AA-CE (0.5 h)	ND	ND
recLDL-[^{14}C]AA-CE (4 h)	1.8 ± 1.0	0.8 ± 0.1
fMet-Leu-Phe (100 nM)		
recLDL-[^{14}C]AA-CE (0.5 h)	6.1 ± 0.6	3.3 ± 0.3
recLDL-[^{14}C]AA-CE (4 h)	4.9 ± 0.6	2.7 ± 0.2
Ca^{2+} ionophore (10 μM)		
recLDL-[^{14}C]AA-CE (0.5 h)	5.8 ± 0.8	3.2 ± 0.1
recLDL-[^{14}C]AA-CE (4 h)	5.8 ± 0.2	3.1 ± 0.0

ND, not detectable. Approximately 2 × 10^6 cells per 35-mm dish were cultured as described in Fig. 1 and incubated with recLDL-[^{14}C]AA-CE at 10 μg/ml as indicated. The culture medium was removed and analyzed for the presence of LTs as described in Fig. 3. The cell monolayer was washed twice with phosphate-buffered saline and incubated in phosphate-buffered saline (for constitutive expression) or with 100 nM fMet-Leu-Phe or 10 μM Ca^{2+} ionophore for 30 min. Similar results were obtained when 10 μM unesterified [^{14}C]AA was used as the substrate and only small or undetectable amounts of LTs were formed in the absence of agonists. Data represent the mean ± SD of duplicate dishes.

from LDL-derived AA. For this purpose, we first incubated the cells with recLDL-[^{14}C]AA-CE for 0.5 or 4 h to allow the [^{14}C]AA of LDL to be incorporated into cellular lipid stores. Then, we removed the culture medium and incubated the cells with fMet-Leu-Phe or Ca^{2+} ionophore. Both compounds stimulated the formation of labeled LTB_4 and LTC_4 and the amounts greatly exceeded those produced in the absence of agonists during the preincubation period (Table 2). These results strongly indicated that the failure of the monocytes to produce high amounts of LTs in response to LDL or AA alone was due to suboptimal 5-lipoxygenase stimulation conditions. The amounts of radiolabeled 5-lipoxygenase products that were formed from LDL-derived AA or from added unesterified AA in response to fMet-Leu-Phe varied for LTC_4 from 5 to 15 ng per 10^6 cells and for LTB_4 from 3 to 10 ng per 10^6 cells in six experiments. Similar to the products of the PGH synthase pathway (Table 1), recLDL-[^{14}C]AA-CE delivered high substrate concentrations to the 5-lipoxygenase reaction (data not shown).

As expected, anti-LDL receptor antibodies completely prevented formation of LTs in response to fMet-Leu-Phe (Fig. 3*A*), whereas control rabbit IgG had no effect (Fig. 3*B*) in two experiments, and chloroquine largely prevented the fMet-Leu-Phe-dependent formation of LTs from recLDL-[^{14}C]AA-CE (Fig. 3*B*) but not from unesterified [^{14}C]AA (data not shown). Finally, the study of the concentration dependency and kinetics of LDL- and fMet-Leu-Phe-dependent LT formation yielded results that were similar to those found for the products of the PGH synthase pathway (Fig. 4).

DISCUSSION

The LDL AA Pathway Couples to Eicosanoid Formation in a Cell-Type-Specific Way and May Be Quantitatively Significant. In response to recLDL-[^{14}C]AA-CE, resting monocytes formed three major products of the PGH synthase pathway: 6-keto-$PGF_{1\alpha}$, TXB_2, and PGE_2 (Figs. 1 and 2 and Table 1). Whereas the synthesis of 6-keto-$PGF_{1\alpha}$ and PGE_2 was similar in both fibroblasts and monocytes, formation of TXB_2 distinguishes these cells, because the former does not express TX synthase activity (7, 17). The present data support the conclusion that the classical LDL pathway of Brown and Goldstein (9, 10) plays a regulatory role in the synthesis of

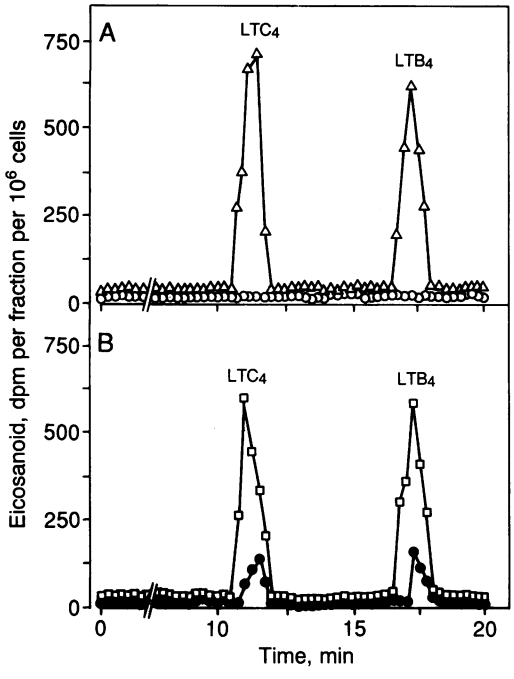

FIG. 3. fMet-Leu-Phe induced LT synthesis in response to recLDL-[^{14}C]AA-CE depends on the LDL receptor pathway. Monocytes were cultured as described in Fig. 1. recLDL-[^{14}C]AA-CE at 20 μg/ml was added for 4 h in the absence (△) or in the presence of an anti-LDL receptor antibody (○), an unrelated antibody (□), or 60 μM chloroquine (●) as described in Fig. 1. fMet-Leu-Phe (100 nM) was added for 10 min. Chromatograms represent single samples from duplicate dishes. Data in *A* and *B* were separated for easier reading.

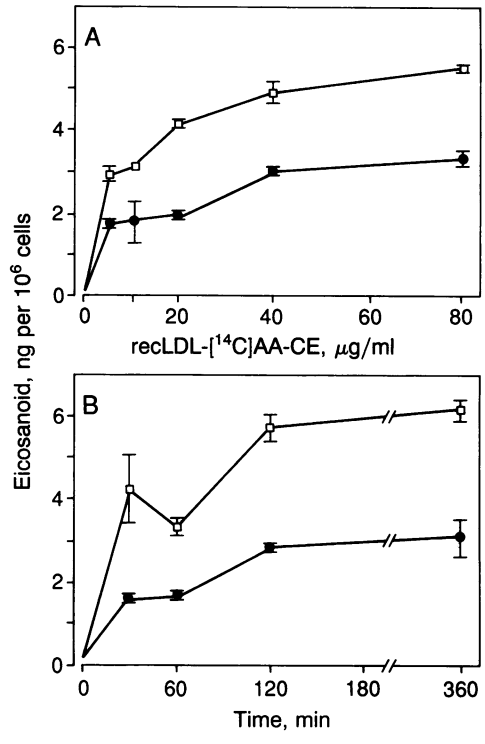

FIG. 4. Concentration dependence and kinetics of fMet-Leu-Phe-dependent labeled LT formation from recLDL-[^{14}C]AA-CE. Approximately 2 × 10^6 monocytes were cultured as described in Fig. 1 and incubated with increasing concentrations of recLDL-[^{14}C]AA-CE for 4 h (*A*) or with recLDL-[^{14}C]AA-CE at 10 μg/ml for increasing time periods (*B*). fMet-Leu-Phe at 100 nM was added for 10 min (*A*) or 30 min (*B*) and radioactivity in LTC_4 (□) and LTB_4 (●) was determined. Data represent the mean ± SD of duplicate dishes.

these eicosanoids, because the amounts of eicosanoids that were formed in response to recLDL-[^{14}C]AA-CE were comparable to or even exceeded those that were formed in response to the addition of 10 μM free [^{14}C]AA (Table 1). One caveat should be kept in mind when considering these results: Although eicosanoid formation was significant at concentrations of recLDL-[^{14}C]AA-CE as low as 0.5 μg/ml, a concentration that is well below LDL receptor saturation in monocytes (12, 20, 21), gas liquid chromatography analyses revealed that recLDL contained 3–7 times more AA molecules per LDL particle than native LDL. Moreover, the use of radiolabeled AA present as CE does not allow precise mass estimates of eicosanoid synthesis, because the intracellular concentration of unesterified AA derived from endogenous lipids is unknown. Although constitutive eicosanoid production of resting monocytes has been reported to be low (5, 7, 19, 22), these considerations may become more relevant when agonist-dependent eicosanoid responses that are associated with cellular phospholipase activation and AA release are considered (Figs. 3 and 4 and Table 2). Thus, further studies will be required to define more thoroughly the quantitative aspects of the LDL–AA pathway in monocytes.

LDL-Derived AA Is Differentially Metabolized by Two Major Oxidative Pathways. Our study of LDL-dependent eicosanoid formation in monocytes supports a second major conclusion, that recLDL preferentially promotes the synthesis of products of the PGH synthase pathway as compared with products of the 5-lipoxygenase pathway. This type of preferentiality has, to our knowledge, not been observed previously in traditional model systems with agonists that activate cell-surface-receptor-coupled phospholipases (19, 22). It probably depended on the unique characteristics of the 5-lipoxygenase that initiates LT synthesis, rather than on the LDL or PGH synthase pathways. Unlike all other enzymes of AA metabolism, the 5-lipoxygenase requires complex activation conditions, including an increase in the concentration of intracellular free Ca^{2+} ions and the action of a 5-lipoxygenase-activating protein (23). Such activation conditions are elicited by unspecific stimuli such as the Ca^{2+} ionophore or by receptor-coupled agonists such as fMet-Leu-Phe or the complement peptide C5a (19). It has been reported that native LDL within 60 sec induces an increase in the concentrations of inositol trisphosphate, diacylglycerol, and intracellular free Ca^{2+} in rat vascular smooth muscle cells (24), and another study of human skin fibroblasts showed that native LDL had similar activities in LDL-receptor-negative fibroblasts (25). However, our attempts to detect recLDL- or native LDL-dependent changes in the concentration of intracellular free Ca^{2+} using fura-2 as a fluorescent probe in monocytes yielded negative results when studied for 10 min after LDL addition. Furthermore, recLDL, like added unesterified AA, was unable to induce synthesis of significant amounts of LTs, but significant amounts of LTs were formed when monocytes were preincubated with recLDL and subsequently stimulated with fMet-Leu-Phe or Ca^{2+} ionophore or when unesterified AA was added with Ca^{2+} ionophore (Figs. 3 and 4, Table 2, and data not shown). That native LDL can stimulate the formation of PGE_2 and LTB_4 in human blood-derived monocytes in the absence of an additional stimulus has been reported (26, 27), but the source of the substrate for eicosanoid synthesis has not been determined. Other studies showed strong stimulatory effects of oxidized LDL, acetylated LDL, and malondialdehyde-modified LDL in mouse peritoneal macrophages or human blood-derived monocytes (28, 29). The molecular mechanisms of some of the effects of both native and modified LDLs in various cell types and species remain to be identified. We assume that LDL delivers AA to both the PGH synthase and 5-lipoxygenase pathways, but that the low activation state of the

5-lipoxygenase of resting monocytes (8, 22, 23, 26–29) precludes the conversion of significant amounts of AA into LTs. However, the same AA is readily converted into LTs when the 5-lipoxygenase is activated by second messengers that are derived from signaling pathways such as those generated by fMet-Leu-Phe or Ca^{2+} ionophore. Thus, LDL-dependent eicosanoid metabolism in monocytes can be distinguished from eicosanoid synthesis that occurs in response to activation of cell surface receptors (30) by at least two criteria: (*i*) LDL delivers substrate AA to cells and (*ii*) LDL does not appear to acutely increase the concentration of free intracellular Ca^{2+} ions. Thus, when LDL-derived AA is released from the lysosomal compartment of resting monocytes, the 5-lipoxygenase appears to remain in its inactive state while oxidative AA metabolism through the PGH synthase reaction can proceed unimpaired (5, 8).

In view of the differential expression of the LDL–AA pathway in several cell types, the question of whether LDL-derived eicosanoids exert autocrine and/or paracrine biological activities in the producer cells themselves or in other target cells should be addressed.

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