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α-Linolenic acid reduces the lovastatin-induced rise in arachidonic acid and elevates cellular and lipoprotein eicosapentaenoic and docosahexaenoic acid levels in Hep G2 cells

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Increased plasma arachidonic acid (20:4ω6) levels have been reported in patients undergoing 3-hydroxy-3-methyl coenzyme A (HMG-CoA) reductase inhibitor therapy. We have previously shown that this effect is related to an increased conversion of linoleic acid (18:2ω6) to 20:4ω6 via the fatty acid desaturases and results in an increased formation of biologically potent eicosanoids. Because fatty acid desaturases also act on ω3 fatty acids, and because the long chain ω3 fatty acids counterbalance many of the physiological effects of ω6 fatty acids, we now examine the effects of α-linolenic acid (18:3ω3) supplementation on the lovastatin-induced changes in cellular and lipoprotein fatty acid levels. Human hepatoma Hep G2 cells were incubated with lovastatin (10 μmol/L) or its carrier dimethyl sulphoxide (DMSO, final concentration 0.1%) for 72 hr and with albumin-bound 18:3ω3 (40 μmol/L) alone or with different ratios of 18:3ω3 to 18:2ω6 for the last 24 hr. In comparison with control cells, lovastatin-treated cells converted more 18:3ω3 into eicosapentaenoic (20:5ω3) and docosahexaenoic (22:6ω3) acids, which were incorporated in increasing amounts in cellular phospholipids and lipids secreted by these cells. The lovastatin-mediated increase in 20:4ω6 levels in cellular and secreted lipids was also significantly reduced in 18:3ω3-supplemented cells. The effect of 18:3ω3 supplementation on the lovastatin-induced changes in ω6 and ω3 fatty acid composition was dependent on the 18:3ω3/18:2ω6 supplementation ratio. The present studies suggest that the previously described effects of HMGCoA reductase inhibitors on polyunsaturated fatty acid metabolism can be modulated by the dietary 18:3ω3/18:2ω6 ratio. (J. Nutr. Biochem. 7:465–471, 1996.)

Keywords: α-linolenic acid; arachidonic acid; ω3 fatty acids; 3-hydroxy-3-methyl coenzyme; A reductase inhibitor, lipoproteins; Hep G2 cells

Introduction

We have recently demonstrated that the 3-hydroxy-3-methyl coenzyme A (HMG-CoA) reductase inhibitor lovastatin (LOV) increases the arachidonic acid (20:4ω6) levels in the cellular phospholipids and lipoproteins secreted by the human hepatoma Hep G2 cells.[1] This finding, which is a consequence of increased fatty acid desaturation activity, may explain the sporadic reports of decreased linoleic acid (18:2ω6) levels and increased 20:4ω6 levels in lipoproteins[2–4] and erythrocytes[5] of patients undergoing HMG-CoA reductase inhibitor therapy. As demonstrated by Habenicht et al.,[6] cholesteryl arachidonate of the LDL lipid

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core is an important source of 20:4ω6 for the formation of eicosanoids in extrahepatic tissues. We have shown in vitro thatthe LOV-induced increase in cellular phospholipid 20:4ω6 is accompanied by increased eicosanoid synthesis.[1] Furthermore, 20:4ω6 and other polyunsaturated fatty acids (PUFAs) are important regulatory components of membrane phospholipids and are increasingly recognized as regulators of gene expression.[7]

The effects of eicosapentanoic (20:5ω3) and docosahexanoic (22:6ω3) acids in cardiovascular and other proliferative, inflammatory diseases have been extensively researched and documented.[8] Epidemiological evidence also points to significant inverse correlations between the dietary ω3/ω6 ratio and cardiovascular and cancer mortality.[9] Furthermore, interest in the potentially protective cardiovascular properties of diets enriched in the ω3 fatty acid presursor α-linolenic acid (18:3ω3) has recently been renewed.[10] Because ω3 fatty acids compete with the ω6 fatty acids for the same desaturation and acylation enzymes,[11] the LOV-induced changes in PUFA metabolism may be modulated by ω3/ω6 precursor ratios. Thus, the present study examined the combined effect of lovastatin and α-linolenic acid on the ω3 and ω6 fatty acid composition of cellular phospholipids and lipoproteins secreted by the human hepatoma Hep G2 cells.

Methods and materials

Materials

Lovastatin was a kind gift of Dr. A. W. Alberts from Merck Sharp and Dome (Rahway, NJ, USA). Free fatty acids, bovine serum albumin (BSA, essentially fatty acid free, A6003), cell culture media, and cell culture ingredients were from Sigma (Munich, Germany). Organic solvents, dimethyl sulphoxide (DMSO) were from Merck (Darmstadt, Germany). Bond Elut solid phase extraction columns (NH_2-aminopropyl, 1 mL bed volume) were from Baker (No. 7088-1, Denveter, Netherlands).

Cell culture

Hep G2 cells (American Tissue Type Culture Collection, Rockville, MD, USA) were grown in 15 ml flasks in Dulbecco's modified Eagle's medium (DMEM, D5405) supplemented with 10% fetal calf serum (FCS), non-essential amino acids (NEAA, 1 mmol/L) and l-glutamine (2 mmol/L) and were passaged (1:6) once a week with trypsin-EDTA (1x).

Preparation of albumin-bound fatty acids

Free fatty acids were dissolved in ethanol and converted to their anion with 0.5 mol/L KOH. Ethanol was evaporated under N_2 and the salt was immediately reconstituted with 2.5 mmol/L BSA dissolved in culture medium to yield a 5 mmol/L fatty acid solution. The pH was adjusted to 7.4 with 1 mol/L NaOH and aliquots were stored at −80°C.

Determination of phospholipid fatty acids

Freshly passaged Hep G2 cells, grown in normal medium for the first 3 days, were treated with LOV (10 μmol/L, dissolved in DMSO) or DMSO alone (final concentration 0.1%), and with albumin-bound 18:3ω3 alone (40 μmol/L) or with different ratios of 18:3ω3/18:2ω6 on days 4 to 8 as described in legends. Cells were then washed (3x5 ml NaCl 0.9%) and cellular lipids were extracted with chloroform/methanol (2:1 vol/vol), containing butylated hydroxytoluene (BHT, 0.2%). Total phospholipids were separated from neutral lipids on aminopropyl-bonded phase Bond-Elut columns.[12] The phospholipid eluate was transesterified with anhydrous 3N methanolic HCL (90°C, 1 hr) in the presence of 17:0 as internal standard. Fatty acid methyl esters were recovered in petroleum benzene and quantified with a Hewlett-Packard 5890A gas chromatogram, using a 2.5 mm x 30 m DB-225 fused silica capillary column.[1]

Determination of fatty acid composition of lipids secreted by Hep G2 cells

Hep G2 cells were kept in growth medium for the first 3 days after passage. On day 4, cells were given fresh growth medium and were treated with LOV 0.4 to 10 μmol/L or DMSO (6 x 145-cm^2 plates per treatment), and with albumin-bound 18:3ω3 and/or 18:2ω6 as described in legends. On day 7, growth medium was removed and cells were washed 3 times with 30 mL Dulbecco's PBS and once with FCS-free DMEM. Fresh FCS-free DMEM (30 mL) containing LOV (0.4 to 10 μmol/L) or DMSO was then added and harvested after 24 hr. This procedure was repeated on day 8. The harvested medium was supplemented with EDTA (1 mg/mL) and gentamycin sulphate (0.1 mg/mL), centrifuged at 1,000 g x 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, Amicon, Witten, Germany). Concentrates were lyophilized and total lipids extracted with chloroform/methanol (2:1vol/vol). Phospholipid, triglyceride, and cholesterol ester fractions were separated on aminopropyl columns,[12] and fatty acids quantified as described.

Statistics

Unless otherwise stated, results are expressed as means ± SEM. The data were analyzed by means of analysis of variance, using the 512+ Statview statistical package (Abacus Concepts, Inc. 1986). Differences between means were assessed with the Scheffe's F-test.

Results

Effect of LOV and 18:3ω3 on cellular phospholipid fatty acid levels of Hep G2 cells

As depicted in *Figure 1*, incubation of control cells with 18:3ω3 (40 μmol/L) resulted in its incorporation into cellular phospholipids and further conversion into 20:5ω3, 22:5ω3 and 22:6ω3. Simultaneous treatment with LOV (10 μmol/L), reduced 18:3ω3 levels by 70% (2.83 ± 0.28 to 0.86 ± 0.09%, $P < 0.01$), whereas 22:6ω3 levels were increased by 47% (3.90 ± 0.33 to 5.70 ± 0.72%, $P < 0.01$). Supplementation with 18:3ω3 also significantly lowered the cellular phospholipid 20:4ω6 levels in both control (6.9 ± 0.4 to 5.5 ± 0.3, $P < 0.05$) and LOV-treated (7.3 ± 0.2 to 6.1 ± 0.3, $P < 0.05$) cells.

In cells not receiving exogenous essential fatty acids, LOV treatment alone reduced 18:2ω6 levels in cellular phospholipids (2.09 ± 0.39 in control vs 1.46 ± 0.17 in LOV-treated cells, $P < 0.05$), but did not significantly alter the levels of 20:4ω6 (6.89 ± 0.42 versus 7.30 ± 0.15), 18:

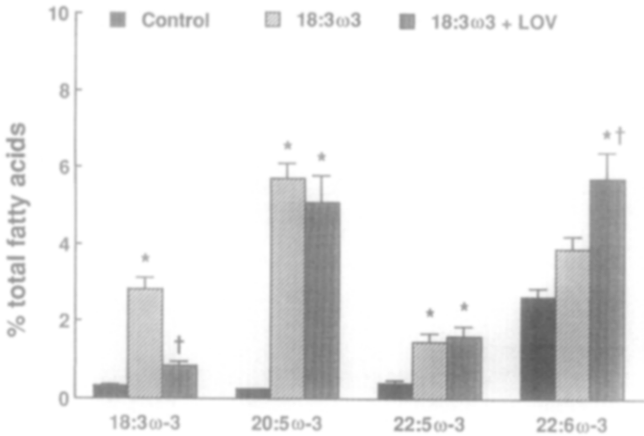

Figure 1 Effect of 18:3ω3 supplementation and LOV on cellular phospholipid ω3 fatty acid content of Hep G2 cells. Cells in normal growth medium were treated with DMSO alone (control), BSA-bound 18:3ω3 (40 µmol/L) or BSA-bound 18:3ω3 (40 µmol/L) and LOV (10 µmol/L) on days 4 to 8 after passage. Total lipids were extracted and cellular phospholipid fatty acids were quantified as described under "Methods and materials." Bars represent means ± SEM of three or four experiments. * and † indicate that values are statistically different from control and 18:3ω3-supplemented cells, respectively.

3ω3 (0.32 ± 0.06 versus 0.18 ± 0.03), 20:5ω3 (0.24 ± 0.03 versus 0.29 ± 0.09), 22:5ω3 (0.41 ± 0.05 versus 0.46 ± 0.05), and 22:6ω3 (2.63 ± 0.21 versus 2.81 ± 0.20).

Effect of LOV and 18:3ω3 on fatty acid composition of lipids secreted by Hep G2 cells

To investigate whether the above observed changes in cellular phospholipid fatty acid composition were reflected in lipoproteins synthesized by the Hep G2 cells, we compared the fatty acid composition of lipids secreted into culture medium by cells incubated with increasing concentrations of LOV (0.4, 4 or 10 µmol/L) and supplemented with either 18:3ω3, 18:2ω6 (both 40 µmol/L) or no exogenous fatty acids. In agreement with others,[13,14] the greatest proportion of fatty acids secreted by Hep G2 cells were esterified in phospholipids and triglycerides (56% and 37% of total fatty acids, respectively), whereas only a small amount appeared in cholesterol ester (6.7%). As we have shown previously,[1] LOV produced a significant dose-dependent increase in the 20:4ω6 content of phospholipids (*Table 1*), triglycerides (*Table 2*), and cholesteryl esters (*Table 3*) secreted by Hep G2 cells supplemented with exogenous 18:2ω6. A similar, dose-dependent increase in 20:4ω6 was also observed in the phospholipids secreted by cells receiving essentially no exogenous fatty acids, at LOV doses as low as 0.4 µmol/L (*Table 1*). In contrast, this LOV-mediated increase in 20:4ω6 secreted was blocked by 18:3ω3-supplementation at the lower LOV concentrations (0.4 and 4 µmol/L), and was statistically significant only in the secreted phospholipids at the highest LOV dosage used (10 µmol/L) (*Table 1*).

Cells supplemented with exogenous 18:3ω3 secreted this fatty acid in all three lipid fractions (*Tables 1* to *3*). Relative to cells receiving no exogenous fatty acids and those supplemented with 18:2ω6, these cells also secreted more 20:5ω3 and 22:6ω3, especially in the phospholipid (*Table 1*) and cholesteryl ester (*Table 3*) fractions. The levels of 20:5ω3 and 22:6ω3 were further increased by LOV in a dose-dependent manner. Interestingly, LOV also increased 22:6ω3 levels in the phospholipids secreted by cells receiving no exogenous 18:3ω3 (*Table 1*).

As described previously,[1] 16:0 levels were decreased, whereas 18:0 and 18:1 levels increased after LOV treatment in the lipids secreted by Hep G2 cells, regardless of fatty acid supplementation.

Table 1 Fatty acid composition of phospholipids secreted by Hep G2 cells

LOV	no fatty acid				+18:3ω3				+18:2ω6			
(µmol/L)	0	0.4	4	10	0	0.4	4	10	0	0.4	4	10
16:0	49.88 ± 1.86	49.66 ± 1.67	45.44* ± 0.95	44.60* ± 1.08	48.27 ± 0.96	46.85* ± 0.28	44.90* ± 2.52	42.15* ± 1.68	48.96 ± 0.38	43.80* ± 0.54	41.85* ± 0.64	42.21* ± 0.60
18:0	4.28 ± 0.27	4.92 ± 0.3	5.12* ± 0.25	5.32* ± 0.14	4.39 ± 0.10	4.82 ± 0.46	5.40* ± 1.41	5.63* ± 0.39	4.73 ± 0.32	5.52 ± 0.37	5.77* ± 0.40	6.05* ± 0.22
18:1	40.20 ± 1.97	39.28 ± 1.90	41.30 ± 1.31	42.21 ± 1.39	36.31 ± 0.73	37.50 ± 0.77	37.38 ± 0.43	38.51 ± 0.50	38.07 ± 0.58	39.54 ± 0.82	41.12 ± 1.06	40.04 ± 0.94
18:2ω6	1.16 ± 0.11	1.15 ± 0.12	1.12 ± 0.04	1.10 ± 0.06	1.78 ± 0.22	1.57 ± 0.10	1.64 ± 0.20	1.66 ± 0.07	3.66 ± 0.20	4.07 ± 0.38	3.16 ± 0.40	3.02 ± 0.53
20:4ω6	2.01 ± 0.04	2.79* ± 0.01	3.23* ± 0.16	3.13* ± 0.15	2.20 ± 0.22	2.36 ± 0.05	2.42 ± 0.30	3.20* ± 0.30	2.86 ± 0.15	4.31* ± 0.18	4.68* ± 0.16	4.74* ± 0.33
18:3ω3	n.d.	n.d.	n.d.	n.d.	0.83 ± 0.19	0.58 ± 0.10	0.49 ± 0.17	0.65 ± 0.14	n.d.	n.d.	n.d.	n.d.
20:5ω3	0.07 ± 0.01	0.11 ± 0.01	0.09 ± 0.02	0.03 ± 0.00	2.44 ± 0.11	2.36 ± 0.18	2.98* ± 0.19	2.82* ± 0.09	0.06 ± 0.01	0.11 ± 0.02	0.22 ± 0.07	0.26 ± 0.17
22:6ω3	0.79 ± 0.06	1.06* ± 0.02	1.25* ± 0.00	1.13* ± 0.06	2.40 ± 0.14	2.59 ± 0.11	3.19* ± 0.28	3.34* ± 0.29	0.73 ± 0.06	1.16* ± 0.13	1.22* ± 0.14	1.21* ± 0.19

Freshly passaged Hep G2 cells were incubated with LOV 0.4 to 10 µmol/L on days 4 to 8, with 18:2ω6 or 18:3ω3 (40 µmol/L) on days 4 to 6 and in serum-free DMEM on days 7 and 8. Fatty acid composition of lipids secreted into the medium were quantitated as described under "Methods and materials." Values are means ± 1 SEM from 3 to 5 experiments.
*Value is different from respective DMSO control using ANOVA with Sheffe's F-test for differences between means.
n.d., not detectable or less than 0.1% of total fatty acids.

Table 2 Fatty acid composition of triglycerides secreted by Hep G2 cells

LOV (μmol/L)	no fatty acid				+18:3ω3				+18:2ω6			
	0	0.4	4	10	0	0.4	4	10	0	0.4	4	10
16:0	28.79 ± 0.31	26.92 ± 0.47	25.53* ± 1.27	23.99* ± 1.00	32.40 ± 1.36	30.94 ± 0.66	29.35* ± 1.05	27.67* ± 1.13	32.05 ± 0.76	28.56* ± 0.47	25.86* ± 0.71	25.21* ± 0.28
18:0	3.80 ± 0.12	3.51 ± 0.3	3.87 ± 0.01	3.88 ± 0.10	3.24 ± 0.38	3.28 ± 0.21	3.75* ± 0.39	3.84* ± 0.43	3.07 ± 0.17	3.07 ± 0.30	3.50 ± 0.16	3.56 ± 0.22
18:1	63.94 ± 1.57	66.01 ± 1.35	66.20* ± 0.24	67.92* ± 0.62	58.65 ± 0.97	60.30 ± 1.09	60.99* ± 0.38	62.20* ± 0.79	62.25 ± 1.12	64.72 ± 1.35	66.26 ± 1.84	66.34 ± 1.05
18:2ω6	0.42 ± 0.15	0.37 ± 0.15	0.59 ± 0.04	0.45 ± 0.04	2.01 ± 0.20	1.50 ± 0.28	1.59 ± 0.24	2.00 ± 0.51	1.54 ± 0.12	1.75 ± 0.25	1.75 ± 0.22	1.86 ± 0.42
20:4ω6	0.59 ± 0.19	0.30 ± 0.01	0.48 ± 0.06	0.57 ± 0.02	0.41 ± 0.13	0.46 ± 0.18	0.61 ± 0.27	0.61 ± 0.20	0.36 ± 0.06	0.38 ± 0.04	0.61* ± 0.04	0.69* ± 0.08
18:3ω3	n.d.	n.d.	n.d.	n.d.	0.99 ± 0.14	0.81 ± 0.15	0.83 ± 0.16	1.09 ± 0.34	n.d.	n.d.	n.d.	n.d.
20:5ω3	n.d.	n.d.	n.d.	n.d.	0.34 ± 0.08	0.31 ± 0.06	0.37 ± 0.08	0.55 ± 0.06	n.d.	n.d.	n.d.	n.d.
22:6ω3	0.30 ± 0.01	0.24 ± 0.02	0.31 ± 0.02	0.26 ± 0.01	0.92 ± 0.17	1.05 ± 0.12	1.27 ± 0.35	1.50* ± 0.24	0.17 ± 0.06	0.13 ± 0.05	0.14 ± 0.06	0.21 ± 0.02

Freshly passaged Hep G2 cells were incubated with LOV 0.4 to 10 μmol/L on days 4 to 8, with 18:3ω3 or 18:2ω6 (40 μmol/L) days 4 to 6 and in serum-free DMEM on days 7 and 8. Fatty acid composition of lipids secreted into the medium were quantitated as described under "Methods and materials." Values are means ± 1 SEM from three to five experiments.
*Value is different from respective DMSO control using ANOVA with Sheffe's F-test for differences between means.
n.d., not detectable or less than 0.1% of total fatty acids.

Effect of LOV on cellular and lipoprotein fatty acids of Hep G2 cells supplemented with different 18:3ω3/18:2ω6 ratios

In an attempt to simulate conditions likely to reflect an in vivo situation, the LOV-induced changes in ω6 and ω3 metabolism in Hep G2 cells were next examined under different 18:3ω3/18:2ω6 supplementation ratios. As depicted in *Figure 2,* cellular phospholipid 20:4ω6 levels decreased, whereas 22:6ω3 levels increased with increasing 18:3ω3/18:2ω6 ratios. The simultaneous treatment with LOV resulted in an increase in both 20:4ω6 and 22:6ω3 levels. Significant statistical interactions between 18:3ω3/18:2ω6 supplementation ratios and LOV treatment furthermore indicated that the effect of LOV on fatty acid desaturation was modulated by the 18:3ω3/18:2ω6 precursor availability. The LOV-induced increase in 20:4ω6 and 22:6ω3 levels were most pronounced at the lowest and highest 18:3ω3/18:2ω6 supplementation ratios, respectively. In parallel experiments, a similar interaction between the 18:3ω3/18:2ω6

Table 3 Fatty acid composition of cholesterol esters secreted by Hep G2 cells

LOV (μmol/L)	no fatty acid				+18:3ω3				+18:2ω6			
	0	0.4	4	10	0	0.4	4	10	0	0.4	4	10
16:0	25.98 ± 1.29	27.01 ± 0.47	28.08 ± 1.27	25.08 ± 0.91	29.96 ± 0.62	29.87 ± 2.12	27.26 ± 1.32	27.01 ± 1.74	29.65 ± 0.61	28.95 ± 0.55	26.78 ± 1.06	28.64 ± 1.49
18:0	5.82 ± 1.60	9.35 ± 1.3	6.60 ± 1.55	8.39 ± 3.05	4.80 ± 0.80	7.92 ± 2.43	11.76* ± 0.25	8.22 ± 2.07	4.87 ± 0.27	5.12 ± 0.36	8.36* ± 0.76	9.92* ± 0.62
18:1	56.89 ± 2.95	53.75 ± 1.35	57.97 ± 2.67	58.62 ± 4.65	55.31 ± 3.18	49.53 ± 4.79	44.99 ± 3.32	48.33 ± 7.34	58.54 ± 1.59	56.02 ± 1.56	54.25 ± 1.57	52.56 ± 2.84
18:2ω6	7.83 ± 1.50	4.29 ± 1.15	3.13 ± 0.82	2.83 ± 1.00	2.36 ± 0.36	5.44 ± 1.59	4.07 ± 0.77	2.86 ± 0.81	4.27 ± 0.52	5.89 ± 1.05	4.36 ± 1.33	4.65 ± 1.48
20:4ω6	1.07 ± 0.24	1.27 ± 0.41	1.62 ± 0.40	1.61 ± 0.20	1.16 ± 0.38	1.28 ± 0.73	1.61 ± 0.52	1.55 ± 0.42	1.40 ± 0.23	2.12 ± 0.33	3.90* ± 1.42	3.17* ± 0.64
18:3ω3	n.d.	n.d.	n.d.	n.d.	2.37 ± 0.13	2.11 ± 0.04	3.13 ± 1.02	1.92 ± 0.46	n.d.	n.d.	n.d.	n.d.
20:5ω3	n.d.	n.d.	n.d.	n.d.	0.67 ± 0.11	1.53 ± 0.93	1.78 ± 0.67	1.82* ± 0.56	n.d.	n.d.	n.d.	n.d.
22:6ω3	0.53 ± 0.28	0.57 ± 0.22	0.44 ± 0.22	0.88 ± 0.36	1.10 ± 0.29	0.83 ± 0.23	2.42 ± 1.41	1.00 ± 0.25	0.15 ± 0.10	0.31 ± 0.14	n.d.	n.d.

Freshly passaged Hep G2 cells were incubated with LOV 0.4 to 10 μmol/L on days 4 to 8, with 18:3ω3 or 18:2ω6 (40 μmol/L) on days 4 to 6 and in serum-free DMEM on days 7 and 8. Fatty acid composition of lipids secreted into the medium were quantitated as described under "Methods and materials." Values are means ± 1 SEM from 3 to 5 experiments.
*Value is different from respective DMSO control using ANOVA with Sheffe's F-test for differences between means.
n.d., not detectable or less than 0.1% of total fatty acids.

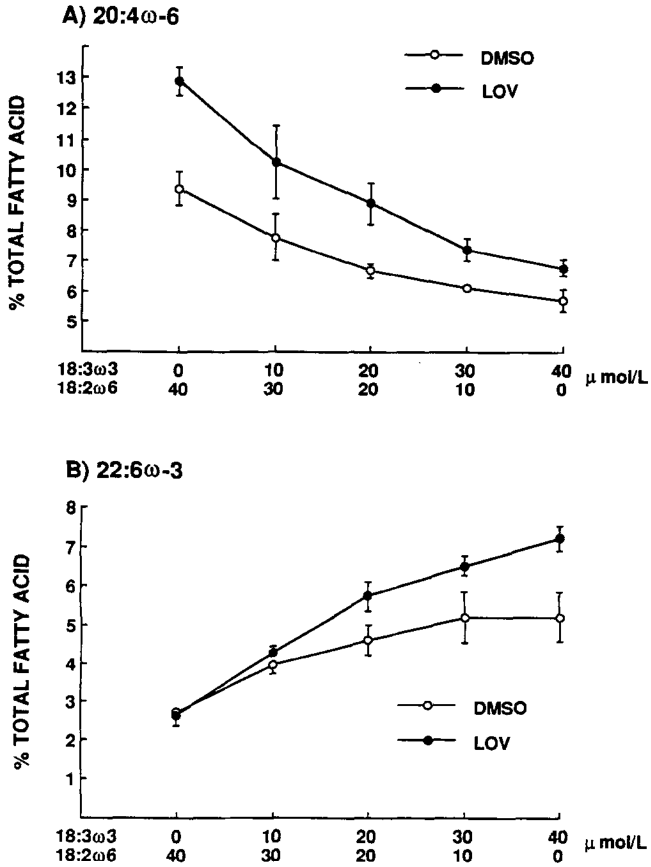

Figure 2 Effect of the 18:3ω3/18:2ω6 supplementation ratio on the LOV-induced changes in 20:4ω6 (A) and 22:6ω3 (B) levels in cellular phospholipids. Cells in normal growth medium was supplemented with different ratios of BSA-bound 18:3ω3/18:2ω6 and treated with LOV (4μmol/L) or its carrier (DMSO) on days 4 to 8 after passage. Total lipids were extracted and cellular phospholipid fatty acids were quantified as described under "Methods and materials." Symbols represent means ± SEM of four experiments. Data were analysed by means of two-factor ANOVA: (A) LOV effect $F(1,30)$ = 43.10, P = .0001; FA effect $F(4,30)$ = 62.03; P = .0001; LOVxFA interaction $F(4,30)$ = 2.41, P = 0.07). (B) LOV effect $F(1,30)$ = 30.63, P = .0001; FA effect $F(4,30)$ = 55.87, P = .0001; LOVxFA interaction $F(4,30)$ = 4.48, P = 0.006.

supplementation ratio and LOV were seen in the fatty acid composition of lipids secreted by the Hep G2 cells (data not shown).

Cell growth and viability

As reported previously,[1] LOV concentration up to 10 μM did not significantly influence cell proliferation, as judged by of cell protein measurement. Cell viability, assessed by means of ethidium bromide/acridin orange fluorescence, was >90% under all conditions.

Discussion

Within the last 5 years, several clinical studies have suggested that HMG-CoA reductase inhibitor therapy may alter essential fatty acids metabolism, resulting in decreased 18:2ω6 and increased 20:4ω6 levels in plasma lipoproteins[2–4] and erythrocytes.[5] We have recently shown in vitro, that this effect is most likely related to an increased conversion of 18:2ω6 to 20:4ω6 via the fatty acid desaturases.[1] Whether the drug-induced increase in cellular and systemic 20:4ω6 levels could alter cellular function in hypercholesterolemic patients, remains to be addressed in clinical studies. However, our present in vitro findings in the human hepatoma Hep G2 cell line suggest that the LOV-induced fatty acid changes in fatty acid metabolism are modulated by the 18:3ω3/18:2ω6 dietary precursor ratio.

The hepatoma Hep G2 cell line has been used extensively in studies of human liver lipid and lipoprotein metabolism.[15] As previously described for 18:2ω6,[1] 18:3ω3 either albumin-bound or incorporated into LDL (data not shown), is readily metabolized to its longer chain derivatives 20:5ω3, 22:5ω3, 22:6ω3, which are then esterified into cellular and lipoprotein lipids. Furthermore, LOV increased 22:6ω3 levels in cellular phospholipids of Hep G2 cells supplemented with albumin-bound 18:3ω3. Our data thus suggest that analogous to its effect on the metabolism of ω6 fatty acids,[1] LOV increases the desaturation and elongation of 18:3ω3. The specific desaturation enzyme stimulated by LOV could not be elucidated from the present data. However, the simultaneous reduction in 18:3ω3 and increase in 22:6ω3 levels in LOV-treated cells suggest an induction of Δ^6 desaturase, the enzyme involved in both the initial desaturation of 18:3ω3 and the synthesis of 22:6ω3.[16] Our data also cannot exclude the possibility that LOV-related effects on fatty acid esterification into cellular and lipoprotein lipids contributed to the changes in fatty acid composition observed. The supplementation of cells with albumin-bound 18:3ω3 decreased 20:4ω6 levels in the phospholipids of control cells and reduced the LOV-induced increase in 20:4ω6 in LOV-treated cells, presumably by competing with 18:2ω6 in cellular desaturation and acylation reactions.[11,17] The effect of LOV on cellular ω6 and ω3 fatty acid composition was thus modulated by the 18:3ω3/18:2ω6 precursor ratio.

The changes in cellular phospholipid fatty acids resulting from LOV and fatty acid supplementation were reflected in the fatty acid pattern of lipids secreted by the Hep G2 cells. Supplementation of control cells with 18:3ω3 increased 20:5ω3 and 22:6ω3 levels in all three lipid fractions secreted. Cotreatment with LOV further increased 20:5ω3 and 22:6ω3 levels in all three lipid fractions, in a dose-dependent manner. As reported previously,[1] LOV increased 20:4ω6 levels of lipids secreted by the Hep G2 cells. This increase was the highest in cells supplemented with exogenous 18:2ω6, but was also significant in unsupplemented cells at LOV concentrations as low as 0.4 μmol/mL, a dose equivalent to plasma concentrations of patients undergoing LOV therapy.[18] This rise in 20:4ω6 levels was blocked with 18:3ω3 at the lower LOV concentrations, and was seen only with the highest LOV dose of 10 μM. This suppressive effect of 18:3ω3 increased with increasing 18:3ω3/18:2ω6 ratios.

In contrast to Hep G2 cells supplemented with exogenous 18:3ω3, the cellular levels of ω3 fatty acids were not significantly altered by LOV in fatty acid unsupplemented cells. This most likely reflects the low levels of endogenous 18:3ω3 in the cellular lipids of unsupplemented Hep G2 cells. It has been previously proposed that cultured cells in general are deplete of essential fatty acids due to their continuous growth in essential fatty acid poor medium.[19] On the other hand, the small but statistically significant increase in both 20:4ω6 and 22:6ω3 in the phospholipids secreted by the LOV-treated, fatty acid unsupplemented cells suggest that the newly synthesized PUFAs were preferentially utilized in the assembly of lipoproteins.

Because greater increases in plasma levels of 20:5ω3 and 22:6ω3 can be achieved through the consumption of large amounts of fatty acid or through fish oil supplements, it has been argued that 18:3ω3 is an inefficient source of the long-chain ω3 fatty acids in humans.[20] Only modest increases in 20:5ω3 but not 22:6ω3 have been documented in most human studies of 18:3ω3 supplementation.[21–28] Nevertheless, it has recently been estimated that the conversion of 18:3ω3 in the U.S. diet could contribute substantially to the requirements for long-chain ω3 fatty acids of healthy adults.[29] The extent of in vivo conversion of 18:3ω3 into 20:5ω3,[29] the incorporation of 20:5ω3 into cellular membranes,[30] as well as the 18:3ω3-mediated suppression of 20:4ω6[18] levels depends on the fatty acid composition of the background diet. Thus, diets that partially replace 18:2ω6 with 18:3ω3 result in larger increases in plasma and cellular 20:5ω3 levels,[21] than those where 18:3ω3 is supplemented to diets also high in 18:2ω6.

Recently, renewed interest in diets high in 18:3ω3 has been stimulated by the apparent antiatherogenic properties of the "Mediterranean" diet, which in addition to being enriched in antioxidative vitamins and oleic acid, contains appreciable amounts of 18:3ω3.[10] Our present results in the Hep G2 cells indicate that the effect of HMG-CoA reductase inhibitors on the hepatic metabolism of ω6 and ω3 fatty acids can be modulated by the 18:3ω3/18:2ω6 precursor ratio. Thus, a substitution of the commonly used vegetable oils that are rich in 18:2ω6 but contain little 18:3ω3, with those with higher 18:3ω3/18:2ω6 ratios (soya, linseed, rapeseed, walnut oils) may be of benefit to hypercholesterolemic patients on HMG-CoA reductase therapy. Such a strategy, in addition to lowering blood lipid levels, could reduce the drug-related increase in 20:4ω6 levels and provide a more favourable ω6/ω3 fatty acid ratio.

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References

1 Hrboticky, N., Tang, L., Zimmer, B., Lux, I., and Weber, P.C. (1994). Lovastatin increases arachidonic acid levels and stimulates thromboxane synthesis in human liver and monocytic cell lines. *J. Clin. Invest.* **93,** 195–203

2 Agheli, N. and Jacotot, B. (1991). Effect of simvastatin and fenofibrate on the fatty acid composition of hypercholesterolaemic patients. *Br. J. Clin. Pharmacol.* **32,** 423–428

3 Hong, C.Y., Lin, S.J., Chang, B.N., Shen, P.M., and Shiao, M.S. (1993). Effect of Pravastatin on fatty acid profile of low density lipoprotein in patients with hypercholesterolemia. *Prost. Leukotr. Essential Fatty Acids* **48,** 155–158

4 Kleinveld, H.A., Demacker, P.N.M., de Haan, A.F.J., and Stalenhoef, A.F.H. (1993). Decreased in vitro oxidizability of low-density lipoprotein in hypercholesterolaemic patients treated with 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors. *Eur. J. Clin. Invest.* **23,** 289–295

5 Doormaal, van J.J., Bos, W.J.W., Muskiet, F.A.J., and Doorenbos, H. (1989). Simvastatin influences linoleic acid metabolism. *Pharmaceut. Weekblat* **11,** 134–135

6 Habenicht, A.J.R., Salbach, P., Goerig, M., Zeh, W. Janssen-Timmen, U., Blattner, C., King, W.C., and Glomset, J.A. (1990). The LDL receptor pathway delivers arachidonic acid for eicosanoid formation in cells stimulated by platelet-derived growth factor. *Nature* **345,** 634–636

7 Clark, S.D. and Jump, D.B. (1994). Dietary polyunsaturated fatty acid regulation of gene transcription. *Annu. Rev. Nutr.* **14,** 83–98

8 Leaf, A. and Weber, P.C. (1988). Cardiovascular effects of n-3 fatty acids. *N. Engl. J. Med.* **318,** 549–557

9 Dolecek, T.A. (1992). Epidemiological evidence of relationships between dietary polyunsaturated fatty acids and mortality in the multiple risk factor intervention trial. *Proc. Soc. Exp. Biol. Med.* **200,** 177–182

10 de Lorgeril, M., Renaud, S., Mamelle, N., Salen, P., Martin, J.L., Monjaud, I., Guidollet, J., Touboul, P., and Delaye, J. (1994). Mediterranean alpha-linolenic acid-rich diet in secondary prevention of coronary heart disease. *Lancet* **343,** 1454–1459

11 Brenner, R.R. (1974). The oxidative desaturation of unsaturated fatty acids in animals. *Mol. Cell. Biochem.* **3,** 41–52

12 Kaluzny, M.A., Duncan, L.A., Merritt, M.V., and Epps, D.E. (1985). Rapid separation of lipid classes in high yield and purity using bonded phase columns. *J. Lipid Res.* **26,** 135–144

13 Thrift, R.N., Forte, T.M., Cahoon, B.E., and Shore, V.G. (1986). Characterization of lipoproteins produced by the human liver cell line, Hep G2, under defined conditions. *J. Lipid Res.* **27,** 236–250

14 Forte, T.M., McCall, M.R., Knowles, B.B., and Shore, V.G. (1989). Isolation and characterization of lipoproteins produced by human hepatoma-derived cell lines other than Hep G2. *J. Lipid Res.* **30,** 817–829

15 Javitt, N.B. (1990). Hep G2 cells as a resource for metabolic studies: lipoprotein, cholesterol and bile acids. *F.A.S.E.B.* **4,** 161–168

16 Sprecher, H., Luthria, D.L., Mohammed, B.S., and Baykousheva, S.P. (1995). Reevaluation of the pathways for the biosynthesis of polyunsaturated fatty acids. *J. Lipid Res.* **36,** 2471–2477

17 Garg, M.L., Thomson, A.B.R., and Clandinin, M.T. (1990). Interactions of saturated, n-6 and n-3 polyunsaturated fatty acids to modulate arachidonic acid metabolism. *J. Lipid Res.* **31,** 271–277

18 Pan, H.Y., DeVault, A.R., Wang-Iverson, D., Ivashkiv, E., Swanson, B.N., and Sugerman, A.A. (1990). Comparative pharmacokinetics and pharmacodynamics of pravastatin and lovastatin. *J. Clin. Pharmacol.* **30,** 1128–1135

19 Gallela, G., Marangoni, F., Risé, P., Colombo, C., Galli, G., and Galli, C. (1993). n-6 and n-3 fatty acid accumulation in THP-1 cell phospholipids. *Biochim. Biophys. Acta* **1169,** 280–290

20 Dyerberg, J., Bang, H.O., and Aagaard, O. (1980). α-Linolenic acid and eicosapentaenoic acid. *Lancet* **i,** 199

21 Mantzioris, E., James, M.J., Gibson, R.A., and Cleland, L.G. (1994). Dietary substitution with an α-linolenic acid-rich vegetable oil increases eicosapentaenoic acid concentrations in tissues. *Am. J. Clin. Nutr.* **59,** 1304–1309

22 Weaver, B.J., Corner, E.J., Bruce, V.M., McDonald, B.E., and Holub, B.J. (1990). Dietary canola oil: effect on the accumulation of eicosapentaenoic acid in the alkenylacyl fraction of human platelet ethanolamine phosphoglyceride. *Am. J. Clin. Nutr.* **51,** 594–598

23 Seppänen-Laasko, T., Vanhanen, H., Laasko, I., Kohtamäki, H., and Viikari, J. (1992). Replacement of butter on bread by rapeseed oil and rapeseed oil—containing margarine: effects on plasma fatty acid composition and serum cholesterol. *Br. J. Nutr.* **68,** 639–654

24 Sanders, T.A.B. and Younger, K.M. (1981). The effect of dietary

supplements of ω3 polyunsaturated fatty acids on the fatty acid composition of platelets and plasma choline phosphoglycerides. *Br. J. Nutr.* **45,** 613–616
25 Renaud, S. and Nordøy, A. (1983). "Small is beautiful": α-linolenic acid and eicosapentaenoic acid in man. *Lancet* **i,** 1169
26 Kestin, M., Clifton, P., Belling, D.B., and Nestel, P.J. (1990). n-3 Fatty acids of marine origin lower systolic blood pressure and triglycerides but raise LDL cholesterol compared with n-3 and n-6 fatty acids from plants. *Am. J. Clin. Nutr.* **51,** 1028–1034
27 Mest, H.J., Beitz, J., Heinroth, I., Block, H.U., and Förster, W. (1983). The influence of linseed oil diet on fatty acid patterns in phospholipids and thromboxane formation in platelets in man. *Klin. Wochenschr.* **61,** 187–191
28 Ferrier, L.K., Caston, L.J., Leeson, S., Squires, J., Weaver, B.J., and Holub, B.J. (1995). α-Linolenic acid- and docosahexaenoic acid-enriched eggs from hens fed flaxseed: influence on blood lipids and platelet phospholipid fatty acids in humans. *Am. J. Clin. Nutr.* **62,** 81–86
29 Emken, E.A., Adlof, R.O., and Gulley, R.M. (1994). Dietary linolenic acid influences desaturation and acylation of deuterium-labeled linoleic and linolenic acids in young adult males. *Biochim. Biophys. Acta* **1213,** 277–288
30 Cleland, L.G., James, M.J., Neumann, M.A., D'Angelo, M., and Gibson, R.A. (1992). Linoleate inhibits EPA incorporation from dietary fish-oil supplements in human subjects. *Am. J. Clin. Nutr.* **55,** 395–399