Lovastatin Increases Arachidonic Acid Levels and Stimulates Thromboxane Synthesis in Human Liver and Monocytic Cell Lines

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Abstract

The effect of lovastatin (LOV), the inhibitor of 3-hydroxy-3-methyl-glutaryl coenzyme A reductase, on linoleic acid (LA, 18:2n-6) metabolism was examined in human monocytic Mono Mac 6 (MM6) and hepatoma Hep G2 cells. The desaturation of LA was examined after LOV (72 h, 10 μM) or dimethylsulfoxide (LOV carrier, < 0.1%) and [^{14}C]LA (last 18 h, 0.3 μCi, 5 μM). In both cell lines, LOV reduced the percentage of ^{14}C label associated with LA and increased the percentage of label in the 20:4n-6 and the 22:5n-6 fractions. In Hep G2 but not MM6 cells, this effect was fully reversible by means of coincubation with mevalonic acid (500 μM), but not with cholesterol or lipoproteins. In both cell lines, the LOV-mediated increase in LA desaturation resulted in dose-dependent reductions of LA and elevations of AA in cellular phospholipids. The lipids secreted by LOV-treated Hep G2 cells were also enriched in arachidonic acid (AA). In the MM6 cells, LOV increased release of thromboxane upon stimulation with the calcium ionophore A23187. In summary, our findings of higher LA desaturation and AA enrichment of lipids secreted by the Hep G2 cells suggest that LOV treatment may increase the delivery of AA from the liver to extrahepatic tissues. The changes in membrane fatty acid composition can influence a variety of cellular functions, such as eicosanoid synthesis in monocytic cells. The mechanism appears to be related to the reduced availability of intermediates of cholesterogenesis. (*J. Clin. Invest.* 1994. 93:195–203.) Key words: fatty acids • 3-hydroxy-3-methyl coenzyme A reductase inhibitor • lipoproteins • mevalonate • thromboxane

Introduction

Inhibitors of 3-hydroxy-3-methyl-glutaryl CoA(HMGCoA)[1] reductase, such as (LOV), are widely used in the treatment of hypercholesterolemia. They suppress the early conversion step of HMGCoA to mevalonate (MVA) and thus the subsequent synthesis of cholesterol and its precursors. Their main site of action is the liver, where inhibition of cholesterol synthesis leads to the desired up-regulation of LDL receptor-mediated

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1. *Abbreviations used in this paper:* HMGCoA, 3-hydroxy-3-methyl-glutaryl coenzyme A; LA, linoleic acid; LOV, lovastatin; MM6, Mono Mac 6; MVA, mevalonic acid; PUFA, polyunsaturated fatty acids.

removal of plasma cholesterol. The inhibition of intracellular cholesterol synthesis, however, may be associated with an alteration of a variety of other cellular functions. The cholesterol content of membranes plays an essential role in membrane integrity and regulation of membrane-mediated processes (1, 2). Furthermore, the involvement of many cholesterol precursors, such as farnesyl or geranyl pyrophosphates are under intensive investigation regarding their role in multiple facets of cellular function (3, 4).

A number of clinical investigations (5–7) show that the use of HMGCoA reductase inhibitors can modify plasma and erythrocyte long-chain polyunsaturated fatty acids (PUFA). Microsomal acyl CoA desaturases, the rate-determining enzymes of PUFA synthesis (Fig. 1) are known to be influenced by microsomal cholesterol composition (1, 8–10). Furthermore, changes in desaturase activity may occur during cellular events such as differentiation (11, 12) or transformation (13), processes potentially influenced by cholesterol or its precursors. Modification of fatty acid desaturase activity leads to changes in eicosanoid synthesis (10), presumably by influencing eicosanoid precursor availability.

The present study examined the effect of LOV on linoleic acid (LA) metabolism, and cellular phospholipid fatty acid composition in human hepatoma Hep G2 (14) and human monocytic Mono Mac 6 (MM6) (15) cells. Two functional consequences, the fatty acid composition of lipids secreted by the Hep G2 cells and thromboxane release in ionophore A23187–stimulated MM6 cells were also investigated.

Methods

Materials. LOV was a kind gift of Dr. A. W. Alberts from Merck Sharp & Dohme (Rahway, NJ). LA (18:2n-6), MVA lactone, cholesterol, BSA (essentially fatty acid free, A6003), and cell culture media and ingredients were from Sigma Chemical Co. (Munich, FRG). Organic solvents, DMSO, silica 60 TLC (no. 5721), and HPTLC (no. 5641) plates were purchased from E. Merck (Darmstadt, FRG). Bond-Elut solid-phase extraction columns (NH$_2$-aminopropyl, 1-ml bed volume) were from Baker Chemical Co. (no. 7088-1, Denveter, Netherlands) and [1-^{14}C]LA (sp act 1.85–2.26 Bq/mmol) was from Amersham (Braunschweig, FRG).

Cell culture. MM6 cells, obtained from Dr. H.-W. E. Ziegler-Heitbrock (Institute of Immunology, University of Munich), were grown in stationary suspension in 2-ml culture well dishes at 37°C in a humidified atmosphere of 5% CO$_2$ and 95% air and were split every 3–4 d. The medium, RPMI 1640 supplemented with 10% FCS, nonessential amino acids (0.1 mM), oxaloacetate (1 mM), pyruvate (1 mM), penicillin-streptomycin (1%), L-glutamine (2 mM), was ultrafiltered through a Gambro 200 column (Gambro, Hechingen, FRG) to eliminate LPS. Hep G2 cells (American Type Culture Collection, Rockville, MD) were grown in 15-ml flasks in DME medium (Sigma, D5405) supplemented with 10% FCS, nonessential amino acids (1 mM), and L-glutamine (2 mM) and were passaged (1:4) once a week with trypsin-EDTA.

Preparation of albumin-bound LA. LA was dissolved in ethanol and converted to its anion by precipitation with 0.5 N KOH. Ethanol was

18:2n-6 → [Δ 6 Desaturase] → 18:3n-6 ↓ 20:3n-6 → [Δ 5 Desaturase] → 20:4n-6 ↓ 22:4n-6 → [Δ 4 or Δ 6 Desaturase] → 22:5n-6

18:3n-3 ↓ 18:4n-3 ↓ 20:4n-3 ↓ 20:5n-3 ↓ 22:5n-3 ↓ 22:6n-3
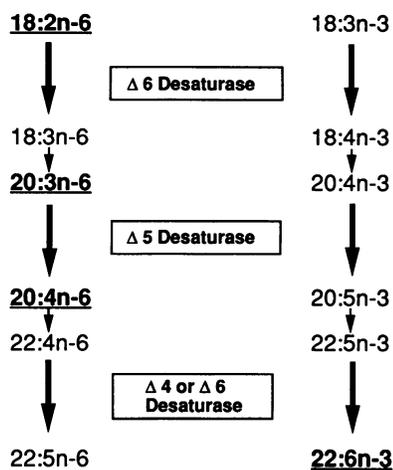
Figure 1. A schematic representation of desaturation and elongation of LA (18:2n-6) and LA (18:3n-3). Highlighted fatty acids represent major species found in animal tissues.

evaporated under N_2 and the salt was immediately reconstituted with 2.5 mM BSA dissolved in RPMI 1640 to yield a 5 mM fatty acid solution. The pH was adjusted to 7.4 with 1 N NaOH and aliquots were stored in liquid N_2.

Preparation of lipoproteins. LDL was isolated from normolipemic fasting plasma in the density interval 1.021–1.063 by sequential preparative ultracentrifugation and dialyzed against 20 liters of buffer containing 9 g/liters Na Cl, 0.09 g/liters EDTA, and 0.05 g/liters chloramphenicol, pH 7.4. Acetyl LDL was prepared as described in Basu et al. (16), washed, and concentrated in the above buffer by means of Centricon filters (CF25, Amicon, Witten, FRG). The extent of LDL acetylation was assessed by agarose-gel electrophoresis (Ciba Corning, Medfield, MA). Protein content was determined (17), and lipoproteins were filter-sterilized (0.22 μm), stored at 4°C, and used within 1 mo.

Determination of phospholipid fatty acids. Newly passaged MM6 (0.2 × 10^6 cells/ml) in the above medium with 10% FCS and Hep G2 cells 72 h after passage and given fresh DME medium with 10% delipidated serum (CPSR-1, Sigma Chemical Co.) were treated with LOV (dissolved in DMSO) or DMSO alone (≤ 0.1%) for 72 h. Albumin-bound LA was added for the last 24 h or for 72 h to the MM6 cells (10 μM) and Hep G2 cells (40 μM), respectively. After incubation, cells were washed (3 × 5 ml NaCl 0.9%) and cellular lipids were extracted with chloroform/methanol (2:1), containing butylated hydroxytoluene. Phospholipids were separated on aminopropyl-bonded phase Bond-Elut columns (18). The phospholipid eluate was transesterified with methanolic HCl (90°C, 1 h) in the presence of 17:0 as internal standard, fatty acid methyl esters were recovered in petroleum benzene and quantified with a Hewlett-Packard Co. (Palo Alto, CA) model 5890A gas chromatograph, using a 0.25-mm × 30-m DB-225 fused silica capillary column. The carrier gas was helium at a flow rate of 5 ml/min. Injection and ionization temperatures were 200°C and 250°C, respectively.

Determination of LA desaturation. Freshly passaged MM6 cells (0.2 × 10^6 cells/mL) and Hep G2 cells (1:4 or 1:3 split) were treated with LOV, MVA, cholesterol, or lipoproteins as described later in detail. At 52 h, cells either given fresh serum-free media containing 0.025% BSA or left in the original 10% FCS media, were incubated with 0.3 μCi (5 μM, dissolved in ethanol, final concentration 0.15%) [^{14}C]18:2n-6 for the last 18 h. At 72 h, cells were washed in PBS, four times with and without 0.1% BSA. The radioactivity in medium and washed cell aliquots was measured to estimate the incorporation of ^{14}C label. Total cellular lipids were extracted with hexane/isopropanol (3:2, vol/vol, containing butylated hydroxytoluene) and transesterified with methanolic HCl (90°C, 1 h). Fatty acid methyl esters were separated on HPTLC plates impregnated with 20% of aqueous $AgNO_3$ or 3% $AgNO_3$ in 93% methanol (19). The percent distribution of ^{14}C label in fatty acids with two to five double bonds was quantified by means of a TLC-linear radioactivity scanner (Berthold, Munich, FRG). R_f values were determined using both radioactive and cold

standards. For the determination of ^{14}C label distribution among lipid subclasses, total lipid was extracted as above and separated into total phospholipid, triglyceride, free fatty acid, and cholesterol ester on aminopropyl-bonded phase Bond-Elut columns (18) or on silica TLC plates developed in petroleum ether, diethyl ether, and acetic acid (85:15:3, vol/vol/vol). The radioactivity in isolated fractions was measured by means of a liquid scintillation counter (model LS 5801; Beckman Instruments, Munich, FRG).

Determination of fatty acid composition of lipids secreted by Hep G2 cells. Freshly passaged Hep G2 cells, grown in DME with 10% FCS, were treated with LOV 0.04–10 μM or DMSO (5–6 175-cm^2 flasks per treatment) for a total of 8 d. On day 4, cells were given fresh growth medium containing 10 μM albumin-bound LA. On day 6, growth medium was removed and cells were washed three times with 30 ml of Dulbecco's PBS and once with serum-free DME. After washing, 30 ml of fresh serum-free DME was added and harvested after 24 h. This procedure was repeated on day 7. The harvested medium was supplemented with EDTA (1 mg/ml) and gentamycin sulfate (0.1 mg/ml), centrifuged at 1,000 g for 30 min at 4°C to remove cell debris, and stored at 4°C. Media from the two harvests were pooled and concentrated to minimal volumes by ultrafiltration (Amicon stirred cell, PM30 membranes). Concentrates were lyophilized and total lipids were extracted with chloroform/methanol (2:1). Phospholipid, triglyceride, and cholesterol ester fractions were separated on aminopropyl columns (18), and fatty acids were quantified as described above.

Determination of thromboxane release. Freshly passaged MM6 cells were incubated with LOV, LA, MVA, and lipoproteins as described later in detail. Medium was removed after centrifugation (800 rpm × 5 min) and cells were resuspended at 10^6 cells/ml in a buffer containing 20 mM Hepes, 120 mM NaCl, 2.7 mM KCl, 1.4 mM $MgSO_4$, 1 mM $CaCl_2$, 1.4 mM KH_2PO_4, 25 mM $NaHCO_3$, and 10 mM glucose, pH 7.4. Cells were then stimulated (37°C, 5 min) with ionophore A23187 (1 μM in DMSO) or DMSO alone (final concentration ≤ 0.1%). Stimulation was terminated at 4°C, and thromboxane release was determined in 50-μl aliquots of supernatant using a highly specific and sensitive antiserum against TXB_2 (gift of Dr. J. Levine, Brandeis University, Waltham, MA) and [^{3}H]TXB_2 as a tracer (20).

Determination of cellular protein and lipids. Aliquots of Hep G2 and MM6 cells were washed and extracted with hexane/isopropanol (3:2) as described above. Total cellular cholesterol (21), phospholipid (22), and triglyceride (kit no 470694, Boehringer Mannheim GmbH, Mannheim) levels were determined in aliquots of the total lipid extract. Protein of extracted cell pellets was measured as in Rodriguez-Vico et al. (23).

Statistics. Unless otherwise stated, results are expressed as means±SEM. Statistical significance was assessed by means of Student's t test for paired and unpaired data and analysis of variance as appropriate.

Results

LOV effects on cellular phospholipid fatty acid composition. In MM6 cells supplemented with LA (10 μM), LOV (0.4, 4, 10, and 20 μM) produced a statistically significant, dose-dependent decrease in cellular phospholipid 18:2n-6 content (10.5±0.8% in control cells vs. 5.6±0.5% in cells treated with 20 μM LOV) (Table I). In contrast, the relative AA content was increased from 9.7±0.5% in control cells to 12.9±0.3% and 14.9±0.4% in cells treated with 10 and 20 μM LOV, respectively (Table I). At the two highest LOV doses, significant increases in the minor longer-chain, more desaturated derivatives of AA, 22:4n-6 and 22:5n-6, were also observed.

Similarly, in Hep G2 cells supplemented with 40 μM of BSA-bound LA, LOV at doses ranging from 0.4 to 40 μM reduced the relative phospholipid 18:2n-6 content (12.5±0.1% control cells to 9.4±0.4% 40 μM LOV) (Table II). The relative

Table I. Phospholipid Fatty Acid Composition of MMG Cells Incubated with LOV and LA

| | DMSO | LOV(μM) | | | |
		0.4	4	10	20
Saturated					
c16:0	40.8±0.8	41.3±1.0	41.7±1.0	41.0±1.0	39.4±0.8
c18:0	8.1±0.4	7.9±0.3	8.6±0.6	7.6±0.4	8.1±0.6
Monoene					
c18:1‡	23.4±0.8	22.8±0.8	22.5±0.7	23.6±1.0	22.4±1.0
Polyunsaturated					
n-6 series					
18:2	10.5±0.8	9.0±0.5	7.7±0.5*	6.4±0.5*	5.6±0.5*
20:3	1.8±0.1	1.9±0.1	2.0±0.1	2.0±0.1	2.1±0.2
20:4	9.7±0.5	11.2±0.4	11.7±0.5	12.9±0.3*	14.9±0.4*
22:4	0.6±0.06	0.7±0.07	0.9±0.07	1.2±0.10*	1.8±0.14*
22:5	0.4±0.05	0.4±0.04	0.5±0.05	0.6±0.1	0.7±0.05*
n-3 series					
20:5	0.7±0.03	0.7±0.03	0.7±0.03	0.6±0.04	0.6±0.05
22:5	1.3±0.2	1.3±0.1	1.3±0.1	1.5±0.2	1.6±0.2
22:6	2.5±0.2	2.5±0.1	2.5±0.1	2.8±0.3	2.9±0.3

Freshly passaged MM6 cells were incubated with 0.4–20 μM LOV or DMSO ≤ 0.1% for 72 h and 10 μM LA for the last 24 h. Cells were extracted, phospholipids separated and fatty acid methyl esters quantitated as described in Methods. Values (μM) are means±1 SEM from five to eight experiments. * Value is different from DMSO control using one-factor ANOVA with Sheffe F test for differences between means. ‡ Value represents sum of 18:1 isomers.

content of 20:4n-6 was increased from 6.0±0.3% in control cells to 7.3±0.3%, 7.7±0.1%, and 7.3±0.3% in cells treated with 4, 10, and 40 μM LOV, respectively (Table II).

Lovastatin had no effect on the relative content of phospholipid saturated and monoene fatty acids in the two cell lines studied (Tables I and II). The levels of n-3 fatty acids were also not altered by LOV, most likely due to the low levels of the precursor (18:3n-3) present.

The effect of LOV (10 μM, 72 h) on cellular phospholipid composition was also examined in Hep G2 cells coincubated with LDL (100 μg/ml, last 24 h). LDL incubation produced a large increase in LA content in cellular phospholipids

Table II. Phospholipid Fatty Acid Composition of Hep G2 Cells Incubated with LOV and LA

| | DMSO (0.025%) | LOV(μM) | | | | |
		0.04	0.4	4	10	40
Saturated						
c16:0	32.4±2.2	31.4±1.3	32.3±1.4	31.8±1.2	31.1±1.1	30.6±0.9
c18:0	6.5±0.3	6.2±0.5	6.6±0.6	7.4±1.3	6.5±0.4	6.7±0.3
Monoene						
c18:1‡	40.5±1.0	41.2±1.0	40.3±1.9	40.7±1.8	42.4±1.2	42.5±0.9
Polyunsaturated						
n-6 series						
18:2	12.5±0.1	11.9±0.1	10.5±0.1*	9.6±0.3*	8.9±0.1*	9.4±0.4*
20:3	1.8	1.9	2.0	2.2	2.2	2.7
20:4	6.0±0.3	6.2±0.2	6.6±0.1	7.3±0.3*	7.7±0.1*	7.3±0.3*
22:4	0.1±0.02	0.06±0.02	0.03±0.03	0.1±0.05	0.1±0.03	0.1±0.03
22:5	0.4±0.1	0.4±0.1	0.2±0.1	0.3±0.2	0.5±0.1	0.5±0.1
n-3 series						
20:5	0.2±0.01	0.2±0.01	0.2±0.01	0.2±0.01	0.2±0.02	0.2±0.0
22:5	0.1±0.05	0.1±0.05	0.2±0.00	0.1±0.1	0.1±0.05	0.2±0.1
22:6	1.7±0.2	1.7±0.2	1.7±0.02	1.7±0.2	1.7±0.2	1.7±0.2

He G2 cells, 72 h after passage, were incubated in fresh DME medium containing 10% delipidated serum, 40 μM LA and LOV 0.04–40 μM or DMSO alone (≤ 0.1%) for 72 h. Cells were extracted, phospholipids separated, and fatty acid methyl esters quantitated as described in Methods. Values (μM) are means±1 SEM from three experiments. * Value is different from DMSO control using one-way repeated measures ANOVA with Sheffe's F test for differences between means. ‡ Value represents the sum of 18:1 isomers.

(2.4±0.1% vs. 10.9±0.1%), indicating that LDL served as an efficient source of this AA precursor (Table III). LA was incorporated mainly at the expense of oleic acid, while the saturated fatty acids were not affected. Lovastatin treatment increased the relative AA content (from 6.9±0.1% to 8.3±0.3%) and decreased LA content (from 10.9±0.1% to 8.6±0.3%) in the LDL-loaded cells.

LOV effects on the metabolism of LA. In order to confirm the above described LOV-induced compositional changes in the cellular phospholipid n-6 polyunsaturated fatty acids, the metabolism of [^{14}C]18:2n-6 in both cell lines was followed. In the Hep G2 cell line, LOV treatment (10 μM, 72 h) resulted in an apparent increase in the desaturation of LA (Fig. 2 *A*). Relative to cells treated with DMSO (< 0.1%), the percentage of ^{14}C label appearing as 18:2n-6 decreased, while the proportion of label in fatty acid fractions with three, four, and five double bonds increased after 18 h of incubation with [^{14}C]LA (5 μM, 0.3 μCi). As illustrated in Fig. 2, the maximal change in LA metabolism was seen when LOV was given to freshly passaged cells (72 h, 10 μM).

In control cells ($n = 3$), 87.7±0.6% of the total label was found in phospholipids while 9.3±0.9%, 2.2±1.2%, and 0.7±0.1% were associated with the triglycerides, free fatty acids, and cholesterol esters, respectively. After LOV treatment (10 μM, 72 h), a marginal decrease in the percentage of label was found in the phospholipid (81.9±1.5%), free fatty acid (1.4±0.6%), and cholesterol ester (0.3±0.01%) fractions, while more label was associated with the triglycerides (16.4±1.4%).

Under the identical experimental conditions, control MM6 cells (DMSO ≤ 0.1%) desaturated proportionally more [^{14}C]18:2n-6 than did the control Hep G2 cells (Fig. 2 *B*). In

Table III. Phospholipid Fatty Acid Composition of Hep G2 Cells Incubated with LOV and LDL

	DMSO	DMSO + LDL	LOV + LDL
Saturated			
c16:0	35.2±0.9	34.1±0.3	35.6±0.8
c18:0	6.8±0.4	6.7±0.3	6.9±0.3
Monoene			
c18:1[§]	44.9±0.6	36.9±0.7[‡]	35.1±0.4[‡]
Polyunsaturated			
n-6 series			
18:2	2.4±0.1	10.9±0.1[‡]	8.6±0.3[*‡]
20:3	0.8±0.03	1.3±0.02	1.7±0.08
20:4	6.6±0.4	6.9±0.2	8.3±0.3[*‡]
22:4	0.1±0.01	0.1±0.01	0.1±0.02
22:5	0.4±0.04	0.3±0.02	0.4±0.04
n-3 series			
20:5	0.4±0.04	0.6±0.02	0.5±0.04
22:5	0.3±0.06	0.2±0.03	0.3±0.02
22:6	2.2±0.2	2.2±0.1	2.6±0.1

Freshly passaged Hep G2 cells were incubated with LOV (10 μM) or DMSO alone (≤ 0.1%) for 72 h and with LDL (100 μg/ml) for the last 24 h. Cells were extracted, phospholipids separated, and fatty acid methyl esters quantitated as described in Methods. Values are means±1 SEM from three experiments. Significant effects of [‡]LOV and [*]LDL incubation respectively, using one-way repeated measures ANOVA with Sheffe's F test for differences between means. [§] Value represents the sum of 18:1 isomers.
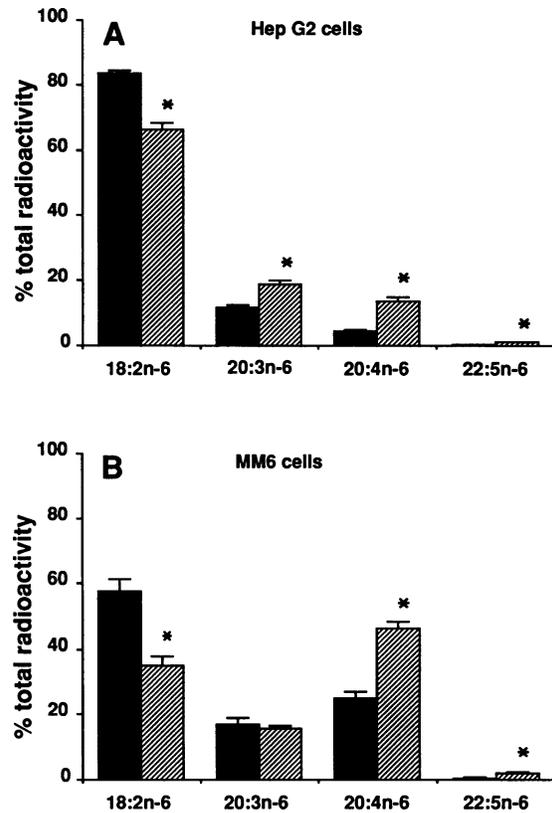
Figure 2. Effect of LOV on the desaturation of [^{14}C]LA. Freshly passaged (*A*) Hep G2 cells and (*B*) MM6 cells were incubated with 10 μM LOV (*hatched bars*) or DMSO (≤ 0.1%, *solid bars*) for 72 h and with [^{14}C]LA (0.3 μCi, 5 μM) for the last 18 h. Total lipids were extracted and the distribution of label in fatty acid fractions containing two, three, four, or five double bonds was determined by argentation chromatography as described in Methods. The 20:3n-6 and 20:4n-6 fractions also include the minor 18:3n-6 and 22:4n-6 fatty acids, respectively. Bars represent means±1 SEM of duplicate determinations from 15 (Hep G2) and 8 (MM6) experiments. *Value is significantly different from DMSO-treated control by means of paired t test, $P \leq 0.01$.

this cell line, LOV (10 μM, 72 h) also significantly decreased the proportion of ^{14}C label in 18:2n-6 while increasing the percentage of ^{14}C label appearing in fatty acids containing four and five double bonds (Fig. 2 *B*).

In both cell lines, the cellular uptake of [^{14}C]18:2n-6 after the 18-h incubation period was ≤ 85% and was not significantly affected by LOV treatment. DMSO in the concentrations used had no effect on [^{14}C]18:2n-6 metabolism (data not shown). Furthermore, the LOV effect was similar in cells given [^{14}C]18:2n-6 in the presence or absence of 10% FCS in medium.

Reversibility of the LOV effect on LA metabolism. In order to explore the possible mechanisms behind the LOV-induced changes in LA metabolism, their reversibility after the coincubation with MVA, cholesterol, or lipoproteins was examined in both cell lines. Our results indicate differences in the response to this treatment between the two cell lines.

In the Hep G2 cell line, the LOV effect on LA desaturation could be reversed by means of coincubation with MVA (500 μM, Fig. 3). The maximal reversal was obtained in LOV-treated cells coincubated with MVA for the entire 72-h period.

Ethanol alone (0.1%) had no such effect. Furthermore, MVA alone (500 μM) produced no changes in the metabolism of LA in control cells (data not shown). The simultaneous coincubation of LOV-treated cells (10 μM, 72 h) with either free cholesterol (15 μg/ml, dissolved in ethanol, final concentration 0.1%), LDL, or acetyl LDL (100 μg/ml protein) did not reverse the LOV effect on LA metabolism (Table IV*A*).

In contrast to the Hep G2 cells, coincubation of LOV-treated MM6 cells with neither MVA in concentrations up to 1 mM, nor with cholesterol, LDL, or acetyl LDL could significantly reverse the LOV effect (Table V*A*).

The potential for other inhibitors of HMGCoA reductase to stimulate the desaturation of LA was investigated in control cells incubated with cholesterol, LDL, or acetyl LDL. In both the Hep G2 and MM6 cell lines, none of these sources of cholesterol influenced the metabolism of linoleic acid (Tables IV*B* and V*B*, respectively). This lack of effect may reflect the fundamental difference between the sterol-mediated transcriptional and the LOV-mediated post-transcriptional regulation of HMGCoA reductase activity (3, 4).

Fatty acid composition of lipids secreted by Hep G2 cells. In order to investigate whether the LOV-mediated increase in hepatocyte LA desaturation was reflected in in situ synthesized lipoproteins, we examined the fatty acid composition of total phospholipids, triglycerides, and cholesterol esters secreted into culture medium by the Hep G2 cells. As shown in Table VI, LOV increased the relative content of AA in all three fractions in a dose-dependent manner. This effect was best seen in the phospholipids, the most abundant lipid secreted by this cell line (24, 25), where statistically significant changes occurred with as little as 0.4 μM LOV. In the phospholipid fraction, a significant increase in the minor 22:4n-6 and the PUFA n-3 fatty acids was also observed. Furthermore, LOV reduced palmitic acid content in the secreted phospholipid and triglyceride
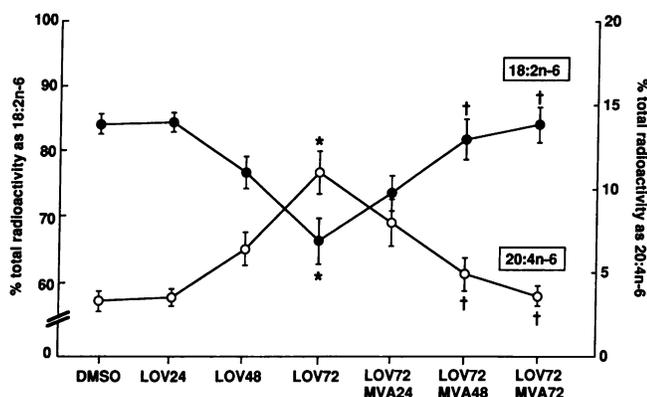
Figure 3. Metabolism of LA in Hep G2 cells incubated with LOV and MVA for different time periods. Freshly passaged Hep G2 cells were incubated with 10 μM lovastatin for the last 24, 48, or 72 h, or with 10 μM LOV for 72 h and 500 μM MVA for the last 24, 48, or 72 h and [^{14}C]18:n-6 (0.3 μCi, 5 μM) for the last 18 h. Total lipids were extracted and the distribution of label in fatty acids with two to five double bonds were determined by argentation chromatography as described in Methods. Values are means±1 SEM of the percentage of total radioactivity recovered as 18:2n-6 and the sum of 20:4n-6 and the minor 22:4n-6 in duplicate measurements from four experiments. Values are significantly different from the *DMSO-control and †LOV 72-h-treated cells, respectively, using one-factor ANOVA with Sheffe *F* test for differences between means, $P \leq 0.05\%$.

Table IV. Metabolism of LA in DMSO or LOV-treated Hep G2 Cells Coincubated with Various Sources of Cholesterol

	18:2n-6	20:3n-6	20:4n-6
	% total radioactivity		
A.			
LOV only	65.1±3.5	18.3±1.6	15.4±1.9
LOV + CHOL	68.5±3.0	17.5±1.6	13.0±1.5
LOV + LDL	70.9±1.5	15.8±1.2	12.3±1.7
LOV + aLDL	64.8±5.4	15.9±3.0	17.9±2.3
B.			
DMSO only	84.4±1.7	11.0±1.1	4.2±0.6
DMSO + CHOL	84.9±1.9	10.9±1.1	4.0±0.8
DMSO + LDL	89.7±0.5	7.2±0.3	2.8±0.3
DMSO + aLDL	87.6±2.3	7.8±1.6	4.1±1.0

Freshly passaged Hep G2 cells were incubated with (A) 10 μM LOV or (B) 0.1% DMSO and with either cholesterol (CHOL; 15 μg/mL), LDL (100 μg protein/ml), or acetyl LDL (aLDL, 100 μg protein/ml) for 72 h and with [^{14}C]18:2n-6 (0.3 μCi, 5 μM) for the last 18 h. Total lipids were extracted and the distribution of label in fatty acids with two to five double bonds were determined by argentation chromatography as described in Methods. 20:3n-6 and 20:4n-6 fractions also include the minor 18:3n-6 and 22:4n-6 fatty acids, respectively. Values are means±1 SEM from duplicate measurements from three to eight experiments.

and increased stearic acid in the phospholipid and cholesterol ester fractions. Unlike for cellular phospholipids, however, LOV did not decrease the LA content of the secreted lipids.

Table V. Metabolism of LA in DMSO or LOV-treated Mono Mac 6 Cells Coincubated with MVA and Various Sources of Cholesterol

	18:2n-6	20:3n-6	20:4n-6
	% total radioactivity		
A.			
LOV only	37.1±2.3	13.8±0.6	46.3±2.5
LOV + MVA	44.4±5.5	13.9±0.4	40.4±5.1
LOV + CHOL	35.1±4.4	14.4±1.8	48.0±4.6
LOV + LDL	39.8±3.3	12.1±0.8	45.6±2.8
LOV + aLDL	34.6±2.1	13.4±0.8	48.7±1.6
B.			
DMSO only	63.1±3.9	15.6±0.8	20.8±1.6
DMSO + MVA	52.9±8.3	16.6±1.4	29.7±9.4
DMSO + CHOL	62.1±2.8	19.3±1.8	17.9±2.7
DMSO + LDL	65.1±1.9	17.7±1.0	16.5±2.8
DMSO + aLDL	59.6±3.9	16.5±0.8	23.3±4.5

Freshly passaged MM6 cells were incubated with (A) 10 μM LOV or (B) 0.1% DMSO and with either MVA (1 mM), cholesterol (CHOL; 15 μg/ml), LDL (100 μg protein/ml), or acetyl LDL (aLDL; 100 μg protein) for 72 h and with [^{14}C]18:2n-6 (0.3 μCi, 5 μM) for the last 18 h. Total lipids were extracted and the distribution of label in fatty acids with two to five double bonds were determined by argentation chromatography as described in Methods. The 20:3n-6 and 20:4n-6 fractions also include the minor 18:3n-6 and 22:4n-6 fatty acids, respectively. Values are means±1 SEM from duplicate measurements from three or four experiments.

Table VI. Effect of LOV on the Fatty Acid Composition of Lipids Secreted by Hep G2 Cells

| | DMSO | LOV(μM) | | | |
		0.04	0.4	4	10
Phospholipids					
c16:0	49.0±0.4	48.1±0.4	43.8±0.5*	41.9±0.6*	42.2±0.6*
c18:0	4.7±0.3	4.3±0.3	5.5±0.4	5.8±0.4*	6.1±0.2*
c18:1‡	38.1±0.6	39.2±0.4	39.5±0.8	41.1±1.1	40.0±0.9
c18:2n-6	3.7±0.2	3.8±0.2	4.1±0.4	3.2±0.4	3.0±0.5
c20:4n-6	2.9±0.2	3.5±0.3	4.3±0.2*	4.7±0.2*	4.7±0.3*
c22:5n-6	0.15±0.03	0.24±0.01	0.27±0.02	0.34±0.01*	0.41±0.1*
Σc20:5, 22:5, 22:6n-3	0.86±0.08	0.83±0.05	1.46±0.10*	1.58±0.21*	1.82±0.32*
Triglycerides					
c16:0	32.1±0.8	29.7±0.3	28.6±0.5*	25.9±0.7*	25.2±0.3*
c18:0	3.1±0.2	2.7±0.1	3.1±0.3	3.5±0.2	3.6±0.2
c18:1‡	62.3±1.1	65.5±0.1	64.7±1.4	66.3±1.8	66.3±1.1
c18:2n-6	1.5±0.1	1.6±0.02	1.8±0.3	1.8±0.2	1.9±0.4
c20:4n-6	0.36±0.06	0.33±0.03	0.38±0.04	0.61±0.04*	0.69±0.08*
Cholesterol ester					
c16:0	29.7±0.6	29.5±0.5	29.0±0.6	26.8±1.1	28.6±1.5
c18:0	4.9±0.3	4.1±0.1	5.1±0.4	8.4±0.8*	9.9±0.6*
c18:1‡	58.5±1.6	58.0±1.1	56.6±1.7	54.3±1.6	52.6±2.8
c18:2n-6	4.3±0.5	6.9±1.6	5.9±1.1	4.4±1.3	4.7±1.5
c20:4n-6	1.4±0.2	1.3±0.03	2.1±0.3	3.9±1.4*	3.2±0.6*

Freshly passaged Hep G2 cells were incubated with LOV 0–10 μM or DMSO alone (≤ 0.1%) on days 1 through 8, with 10 μM LA on days 4–6 and in serum-free DME on days 7 and 8. Total lipids secreted into the medium were isolated, lipid classes separated, and fatty acid methyl esters quantitated as described in Methods. Values (μM) are means±1 SEM from three to seven experiments. * Value is different from DMSO control using one-way repeated measures ANOVA with Sheffe's F test for differences between means. ‡ Value represents the sum of 18:1 isomers.

Thromboxane synthesis in the MM6 cell line. To investigate the potential functional consequences of a LOV-mediated rise in cellular AA levels, its effect on thromboxane synthesis
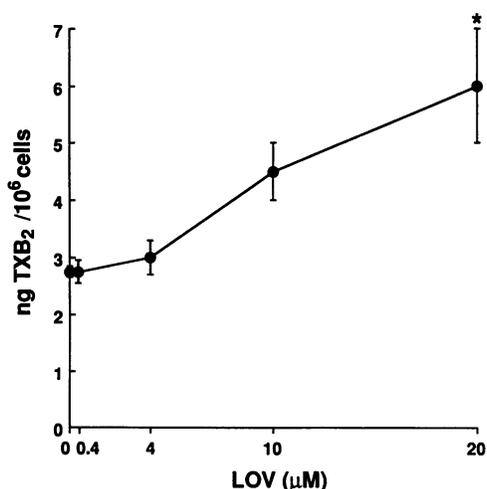
Figure 4. Effect of LOV on thromboxane release by MM6 cells. Freshly passaged MM6 cells were incubated with DMSO (≤ 0.1%) or 0.4–20 μM lovastatin for 72 h, and LA (10 μM) for the last 24 h. Cells were resuspended in buffer and stimulated with 1 μM calcium ionophore A23187 for 5 min, and the thromboxane released was determined as described in Methods. Bars represent means±1 SEM of duplicate measurements from five experiments. *Value is significantly different from the DMSO-treated control using one-factor ANOVA with Sheffe F test for differences between means, $P \leq 0.05$.

was examined in the MM6 cell line. LOV increased the thromboxane synthesis in A23187-stimulated cells in a dose-dependent manner (Fig. 4). Thromboxane release was also marginally higher after treatment with 20 μM LOV in unstimulated cells (5 min, DMSO 0.1%, data not shown). This effect could be reversed by means of coincubation with 500 μM MVA (Fig. 5), while mevalonic acid added to control cells had no influence on thromboxane synthesis (data not shown). In contrast, cells incubated with LOV (10 μM, 72 h) and LDL (100 μg/ml) released more thromboxane than cells treated with LOV alone (Fig. 5). Preliminary results indicate that LDL or acetyl LDL alone also stimulate thromboxane release in control cells (data not shown).

Influence of LOV on cellular lipids. In the Hep G2 cells, 10 and 40 μM LOV (72 h) induced a dose-dependent accumulation of cellular triglycerides, while cellular phospholipids were marginally elevated at 40 μM LOV, $P < 0.10$ (Table VII). In contrast, the cellular cholesterol content of both Hep G2 (Table VII) and MM6 (data not shown) cells was not affected.

Influence of LOV on cell proliferation and viability. In the MM6 cell line, LOV produced a dose-dependent suppression of cellular proliferation which was statistically significant at the 20 μM dose level (Fig. 6). Cell proliferation was partially recovered by means of coincubation with MVA (500 μM), while cell proliferation after LDL (100 μg/ml) was further reduced. The cell viability, as judged by ethidium bromide/acridin orange fluorescence, was 91% in control cells and 86 and 76% in cells treated with 10 and 20 μM LOV, respectively. Cells coincubated with 20 μM LOV and MVA were 89% viable, while the viability of cells coincubated with LOV and LDL was further
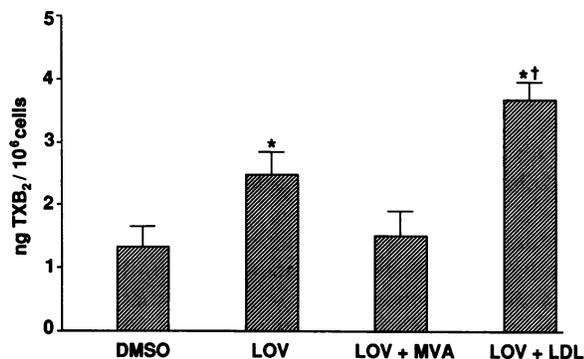
Figure 5. Effect of MVA and LDL on thromboxane release by LOV-treated MM6 cells. Freshly passaged MM6 cells were incubated with DMSO ($\leq$ 0.1%), LOV alone (10 μM) or with LOV (10 μM) and MVA (500 μM) or LDL (100 μg/ml) for 72 h and with 10 μM LA for the last 24 h. Cells were resuspended in buffer and stimulated with calcium ionophore A23187 (1 μM) for 5 min. Thromboxane release was determined as described in Methods. Values are means±SEM of duplicate measurements from three or four experiments. Values are different from *DMSO-treated and [†]LOV-treated cells respectively, by Fisher PLSD test.

reduced. Lipoproteins added to control cells (DMSO < 0.1%) had no effect on cell viability.

In the Hep G2 cell line, a LOV concentration up to 10 μM did not influence cell proliferation, as determined by means of cellular protein measurement, while 40 μM LOV reduced proliferation by 20% (data not shown). The cell viability was > 90% at 0–40 μM LOV.

Discussion

Most clinical studies of HMGCoA reductase inhibitors focus on drug-mediated changes on total lipid or lipoprotein profiles as underlying mechanisms for their therapeutic effects. Less attention has been given to effects of these agents on fatty acid metabolism. However, Doormaals et al. (5) have found decreased levels of LA and increased levels of AA in erythrocytes of patients receiving simvastatin therapy. Similarly, a decrease in LA content of LDL triglyceride and cholesterol ester (7), as well as an increase in total PUFA content of VLDL, LDL, and

Table VII. Effect of LOV on Cellular Triglyceride, Phospholipid, and Cholesterol Content of Hep G2 Cells

	Triglyceride	Phospholipid	Cholesterol
	μg/mg protein	μmol/mg protein	μg/mg protein
DMSO (0.1%)	106.0±14.8	0.49±0.02	26.45±1.02
LOV (10 μM)	170.6±8.6*	0.47±0.01	24.52±1.89
LOV (40 μM)	239.1±13.9*[‡]	0.61±0.06	27.84±2.71

Freshly passaged Hep G2 cells, grown in DME medium containing 10% FCS, were incubated with DMSO (0.1%), or LOV (10 or 40 μM) for 72 h. Subsequently, cells were washed and extracted, and total cellular triglyceride, phospholipid, and cholesterol were measured as described in Methods. Values are means±SEM of triplicate measurements from four experiments. Value is different from * DMSO control and [‡] from LOV 10 μM using one-way repeated measures ANOVA with Sheffe's F test for differences between means.
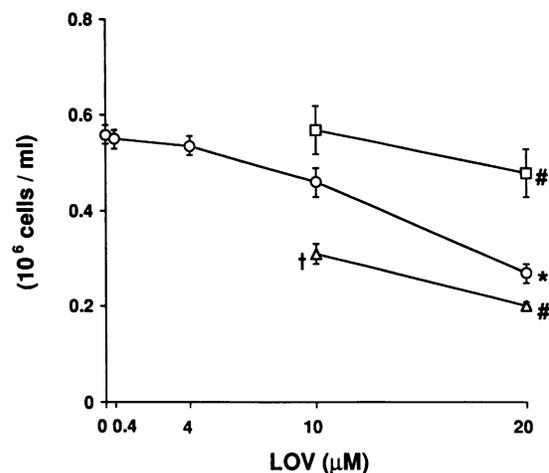
Figure 6. Freshly passaged MM6 cells were seeded at 0.2 × 10^{6} cells/ml and incubated with DMSO (0.1%), LOV (0.4–20 μM, o) or 20 μM LOV with MVA (1 mM, □) or LDL (100 μg protein/ml, △) for 72 h and with 10 μM LA for the last 24 h. Cells were counted in the original medium. Symbols are means±1 SEM from three to eight experiments. *Value is significantly different from DMSO-treated control using one-factor ANOVA with Sheffe F test for differences between means, $P \leq 0.05$. Values are different from [†]LOV 10 μM and *LOV 20 μM-treated cells, respectively, by Student's t test.

HDL triglyceride (6) in patients undergoing HMGCoA reductase inhibitor therapy has been reported. The present study was undertaken to examine the effects of LOV on the metabolism of LA in the human hepatoma (Hep G2) and monocytic MM6 cell lines. We demonstrate that LOV increases the desaturation of LA and thus the cellular phospholipid content of AA. The higher availability of cellular AA in turn results in higher AA content of lipids secreted by the Hep G2 cells and increased release of thromboxane in the MM6 cells.

HMGCoA reductase inhibitors may influence fatty acid desaturase activity secondary to their specific inhibition of the cholesterol biosynthetic pathway or through their nonspecific action on systems related to drug metabolism. With respect to the first possibility, manipulation of membrane cholesterol content is known to have profound effects on membrane fluidity and function of membrane-associated enzymes (1, 2). Increased microsomal membrane fluidity, AA content and enhanced Δ 5 and 6 desaturase activities have been documented in liver microsomes of rats fed a sterol-free diet (9). The feeding of cholesterol-rich diets, on the other hand, can lead to decreased liver microsomal Δ 5 and 6 desaturase activity and AA content (8, 10, 26). Although our findings of increased desaturation of LA in LOV-treated cells tend to parallel the above results, the effect observed does not appear to be closely related to cellular cholesterol content. As previously described in other cell culture systems (27), we find no significant reductions in cellular cholesterol levels after LOV treatment. We assume that the reduction of de novo cholesterol synthesis, produced by LOV, is sufficiently compensated for by increased uptake of cholesterol from the cell culture media, both of which contained 10% FCS. In support of this, both the Hep G2 (14) and MM6 (our own observation) cell lines have functional LDL receptors. Furthermore, coincubation of LOV-treated cells with free cholesterol as well as with lipoproteins did not reverse the LOV-induced changes in LA metabolism.

Alternatively, it is becoming increasingly clear that early as well as later intermediates of the cholesterol biosynthetic pathway are vital for diverse cellular functions (3, 4). The inhibition of the synthesis of early metabolites of cholesterogenesis such as farnesyl or geranyl pyrophosphates, leading to decreased prenylation of a variety of proteins, affect cell growth (28, 29), chemotaxis (27), and signal transduction (28, 30). With respect to the later metabolites of cholesterogenesis, dolichol phosphates are required for glycoprotein synthesis, while ubiquinone and heme are involved in the electron transport chain (4).

In our experiments, the LOV-mediated changes in LA metabolism in the Hep G2 cell line were completely reversed by the addition of MVA. In the MM6 cell line, the LOV-induced increase in thromboxane release was reduced with MVA, although under the experimental conditions used, the conversion of LA to AA was not. Coincubation of LOV-treated cells with LDL, on the other hand, enhanced thromboxane release. Similar effects of LDL on thromboxane release has been seen in endothelium (31) and platelets (32). Taken together, our data strongly suggest that a decrease in the availability of one or more of the early intermediates of cholesterogenesis were responsible for the observed cellular effects of LOV on the microsomal desaturation of PUFA and the subsequent synthesis of eicosanoids.

Treatment with LOV and related compounds results in a marked accumulation of the microsomal inactive HMGCoA reductase enzyme and proliferation of the endoplasmic reticulum (33). Peroxisomal hyperplasia with concomitant increases in fatty acid synthesis and triglyceride and phospholipid accumulation in cultured human keratinocytes after LOV has also been recently reported (34). We also observe accumulation of cellular triglyceride and increased uptake of fatty acid into the triglyceride lipid fraction in our LOV-treated Hep G2 cells. Thus, LOV may act similarly to other lipid-lowering drugs such as the fibrates, for which peroxisomal hyperplasia and induction of endoplasmic reticulum enzymes (35, 36), as well as perturbations in fatty acid metabolism (37–39) have been reported. Induction of fatty acid desaturation in proliferating hepatic endoplasmic reticulum in response to treatment with polychlorinated biphenyls suggests that fatty acid desaturases are cytochrome P450–dependent monooxygenases (40). Cytochrome P450 enzymes are also responsible for the oxidative metabolism of lovastatin (41). Thus HMGCoA reductase inhibitors may stimulate LA desaturation through the induction of the endoplasmic reticulum. Recently, however, no nonspecific activation of several cytochrome P450-linked enzymes has been detected in rats treated with simvastatin, despite elevations in microsomal AA acid content (42). As in the present study, some of the above cited inhibitor-induced effects in human keratinocytes were reversible with MVA as well as with lipoproteins (34). Thus even in this case, LOV would appear to act specifically via its suppression of the cholesterol synthesis pathway.

The hepatoma Hep G2 cell line, used extensively in studies of liver lipid metabolism (14), is an appropriate cell model in the study of effects of HMGCoA reductase inhibitors in the liver, the target organ of these drugs. We show increases in arachidonic acid content in lipids secreted by the Hep G2 cells at lovastatin doses equivalent to plasma LOV concentrations of patients undergoing LOV therapy (43). Even higher transient concentrations may be present in the liver, where LOV is cleared in the first passage. Our findings in this cell line support in vivo studies (5, 42) that indicate that the use of HMGCoA inhibitors leads to an increased availability of AA in hepatic and extrahepatic tissues. Our findings in Hep G2 cells coincubated with LDL also suggest that the LOV-mediated increase in hepatic lipoprotein clearance, and thus increased delivery of LA-rich cholesterol ester to the liver cell, may contribute to this effect.

Our findings in the MM6 cell line, considered to be a good model for the peripheral blood monocyte (15), further suggest potential functional consequences of LOV in the periphery, namely the increased synthesis of thromboxane. This may be the consequence of a direct LOV action in the periphery or an increased supply of AA via lipoproteins secreted by the liver, to eicosanoid-producing tissues.

In summary, the present in vitro finding of increased conversion of LA to AA by the Hep G2 and MM6 cells, and the concomitant increase in AA content of lipids secreted by the Hep G2 cells support clinical findings of increased systemic AA levels in patients undergoing HMGCoA reductase inhibitor therapy. At the cellular level, this effect appears to be strongly linked to a decrease in the availability of early nonsterol intermediates of the cholesterol synthesis pathway, and thus possibly to the decreased prenylation of cellular proteins. This may have impact on the composition of cellular fatty acids and on the systemic synthesis of eicosanoids. The elucidation of the mechanisms involved, and their physiological significance are worthy of further study.

Acknowledgments

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