Dietary α-linolenic acid reduces COX-2 expression and induces apoptosis of hepatoma cells

A. Vecchini,* V. Ceccarelli,* F. Susta,* P. Caligiana,* P. Orvietani,* L. Binaglia,[1,*] G. Nocentini,[†] C. Riccardi,[†] G. Calviello,[§] P. Palozza,[§] N. Maggiano,[§] and P. Di Nardo**

Department of Internal Medicine, Section of Biochemistry,* and Department of Clinical and Experimental Medicine, Section of Pharmacology,[†] University of Perugia, Perugia, Italy; Institute of General Pathology,[§] Catholic University, Rome, Italy; and Department of Internal Medicine, Laboratory of Cellular and Molecular Cardiology,[**] University of Roma "Tor Vergata," Rome, Italy

Abstract **Fatty acid synthetase (FAS) is overexpressed in various tumor tissues, and its inhibition and/or malonyl-CoA accumulation have been correlated to apoptosis of tumor cells. It is widely recognized that both ω-3 and ω-6 polyunsaturated fatty acids (PUFAs) depress FAS expression in liver, although epidemiological and experimental reports attribute antitumor properties only to ω-3 PUFA. Therefore, we investigated whether lipogenic gene expression in tumor cells is differently regulated by ω-6 and ω-3 PUFAs. Morris hepatoma 3924A cells were implanted subcutaneously in the hind legs of ACI/T rats preconditioned with high-lipid diets enriched with linoleic acid or α-linolenic acid. Both-high lipid diets depressed the expression of FAS and acetyl-CoA carboxylase in tumor tissue, this effect correlating with a decrease in the mRNA level of their common sterol regulatory element binding protein-1 transcription factor. Hepatoma cells grown in rats on either diet did not accumulate malonyl-CoA. Apoptosis of hepatoma cells was induced by the α-linolenic acid-enriched diet but not by the linoleic acid-enriched diet. Therefore, in this experimental model, apoptosis is apparently independent of the inhibition of fatty acid synthesis and of malonyl-CoA cytotoxicity. Conversely, it was observed that apoptosis induced by the α-linolenic acid-enriched diet correlated with a decrease in arachidonate content in hepatoma cells and decreased cyclooxygenase-2 expression.**—Vecchini, A., V. Ceccarelli, F. Susta, P. Caligiana, P. Orvietani, L. Binaglia, G. Nocentini, C. Riccardi, G. Calviello, P. Palozza, N. Maggiano, and P. Di Nardo. **Dietary α-linolenic acid reduces COX-2 expression and induces apoptosis of hepatoma cells.** ***J. Lipid Res.*** **2004.** 45: **308–316.**

Supplementary key words cyclooxygenase-2 • polyunsaturated fatty acids • sterol regulatory element binding protein 1

High expression of fatty acid synthetase (FAS) and upregulation of fatty acid synthesis are typical features of tumor tissues (1–5). Population studies of human cancer suggest that high FAS expression and tumor virulence are closely related (6).

Although the mechanisms underlying FAS overexpression in cancer are largely unknown, recent evidence (7–10) strongly suggests that, as in liver, FAS gene transcription in tumor cells is modulated by the sterol regulatory element binding protein-1 (SREBP-1).

SREBP-1 is synthesized as an integral protein of the endoplasmic reticulum membranes. As its C-terminal regulatory domain interacts with SREBP cleavage-activating protein (SCAP), the SREBP/SCAP complex migrates to the Golgi membranes, where a two-step cleavage catalyzed by a serine protease [site-1 protease (S1P)] and a metalloprotease [site-2 protease (S2P)] is responsible for the release of the N-terminal sequence of SREBP-1 in the cytoplasm. At the nuclear level, mature SREBP-1, a transcription factor of the basic helix-loop-helix leucine zipper family, activates genes encoding FAS and other lipogenic enzymes by interacting with sterol response elements present in their promoter region (11). Insulin and dietary carbohydrates activate FAS gene transcription by this mechanism, whereas dietary polyunsaturated fatty acids (PUFAs) of both ω-6 and ω-3 series negatively modulate FAS expression by depressing SREBP-1 mRNA level and reducing the cleavage of the native protein (12, 13).

Recently, it was found that pharmacological inhibitors of FAS are selectively cytotoxic to tumor cells in culture and in vivo (14–17). In particular, the FAS inhibitors cerulenin (2*S*,3*R*-epoxy-4-oxo-7*E*,10*E*-dodecadienamide) and C75 (3-carboxy-4-octyl-2-methylenebutyrolactone) induce

Manuscript received 16 September 2003 and in revised form 14 October 2003.

Published, JLR Papers in Press, October 16, 2003.
DOI 10.1194/jlr.M300396-JLR200

Abbreviations: ACC, acetyl-CoA carboxylase; C/EBP, CCAAT enhancer binding protein; COX-2, cyclooxygenase-2; CPT-1, carnitine palmitoyltransferase-1; FAS, fatty acid synthetase; PPARα, peroxisome proliferator-activated receptor-α; SCAP, sterol regulatory element binding protein-1 cleavage activating protein; S1P, site-1 protease; S2P, site-2 protease; SREBP-1, sterol regulatory element binding protein-1.

[1] To whom correspondence should be addressed.
e-mail: binaglia@unipg.it

a rapid decline of fatty acid synthesis in tumor cells, with subsequent reduction of DNA synthesis, cell cycle arrest, and apoptosis (16, 17).

The hypothesized link between the depression of de novo fatty acid synthesis and cytotoxicity to tumor cells is reinforced by the observation that ω-3 PUFAs possess anticancer properties. Indeed, ω-3 PUFAs, whose role as physiological modulators of FAS gene transcription is widely recognized, have been demonstrated to induce necrosis and apoptosis of tumor cells and to affect tumor growth and metastatic invasion (18–24). Nevertheless, it has to be pointed out that although PUFAs of both the ω-3 and ω-6 families act as negative modulators of FAS gene expression (12, 13), only some of them retard tumor cell growth and induce cell death (18–25).

In light of this evidence, a cause-and-effect relationship between the depression of fatty acid synthesis and tumor cell cytotoxicity appears unlikely. This critical observation is reinforced by recent data demonstrating that a depression of fatty acid synthesis per se is not responsible for apoptosis of tumor cells. Indeed, 5-(tetradecyloxy)-2-furoic acid, an inhibitor of acetyl-CoA carboxylase (ACC), depresses fatty acid synthesis but is not cytotoxic to human breast cancer cells (26). Because both FAS and ACC inhibitors depress fatty acid synthesis but only FAS inhibitors induce malonyl-CoA accumulation in the cytoplasm, it has been inferred that malonyl-CoA mediates cytotoxicity induced by FAS inhibitors (26).

This last hypothesis has been taken as the starting point for the present investigation, which aims to examine the possibility that dietary ω-3 PUFAs, but not ω-6 PUFAs, selectively affect apoptosis of hepatoma cells by increasing their cytoplasmic malonyl-CoA level.

In addition to the malonyl-CoA hypothesis, we also considered the possibility that the cytotoxicity of ω-3 PUFAs to hepatoma cells might depend on the modulation of the mechanisms involved in eicosanoid production (27).

MATERIALS AND METHODS

Chemicals

[α-^{32}P]UTP (specific activity, 800 Ci/mmol) was obtained from Amersham Biosciences Europe (Milan, Italy). Rabbit polyclonal antibody against the N-terminal region of SREBP-1 (H-160: sc-8984) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Horseradish peroxidase-conjugated anti-rabbit immunoglobulin secondary antibody and all other chemicals were obtained from Sigma-Aldrich Co. (Milan, Italy).

Animal treatments

The animal-use protocol was approved by the Istituto Superiore della Sanità (Italian National Health Institute), Rome, Italy. Thirty-six male inbred ACI/T rats (~200 g body weight), randomly divided into three groups, were housed individually in cages and placed in light-cycling rooms with alternating 12 h periods of light and darkness. All animals had free access to food and water.

The first group was fed a standard pellet chow diet (Altromin-Rieper, Bolzano, Italy). The diet of the second and third groups was enriched with polyunsaturated lipids containing an excess of ω-6 and ω-3 PUFAs, respectively. The ω-6 PUFA-enriched diet was prepared by milling equal amounts of standard pellet and sunflower seeds (SS diet), whereas the ω-3 PUFA-enriched diet was prepared by milling the standard pellet with an equal amount of linseeds (LS diet). The resulting powders were pelleted again. The gross composition of the three diets is reported in **Table 1**. The fatty acid content of the three experimental diets was evaluated by gas chromatographic analysis of the fatty acid methyl esters obtained by transmethylation of the extracted lipids using methyleptadecanoate as an internal standard. Gas chromatographic analysis of fatty acid methyl esters was performed using a Carlo Erba Instruments (Milan, Italy) model HRGC 5300 gas chromatograph equipped with an SP-2330 capillary column (30 m × 0.25 mm; Supelco, Inc., Bellefonte, PA) and a flame ionization detector.

After 10 days of dietary conditioning, rats were subjected to subcutaneous transplantation of fast-growing Morris hepatoma 3924A cells, as previously described (18). In particular, hepatoma excised from anaesthetized donors was trimmed of fat, muscle, connective tissue, blood, and obvious necrotic tissues and then gently homogenized in 10 vol of 0.9% NaCl. One milliliter aliquots of the cell suspension were inoculated subcutaneously in both hind legs of ACI/T acceptor rats. Tumors became palpable at 11 to 12 days after transplantation. On day 19 after transplantation, rats were killed, tumors were excised, and specimens of each tumor were separately frozen at −80°C for future analyses. The percentage of apoptotic cells was determined using the TUNEL technique (18, 28).

Fatty acid analysis of tumor lipids

Aliquots of the frozen tumor tissue (about 0.5 g fresh weight) were homogenized in 10 vol of 0.25 M sucrose. Lipids were extracted according to Folch, Lees, and Sloane-Stanley (29), and fatty acid methyl esters obtained by acid-catalyzed transmethylation were analyzed by gas-liquid chromatography, as described above.

Fatty acid composition of phosphatidylcholine was analyzed according to the same procedure after chromatographic isolation of the lipid class on SiO_2 plates using chloroform-methanol-water (65:25:4, v/v/v) as a developing mixture.

TABLE 1. Composition of experimental diets

Nutrients	Diets		
	C	SS	LS
Carbohydrates (mg/g pellet)	521.8	343.1	286.8
Lipids	49.1	210.7	212.8
Proteins	204.7	211.2	217.2
Fiber	44.6	90.8	104.3
Energy (kJ/g)	15.82	19.16	18.42
Fatty acid distribution (%)			
16:0	15.8	7.8	8.3
18:0	2.5	3.8	5.0
18:1 ω-9	22.7	24.1	19.7
18:2 ω-6	51.5	63.4	21.1
18:3 ω-3	5.8	0.7	45.7
20:5 ω-3	0.5	0.1	0.1
22:6 ω-3	0.6	0.1	0.1

Rats were fed either standard pellet chow (C) or high-lipid diets obtained by milling equal weights of standard pellet with sunflower seeds (SS) or linseeds (LS). The powdered mixtures were pelleted again and analyzed for composition. Fatty acid distribution in the three diets was assessed by gas chromatographic analysis of fatty acid methyl esters.

RNase protection assays

Total RNA was extracted from frozen specimens of liver and hepatoma tissues (about 0.5 g fresh tissue) by the cesium chloride purification procedure (30).

For RNA probes, fragments of mRNA encoding the proteins listed in **Table 2** were amplified by reverse transcriptase-PCR from ACI/T rat liver RNA using the primers listed in the same table (31–45). The amplified products were subcloned into pCR 2.1 TOPO vector (Invitrogen) and verified by nucleotide sequencing. After plasmid linearization, antisense RNAs were transcribed with [α-^{32}P]UTP (800 Ci/mmol) using T7 RNA polymerase (Promega).

As preliminary tests indicated that β-actin mRNA level in hepatoma cells is significantly affected by dietary manipulations, 18S mRNA was used as an internal control for RNase protection assays (46).

RNA samples were subjected to RNase protection assay using the RPA III™ kit (Ambion, Inc., Austin, TX), 10 μg of RNA from ACI/T rat liver or hepatoma, 100,000 cpm of the specific antisense probe, and 2,000–20,000 cpm of antisense pTRI RNA 18S (80 bp protected fragment; Ambion) with a 5- to 50-fold lower specific activity, to give an 18S signal comparable to that of the test mRNAs. After digestion with RNase A/T1, the protected fragments were separated on 8 M urea/6.0% polyacrylamide gels. The gels were dried and subjected to quantitative analysis using an Instant Imager autoradiography system (Packard BioScience Co., IL).

Immunoelectrophoretic analysis of native and mature SREBP-1

Frozen specimens from ACI/T rat liver and tumor tissue were homogenized in 7% SDS. Aliquots of the homogenates containing 30 μg of protein were added with 0.5 vol of 3× PAGE sample buffer. The resulting suspensions were kept for 1 min at 100°C and centrifuged for 10 min at 3,000 *g*. The supernatants were loaded in the wells of 10% polyacrylamide gels (90 × 90 × 1.5 mm). After the electrophoretic run (30 mA/gel), proteins were blotted on nitrocellulose membranes and stained with Ponceau. After destaining with water, blots were rinsed with PBS and submitted to the reaction with antibody against SREBP-1. The bound antibody was revealed by peroxidase-conjugated, affinity-purified, anti-rabbit IgG antibody using the SuperSignal CL-HRP substrate (Pierce, Rockford, IL) according to the manufacturer's instructions. SDS-PAGE gels were calibrated with molecular weight markers (Bio-Rad, Hercules, CA). Blots were exposed to Kodak X-Omat film at room temperature.

The effect of dietary fat on the nuclear content of mature SREBP-1 was determined by Western blot analysis of nuclear proteins, as described previously (47).

HPLC quantification of malonyl-CoA

Frozen liver and tumor specimens (~0.5 g fresh weight) from rats fed different diets were homogenized in ice-cold 5% sulfosalicylic acid in 50 mM dithioerythritol to obtain 10% (w/v) homo-

TABLE 2. Sequences of PCR primers used for cloning cDNA fragments

Copy RNA Probe	Primer Pair	Primer Sequences	National Center for Biotechnology Information Accession Number	PCR Product	Ref.
				bp	
SREBP-1	5′	5′-AACCAAGACAGTGACTTCC-3′	L16995	220	31
	3′	5′-ACGGGTACATCTTTACAGC-3′			
SREBP-1a	5′	5′-ATGGACGAGCTGCCTTCGGTGAGGCGGCT-3′	NM_011480 (mouse)	247	32
	3′	5′-CCAGAGAGGAGCCCAGAGAAGCAG-3′			
SREBP-1c	5′	5′-GCGCGGACGACGGAGCCAT-3′	L16995	172	31
	3′	5′-GCAGGAGAAGAGAAGCTCTC-3′			
Fatty acid synthase	5′	5′-TTGCCCGAGTCAGAGAACC-3′	M76767	241	33
	3′	5′-CGTCCACAATAGCTTCATAGC-3′			
Acetyl-CoA carboxylase	5′	5′-GTCATGCCTCCGAGAACC-3′	J03808	180	34
	3′	5′-GCCAATCCACTCGAAGACC-3′			
ATP citrate lyase	5′	5′-GATCAAACGTCGAGGAAAGC-3′	J05210	230	35
	3′	5′-CCCTTCATGGTGGAACAGG-3′			
Peroxisome proliferator activated receptor-α	5′	5′-ACTGGATGACAGTGACATTTCC-3′	NM_013196	238	36
	3′	5′-CTTGATGACCTGCACGAGC-3′			
Carnitine palmitoyltransferase-1	5′	5′-CAGAACACGGCAAAATGAGC-3′	L07736	227	37
	3′	5′-GAGGTTGACAGCAAATCCTG-3′			
Acyl-CoA oxidase	5′	5′-CCACATATGACCCCAAGACC-3′	J02752	219	38
	3′	5′-GATATCCCCGACAGTGATGC-3′			
SREBP-1 cleavage-activating protein	5′	5′-CTGAGAGACAGCTCAGGGACC-3′	U67060 (*Cricetus*)	302	39
	3′	5′-GATGGTGTACGAGACCGTCCT-3′			
Site-1 protease	5′	5′-TGCTCACACTTGAAGATCACC-3′	AF094821	297	40
	3′	5′-GACATTAGCACCTGTGTATCC-3′			
Site-2 protease	5′	5′-TGCAACTTATATCACCAGTCCA-3′	AF019611	200	41
	3′	5′-CACAAAAAGGCCTCTGGGTC-3′			
C/EBPα	5′	5′-GGCTGGAGCCCCTGTACGAGC-3′	NM_012524	209	42
	3′	5′-GCAGTGTGCGATCTGGAACTGC-3′			
C/EBPβ	5′	5′-CCTCTTCGCCGACGACTACG-3′	NM_024125	216	43
	3′	5′-CAGGGCGAACGGGAAGCCG-3′			
C/EBPδ	5′	5′-AGAGCGCCATCGACTTCAGC-3′	NM_013154	280	44
	3′	5′-CCAAGCTCACCACTGTCTGC-3′			
Cyclooxygenase-2	5′	5′-AAGATAGTGATCGAAGACTACG-3′	S67722	254	45
	3′	5′-GAATGACTCAACAAAGTGAGC-3′			

C/EBP, CCAAT enhancer binding protein; SREBP-1, sterol regulatory element binding protein-1.

genates. The cellular levels of malonyl-CoA were quantified in the 100,000 *g* supernatant by reversed-phase HPLC on Supelcosil LC-18 columns (Sigma-Aldrich), as previously described (48).

RESULTS

The daily food intake was not significantly different in the three groups of animals

Although the caloric intake of the rats on SS and LS diets exceeded by ~20 and 16%, respectively, that of controls, no significant differences in weight gain were found among the three groups during tumor growth.

Hepatoma cells contain high levels of mRNAs encoding lipogenic enzymes

It has been reported that FAS mRNA is overexpressed in many tumors (1–5). We found that FAS mRNA also is highly expressed in Morris 3924A hepatoma cells compared with liver cells (**Fig. 1**). Also, ACC and ATP citrate lyase (ATP-CL) mRNAs were more abundant in tumor tissue than in liver. The high mRNA levels for enzymes involved in fatty acid biosynthesis in hepatoma cells were paralleled by a high content of SREBP-1 mRNA. In particular, SREBP-1a mRNA, which was barely detectable in liver, was the predominant SREBP-1 isoform in hepatoma tissue, whereas SREBP-1c mRNA, less potent than SREBP-1a as a transcription factor, was expressed at much lower levels in tumor tissue than in liver.

Additionally, genes encoding proteins involved in native SREBP cleavage were overexpressed in hepatoma cells. In particular, SCAP mRNA, S1P mRNA, and S2P mRNA were ~3-, 4-, and 5-fold more abundant in tumor cells than in liver, respectively (Fig. 1).

In parallel with the overexpression of lipogenic genes, hepatoma cells exhibited a low expression of genes involved in fatty acid degradation. Indeed, peroxisome proliferator-activated receptor-α (PPARα) mRNA and acyl-CoA oxidase mRNA were approximately five times less abundant in tumor tissue than in liver, whereas carnitine palmitoyltransferase-1 (CPT-1) mRNA levels were comparable in both tissues (Fig. 1).

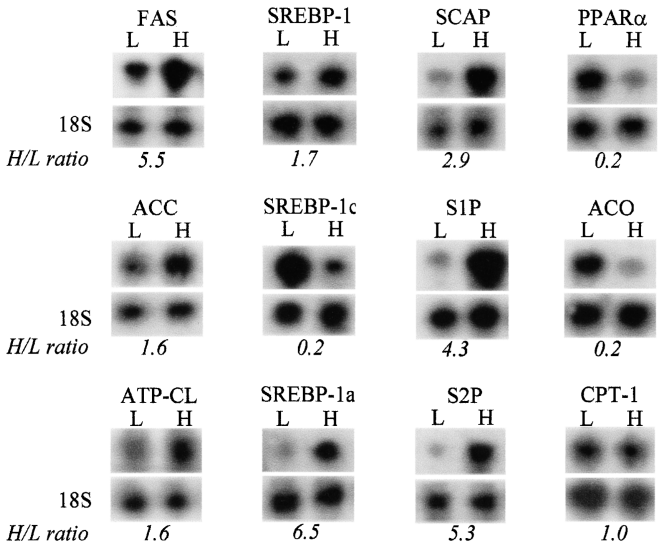

Fig. 1. Expression of genes involved in the synthesis and degradation of fatty acids. Total RNA was isolated from hepatoma tissue and liver of ACI/T rats. Ten microgram aliquots of total RNA were hybridized to ^{32}P-labeled copy RNA (cRNA) probes specific for each protein using 18S cRNA as an internal standard. After RNA digestion, protected fragments were separated by gel electrophoresis and the radioactivity associated with each band was quantified using a PhosphorImager autoradiographic system. For each probe, one representative from six autoradiographic images is shown. The ratio between the mRNA content in hepatoma cells (H) and liver (L) was calculated after correction for loading differences with 18S. The H/L ratio values are reported below each blot. ACC, acetyl-CoA carboxylase; ACO, acyl-CoA oxidase; ATP-CL, ATP citrate lyase; CPT-1, carnitine palmitoyltransferase-1; FAS, fatty acid synthetase; PPARα, peroxisome proliferator-activated receptor-α; SCAP, sterol regulatory element binding protein-1 cleavage-activating protein; S1P, site-1 protease; S2P, site-2 protease; SREBP-1, sterol regulatory element binding protein-1 (total); SREBP-1a, isoform a of SREBP-1; SREBP-1c, isoform c of SREBP-1.

Effect of dietary lipids on apoptosis

It has been reported previously that specific dietary lipids can influence tumor cell growth and apoptosis (18–25). The results obtained in the present study demonstrate that the percentage of apoptotic cells was significantly increased in tumors isolated from rats receiving the α-linolenic acid-enriched diet (LS diet) compared with rats on the control diet (**Fig. 2**). On the contrary, no significant changes in the percentage of apoptotic cells was induced when the diet was enriched with linoleic acid (SS diet).

Dietary PUFAs affect the expression of lipogenic enzymes in hepatoma cells

FAS, ACC, and ATP-CL mRNA content in hepatoma tissue was significantly lower in rats fed high-lipid diets than in rats on the unsupplemented diet (**Fig. 3**). The low expression of lipogenic enzymes in the hepatoma cells grown in rats on PUFA-enriched diets was paralleled by low SREBP-1a and SREBP-1c mRNA levels. On the contrary, dietary PUFA supplements did not induce signifi-

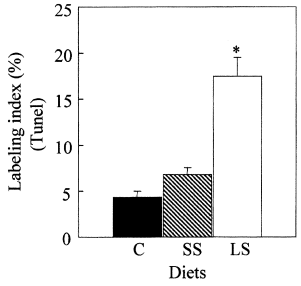

Fig. 2. Effect of high-lipid diets on Morris hepatoma 3924A cell apoptosis. Apoptosis of tumor cells was evaluated by the TUNEL method. Labeling index indicates the percentage of positively labeled cells. Values are means ± SE of six determinations. The asterisk indicates a value significantly different ($P < 0.01$) from those of control animals on the unsupplemented diet (one-way ANOVA followed by Fisher's test). C, control pellet diet; LS, linseed (high-α-linolenic acid diet); SS, sunflower seed (high-linoleic acid diet).

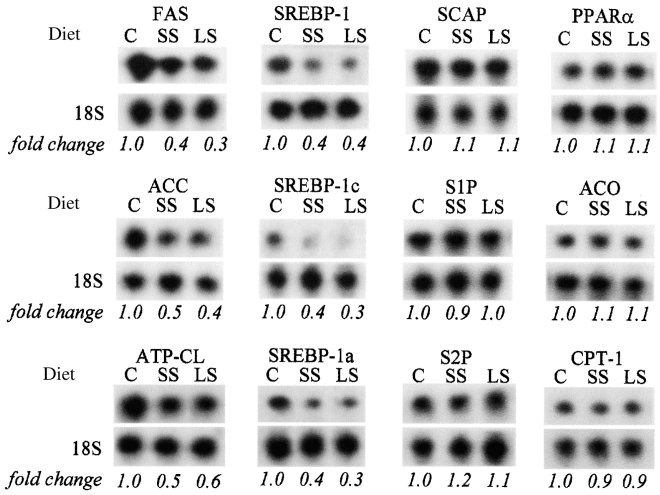

Fig. 3. Effect of high-lipid diets on the mRNA levels of genes involved in the synthesis and degradation of fatty acids. ACI/T rats on the C diet, the SS diet, or the LS diet were killed, and total RNA was isolated from the tumor tissue. For each sample, RNase protection assay was carried out on 10 μg aliquots of total RNA, as described in the legend to Fig. 1. For each probe, one representative from six autoradiographic images is shown. The fold change in mRNA level induced by high-lipid diets with respect to the control diet was calculated after correction for loading differences with 18S, and these values are reported below each blot.

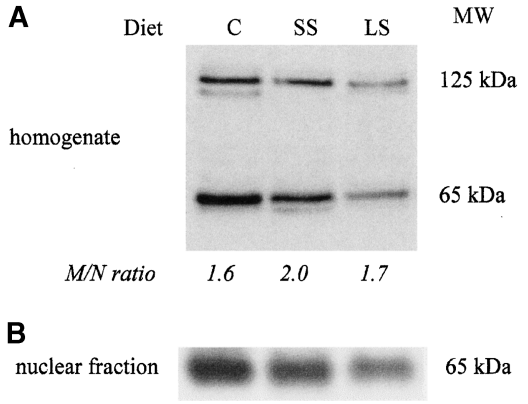

Fig. 4. Western blot analysis of native and mature SREBP-1 protein. A: Whole hepatoma proteins from rats on the C diet, the SS diet, or the LS diet were separated by PAGE (30 μg protein/lane) and blotted to a nitrocellulose membrane. Native (125 kDa) and mature (65 kDa) SREBP-1 protein forms were immunodetected using a polyclonal antibody against the N-terminal domain of the polypeptide. The relative content of the nuclear mature form and the native precursor protein (M/N ratio) was quantified by photodensitometric analysis of the autoradiographic images. One representative from three experiments is shown. B: Nuclear proteins from hepatoma cells grown in rats on different diets were separated electrophoretically and blotted to a nitrocellulose membrane. Only one immunoreactive band with an approximate molecular mass (MW) of 65 kDa was obtained.

cant changes of SCAP, S1P, S2P, PPARα, acyl-CoA oxidase, and CPT-1 mRNA content.

When whole hepatoma proteins from rats fed different diets were submitted to SDS-PAGE and immunoblotting, two electrophoretic bands with approximate molecular masses of 125 and 65 kDa immunoreacted with the antibody against the N-terminal domain of SREBP-1 (**Fig. 4A**). The 125-kDa band is the membrane-bound native form of SREBP-1, whereas the low-molecular-mass band, corresponding to the unique immunoreactive polypeptide revealed when proteins of isolated nuclei were submitted to electrophoresis and immunostaining, represents the mature form of SREBP-1 (Fig. 4B).

The nuclear-to-membrane-bound SREBP-1 signal ratio was not significantly different in hepatoma cells grown in rats fed unsupplemented or high-lipid diets (Fig. 4A), indicating that dietary PUFA did not affect SREBP-1 cleavage in tumor tissue.

Malonyl-CoA levels, assessed by HPLC analysis of the cytosolic fraction (48), were not significantly different in tumors isolated from rats on the control diet (2.2 ± 0.7 nmol/g wet tissue), on the linoleic acid-enriched diet (2.4 ± 0.4 nmol/g wet tissue), and on the α-linolenic acid-enriched diet (2.2 ± 0.6 nmol/g wet tissue).

The fatty acid composition of tumor lipids is changed by dietary conditioning

The fatty acid composition of whole lipids from hepatoma cells grown in rats on different diets is shown in **Table 3**. Both high-lipid diets induced an accumulation of saturated fatty acids in tumor lipids. Moreover, tumors from rats on the linoleic acid-enriched diet (SS diet) accumulated 18:2 ω-6, 20:4 ω-6, and 22:4 ω-6, whereas the LS diet, containing high amounts of α-linolenic acid, induced the accumulation of 18:3 ω-3, 20:5 ω-3, and 22:5 ω-3 and decreases of 20:4 ω-6 and 22:4 ω-6 in hepatoma

TABLE 3. Fatty acid composition of hepatocarcinoma lipids

Fatty Acids in Whole Lipids	Diet		
	C	SS	LS
14:0	2.4 ± 0.2	2.9 ± 0.7	2.6 ± 0.5
16:0	26.3 ± 4.6	33.1 ± 7.8[a]	33.9 ± 6.7[a]
16:1	6.6 ± 1.5	5.6 ± 1.1	7.1 ± 1.4
18:0	18.8 ± 0.9	28.4 ± 3.6[b]	26.3 ± 1.1[b]
18:1 (ω-9)	20.0 ± 0.9	24.7 ± 3.7[a]	26.2 ± 7.8[a]
18:1 (ω-7)	1.9 ± 0.5	1.5 ± 0.4	1.8 ± 0.3
18:2 (ω-6)	15.9 ± 7.1	26.7 ± 2.1[b]	21.8 ± 7.2[a]
18:3 (ω-6)	0.9 ± 0.2	1.4 ± 1.1	1.4 ± 0.2
18:3 (ω-3)	0.4 ± 0.3	0.4 ± 0.2	6.5 ± 2.2[b]
20:0	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1
20:1	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1
20:2	0.5 ± 0.2	0.8 ± 0.4	0.4 ± 0.2
20:3 (ω-6)	0.7 ± 0.2	1.0 ± 0.2[b]	0.6 ± 0.2
20:4 (ω-6)	10.6 ± 1.4	15.0 ± 2.0[b]	6.2 ± 0.5[b]
20:5 (ω-3)	0.3 ± 0.1	0.1 ± 0.1	4.1 ± 0.4[b]
22:1	0.4 ± 0.1	0.5 ± 0.2	0.5 ± 0.2
22:4 (ω-6)	1.2 ± 0.3	2.9 ± 0.4[b]	0.4 ± 0.1[b]
22:5 (ω-6)	0.2 ± 0.1	0.5 ± 0.2[a]	0.1 ± 0.1
22:5 (ω-3)	1.0 ± 0.3	0.6 ± 0.2	3.0 ± 0.2[b]
22:6 (ω-3)	2.2 ± 0.8	1.7 ± 0.5	1.2 ± 0.1[a]

Tumor lipids from rats on the C diet, the SS diet, and LS diet were extracted and transesterified in 3% methanolic sulfuric acid. Fatty acid methyl esters were analyzed by gas-liquid chromatography using methyl eptadecanoate as an internal standard. Data are expressed as micrograms of fatty acid per milligram of protein.

[a] $P < 0.05$ compared with tumors from rats on the C diet.

[b] $P < 0.01$ compared with tumors from rats on the C diet.

tissue. As a consequence, the 20:4 ω-6/20:5 ω-3 molar ratio, which was ~35 and 150 in whole hepatoma lipids from rats fed standard diet and the SS diet, respectively, decreased to 1.5 in rats on the LS diet. Dietary manipulations induced changes of the same molar ratio in phosphatidylcholine. Indeed, the percentage content of 20:4 ω-6 was 5.1 ± 1.1 or 3.7 ± 0.7 in phosphatidylcholine isolated from tumors grown in rats fed the standard or the SS diet, respectively, and was 1.7 ± 0.1 in rats on the LS diet. On the contrary, the percentage content of 20:5 ω-3, which accounted for less than 0.1% in tumors from rats on the control and SS diets, was 1.3 ± 0.1 in tumors from rats on the LS diet. Dietary manipulations did not affect Δ-6 desaturase and Δ-5 desaturase mRNA levels (data not shown).

Dietary α-linolenic acid reduces cyclooxygenase-2 expression in hepatoma cells

Cyclooxygenase-2 (COX-2) was overexpressed in hepatoma cells compared with control liver (**Fig. 5A**). Because COX-2 expression is modulated by members of the CCAAT enhancer binding protein (C/EBP) family of transcription factors (49, 50), C/EBPα, C/EBPβ, and C/EBPδ mRNA levels were determined in hepatoma tissues from rats on different diets.

C/EBPα mRNA was significantly less abundant in hepatoma cells than in liver, whereas the expression of C/EBPβ and C/EBPδ was approximately five and six times higher, respectively, in tumor cells than in the liver of animals on the control diet (Fig. 5A).

Dietary conditioning with high-lipid diets did not affect C/EBPα and C/EBPδ mRNA abundance in hepatoma cells. On the contrary, the levels of COX-2 mRNA and of C/EBPβ mRNA in hepatoma cells were significantly reduced (Fig. 5B) when the diet was enriched with α-linolenic acid (LS diet).

The linoleic acid-rich diet, although not significantly affecting the mRNA level of C/EBP isoforms, induced a high increase of COX-2 expression.

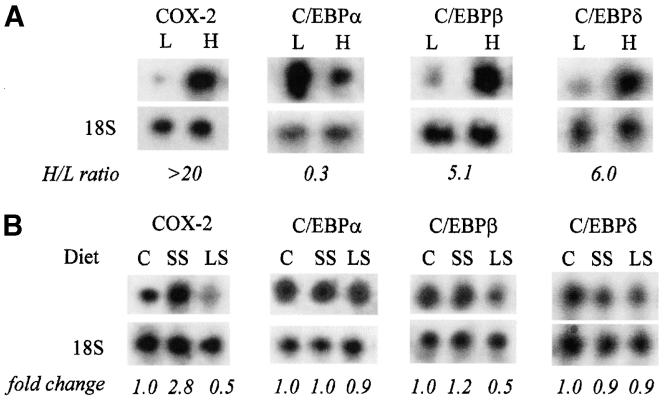

Fig. 5. Abundance of cyclooxygenase-2 (COX-2) mRNA and of CCAAT enhancer binding proteins (C/EBPs) in hepatoma cells grown in rats on different diets. Total RNA was isolated from liver and hepatoma tissues from rats on the C diet, the SS diet, or the LS diet. RNase protection assay was carried out as described in the legend to Fig. 1. For each probe, one representative from six autoradiographic images is shown. A: For each gene, the mRNA content was measured in hepatoma tissue (H) and liver (L) of ACI/T rats on the control diet. H/L ratio was calculated after correction for loading differences. B: The changes induced in tumor tissue by the SS and LS diets on mRNA levels were examined. The values reported below each blot represent the ratio between the mRNA content in tumors from rats on the SS or LS diet and that measured in rats on the C diet.

DISCUSSION

In the present study, evidence is given that FAS and other lipogenic enzymes are highly expressed in Morris hepatoma 3924A cells compared with normal liver. The high expression of lipogenic genes correlates with the high expression of SREBP-1a and of proteins (SCAP, S1P, and S2P) involved in SREBP maturation.

High FAS expression is not a unique feature of hepatoma cells, because most tumors exhibit high levels of both FAS mRNA and the encoded protein (1–5). The role played by FAS overexpression in tumorigenesis has been greatly emphasized because of the apparent link between FAS expression level and tumor malignancy (6). This role appears to be validated by the evidence that some FAS inhibitors, besides depressing fatty acid synthesis, possess anti-tumor activity (14–17).

Nevertheless, further studies made clear that inhibition of fatty acid synthesis per se could be not responsible for apoptosis and could not affect tumor cell proliferation. In fact, ACC inhibitors, although reducing fatty acid synthesis, do not exhibit antitumor properties (26). Because FAS inhibition causes an increase in the cytoplasmic malonyl-CoA concentration, it was postulated that malonyl-CoA accumulation, rather than the depression of fatty acid synthesis, is responsible for the cytotoxicity of FAS inhibitors. A possible outcome of this postulate is that any manipulations able to increase the malonyl-CoA level by acting on the FAS/ACC activity ratio might elicit the apoptotic response of tumor cells.

Based on these premises, the present work was aimed at investigating the mechanism underlying the cytotoxicity of ω-3 PUFAs to Morris 3924A hepatoma cells. The working hypothesis was that FAS and ACC expression in hepatoma cells is differently modulated by dietary ω-3 and ω-6 PUFAs and that ω-3 PUFAs, but not ω-6 PUFAs, induce malonyl-CoA accumulation in the cytoplasm of hepatoma cells.

With this aim, Morris hepatoma 3924A cells were grown subcutaneously in rats on control diet and in rats fed high-lipid PUFA-enriched diets. Two high-lipid diets were used throughout the study: the SS diet was mainly enriched with linoleic acid (18:2 ω-6), and α-linolenic acid (18:3 ω-3) was the most abundant fatty acid in the LS diet.

Twenty days after the inoculation of hepatoma cells in rats on different diets, the cytoplasmic malonyl-CoA concentration and FAS mRNA level in hepatoma cells were evaluated. Although FAS mRNA content was similarly depressed in tumors grown in rats on either high-lipid diet, the percentage of apoptotic cells was significantly in-

creased only in tumors grown in rats on the LS diet. This finding, although corroborating previous evidence that ω-3 PUFAs interfere with tumor cell dynamics, also implies the lack of a relationship between FAS expression and hepatoma cell apoptosis. In addition, it was found that cytoplasmic malonyl-CoA levels were not significantly different in tumors grown in rats on the different diets. This result was expected because the enzymes involved in malonyl-CoA production (ACC) and utilization (FAS) were similarly affected by both PUFA-enriched diets.

In conclusion, at least in the experimental model used throughout the present investigation, apoptosis of hepatoma cells might be independent of the PUFA-induced depression of fatty acid synthesis and of the cytoplasmic malonyl-CoA concentration.

The apparent link between enhanced apoptosis and dietary ω-3 fatty acids was further investigated by evaluating the changes induced by high-lipid diets on the fatty acid composition of hepatoma lipids and on the expression of COX-2 and of Δ-5 and Δ-6 fatty acid desaturase in tumor tissue.

This part of the experimental work was based on the following points: *a*) an aberrant expression of COX-2 has been implicated in the pathogenesis of cancer (51) and in the early stages of human liver carcinogenesis (27, 52–58), whereas its inhibition retards cell growth and increases the apoptosis rate and the apoptotic index of human liver tumor cells (59–62); *b*) a high dietary intake of α-linolenic acid could enrich tumor lipids with eicosapentaenoic acid (20:5 ω-3), which competes with arachidonic acid for COX-2; and *c*) dietary α-linolenic acid could compete with linoleic acid for Δ-5 and Δ-6 fatty acid desaturases in hepatoma cells, thus inhibiting the biosynthesis of arachidonic acid, the precursor of eicosanoids that affect cell proliferation, immune response, tumor cell invasion, and metastasis (27, 52–54).

The results obtained in the present investigation demonstrate that diets enriched with linoleic or α-linolenic acid induce significant changes of the fatty acid composition of whole hepatoma lipids. In particular, tumor lipids from SS-fed rats accumulated ω-6 tetraenoic fatty acids (20:4 and 22:4), whereas the content of the same PUFAs decreased in rats on the LS diet. On the contrary, tumor lipids from rats on the α-linolenic acid-enriched diet (LS diet) exhibited a high content of ω-3 pentaenoic PUFAs (20:5 and 22:5) compared with those from rats on the control diet. Therefore, the 20:4 ω-6/20:5 ω-3 ratio was very low in whole tumor lipids isolated from rats fed the LS diet compared with rats on the control or SS diet. The 20:4 ω-6/20:5 ω-3 molar ratio was very low also in 1,2-diradyl-sn-glycerophosphocholine isolated from tumors grown in rats on the LS diet compared with the control diet. In addition, it was found that these compositional changes were not attributable to changes of Δ-5 and Δ-6 desaturase expression in tumor tissue.

Besides changing the fatty acid composition of tumor lipids, high-lipid diets also significantly affected COX-2 expression in hepatoma tissue. In fact, COX-2 mRNA content was higher in hepatoma cells grown in rats on the SS diet than in tumors grown in rats on the unsupplemented diet. On the contrary, the mRNA content for the same gene was significantly reduced in rats fed the LS diet.

Therefore, considering that the LS diet reduced the cellular level of arachidonic acid, depressed COX-2 expression, and selectively induced apoptosis of hepatoma cells, we hypothesize that the modulation of metabolic pathways leading to the biosynthesis of antiapoptotic prostaglandins might justify the cytotoxic activity of α-linolenic acid to hepatocarcinoma cells.

As far as the factors involved in COX-2 expression are concerned, it is known that transcriptional modulation of the encoding gene is cell-specific (62). In the liver, COX-2 expression is mainly regulated by C/EBPs, C/EBPα playing a prominent role (50). In particular, it has been shown that C/EBPα induces growth arrest and differentiation and negatively modulates COX-2 expression, whereas C/EBPβ and C/EBPδ are positive modulators of COX-2 expression (63). The mRNA levels of the three C/EBP isoforms were measured in tumors from rats on the different diets. Tumors from rats on the α-linolenic acid-enriched diet (LS) contained low C/EBPβ mRNA levels compared with tumors from rats on the control diet. On the contrary, C/EBPβ mRNA level was significantly higher in rats on the SS diet than in rats on the control diet. C/EBPα and C/EBPδ mRNA levels were unaffected by dietary conditioning.

Although the hypothesis that dietary α-linolenic acid cytotoxicity to hepatocarcinoma cells is mediated by a depression of antiapoptotic prostaglandin biosynthesis is in good agreement with the recent evidence that prostaglandin E_2 level decreases in livers and tumors of rats on a linseed oil-enriched diet (64), the role of C/EBPs in the regulation of COX-2 expression in hepatoma cells needs further investigation.

This work was supported by a grant from the Italian Space Agency and by the Fondazione Cassa di Risparmio di Perugia.

REFERENCES

1. Alo, P. L., P. Visca, A. Marci, A. Mangoni, C. Botti, and U. Di Tondo. 1996. Expression of fatty acid synthase (FAS) as a predictor of recurrence in stage I breast carcinoma patients. *Cancer.* **77:** 474–482.
2. Visca, P., V. Sebastiani, E. S. Pizer, C. Botti, P. De Carli, S. Filippi, S. Monaco, and P. L. Alo. 2003. Immunohistochemical expression and prognostic significance of FAS and GLUT1 in bladder carcinoma. *Anticancer Res.* **23:** 335–339.
3. Pizer, E., S. Lax, F. Kuhajda, G. Pasternack, and R. Kurman. 1998. Fatty acid synthase expression in endometrial carcinoma: correlation with cell proliferation and hormone receptors. *Cancer.* **83:** 528–537.
4. Rashid, A., E. S. Pizer, M. Moga, L. Z. Milgraum, M. Zahurak, G. R. Pasternack, F. P. Kuhajda, and S. R. Hamilton. 1997. Elevated expression of fatty acid synthase and fatty acid synthetic activity in colorectal neoplasia. *Am. J. Pathol.* **150:** 201–208.
5. Swinnen, J. V., T. Roskams, S. Joniau, H. Van Poppel, R. Oyen, L. Baert, W. Heyns, and G. Verhoeven. 2002. Overexpression of fatty acid synthase is an early and common event in the development of prostate cancer. *Int. J. Cancer.* **98:** 19–22.
6. Yang, Y-A., W. F. Han, P. J. Morin, F. J. Chrest, and E. S. Pizer. 2002.

Activation of fatty acid synthesis during neoplastic transformation: role of mitogen-activated protein kinase and phosphatidylinositol 3-kinase. *Exp. Cell Res.* **279:** 80–90.
7. Yang, Y-A., P. J. Morin, W. F. Han, T. Chen, D. M. Bornman, E. W. Gabrielson, and E. S. Pizer. 2003. Regulation of fatty acid synthase expression in breast cancer by sterol regulatory element binding protein-1c. *Exp. Cell Res.* **282:** 132–137.
8. Swinnen, J. V., H. Heemers, L. Deboel, F. Foufelle, W. Heyns, and G. Verhoeven. 2000. Stimulation of tumour-associated fatty acid synthase expression by growth factor activation of the sterol regulatory element-binding protein pathway. *Oncogene.* **19:** 5173–5181.
9. Heemers, H., B. Maes, F. Foufelle, W. Heyns, G. Verhoeven, and J. V. Swinnen. 2001. Androgens stimulate lipogenic gene expression in prostate cancer cells by activation of the sterol regulatory element-binding protein cleavage activating protein/sterol regulatory element-binding protein. *Mol. Endocrinol.* **15:** 1817–1828.
10. Li, J. N., M. A. Mahmoud, W. F. Han, M. Ripple, and E. S. Pizer. 2000. Sterol regulatory element-binding protein-1 participates in the regulation of fatty acid synthase expression in colorectal neoplasia. *Exp. Cell Res.* **261:** 159–165.
11. Nohturfft, A., R. A. DeBose-Boyd, S. Scheek, J. L. Goldstein, and M. S. Brown. 1999. Sterols regulate cycling of SREBP cleavage-activating protein (SCAP) between endoplasmic reticulum and Golgi. *Proc. Natl. Acad. Sci. USA.* **96:** 11235–11240.
12. Worgall, T. S., S. L. Sturley, T. Seo, T. F. Osborne, and R. J. Deckelbaum. 1998. Polyunsaturated fatty acids decrease expression of promoters with sterol regulatory elements by decreasing levels of mature sterol regulatory element-binding protein. *J. Biol. Chem.* **273:** 25537–25540.
13. Moon, Y. S., M-J. Latasa, M. J. Griffin, and H. S. Sul. 2002. Suppression of fatty acid synthase promoter by polyunsaturated fatty acids. *J. Lipid Res.* **43:** 691–698.
14. Pizer, E. S., C. Jackisch, F. D. Wood, G. R. Pasternack, N. E. Davidson, and F. P. Kuhajda. 1996. Inhibition of fatty acid synthesis induces programmed cell death in human breast cancer cells. *Cancer Res.* **56:** 2745–2747.
15. Kuhajda, F. P., E. S. Pizer, J. N. Li, N. S. Mani, G. L. Frehywot, and C. A. Townsend. 2000. Synthesis and antitumor activity of an inhibitor of fatty acid synthase. *Proc. Natl. Acad. Sci. USA.* **97:** 3450–3454.
16. Li, J. N., M. Gorospe, F. J. Chrest, T. S. Kumaravel, M. K. Evans, W. F. Han, and E. S. Pizer. 2001. Pharmacological inhibition of fatty acid synthase activity produces both cytostatic and cytotoxic effects modulated by p53. *Cancer Res.* **61:** 1493–1499.
17. Pizer, E. S., F. J. Chrest, J. A. DiGiuseppe, and W. F. Han. 1998. Pharmacological inhibitors of mammalian fatty acid synthase suppress DNA replication and induce apoptosis in tumor cell lines. *Cancer Res.* **58:** 4611–4615.
18. Calviello, G., P. Palozza, E. Piccioni, N. Maggiano, A. Frattucci, P. Franceschelli, and G. M. Bartoli. 1998. Dietary supplementation with eicosapentaenoic and docosahexaenoic acid inhibit growth of Morris hepatocarcinoma 3924A in rats: effects on proliferation and apoptosis. *Int. J. Cancer.* **75:** 699–705.
19. Diggle, C. P. 2002. In vitro studies on the relationship between polyunsaturated fatty acids and cancer: tumour or tissue specific effects? *Prog. Lipids Res.* **41:** 240–253.
20. Hirose, M., A. Masuda, N. Ito, K. Kamano, and H. Okuyama. 1990. Effects of dietary perilla oil, soybean oil and safflower oil on 7,12-dimethylbenz[a]anthracene (DMBA) and 1,2-dimethyl-hydrazine (DMH)-induced mammary gland and colon carcinogenesis in female SD rats. *Carcinogenesis.* **11:** 731–735.
21. Reddy, B. S., C. Burill, and J. Rigotty. 1991. Effect of diets high in omega-3 and omega-6 fatty acids on initiation and postinitiation stages of colon carcinogenesis. *Cancer Res.* **51:** 487–491.
22. Karmali, R. A., J. Marsh, and C. Fuchs. 1984. Effect of omega-3 fatty acids on growth of a rat mammary tumor. *J. Natl. Cancer Inst.* **73:** 457–461.
23. Hudson, E. A., S. A. Beck, and M. J. Tisdale. 1993. Kinetics of the inhibition of tumour growth in mice by eicosapentaenoic acid-reversal by linoleic acid. *Biochem. Pharmacol.* **45:** 2189–2194.
24. Sauer, L. A., and R. T. Dauchy. 1992. The effect of omega-6 and omega-3 fatty acids on ^{3}H-thymidine incorporation in hepatoma 7288CTC perfused in situ. *Br. J. Cancer.* **66:** 297–303.
25. Horrobin, D. F. 1990. Essential fatty acids, lipid peroxidation, and cancer. *In* ω-6 Essential Fatty Acids. D. F. Horrobin, editor. Wiley-Liss, New York. 351–378.
26. Pizer, E. S., J. Thupari, W. F. Han, M. L. Pinn, F. J. Chrest, G. L. Frehywot, C. A. Townsend, and F. P. Kuhajda. 2000. Malonyl-coenzyme A is a potent mediator of cytotoxicity induced by fatty acid synthase inhibition in human breast cancer cells and xenografts. *Cancer Res.* **60:** 213–218.
27. Bagga, D., L. Wang, R. Farias-Eisner, J. A. Glaspy, and S. T. Reddy. 2003. Differential effects of prostaglandin derived from omega-6 and omega-3 polyunsaturated fatty acids on COX-2 expression and IL-6 secretion. *Proc. Natl. Acad. Sci. USA.* **100:** 1751–1756.
28. Gavriely, Y., Y. Sherman, and S. A. Sasson. 1992. Identification of programmed cells death in situ via specific labelling DNA fragmentation. *J. Cell Biol.* **119:** 493–501.
29. Folch, J., M. Lees, and G. M. Sloane-Stanley. 1957. A simplified method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226:** 497–509.
30. Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry.* **18:** 5294–5299.
31. Kim, J., P. Tontoz, and B. Spiegelman. 1993. Add1: a novel helix-loop-helix protein associated with adipocyte determination and differentiation. *Mol. Cell. Biol.* **13:** 4753–4759.
32. Inoue, J., and R. Sato. 1999. A novel splicing isoform of mouse sterol regulatory element-binding protein-1 (SREBP-1). *Biosci. Biotechnol. Biochem.* **63:** 243–245.
33. Amy, C. M., A. Witkowski, J. Naggert, B. Williams, Z. Randhawa, and S. Smith. 1989. Molecular cloning and sequencing of cDNAs encoding the entire rat fatty acid synthase. *Proc. Natl. Acad. Sci. USA.* **86:** 3114–3118.
34. Lopez-Casillas, F., D. H. Bai, X. N. Luo, I. S. Kong, M. A. Hermodson, and K. H. Kim. 1988. Structure of the coding sequence and primary amino acid sequence of acetyl-coenzyme A carboxylase. *Proc. Natl. Acad. Sci. USA.* **85:** 5784–5788.
35. Elshourbagy, N. A., J. C. Near, P. J. Kmetz, G. M. Sathe, C. Southan, J. E. Strickler, M. Gross, J. F. Young, T. N. Wells, and P. H. Groot. 1990. Rat ATP citrate-lyase. Molecular cloning and sequence analysis of a full-length cDNA and mRNA abundance as a function of diet, organ and age. *J. Biol. Chem.* **265:** 1430–1435.
36. Goettlicher, M., E. Widmark, Q. Li, and J. A. Gustafsson. 1992. Fatty acid activate a chimera of the clofibric acid-activated receptor and glucocorticoid receptor. *Proc. Natl. Acad. Sci. USA.* **89:** 4653–4657.
37. Esser, V., C. H. Britton, B. C. Weis, D. W. Foster, and J. D. McGarry. 1993. Cloning, sequencing and expression of a cDNA encoding rat liver carnitine palmitoyltransferase I. Direct evidence that a single polypeptide is involved in inhibitor interaction and catalytic function. *J. Biol. Chem.* **268:** 5817–5822.
38. Miyazawa, S., H. Hayashi, M. Hijikata, N. Ishii, H. Kagamiyama, T. Osumi, and T. Hashimoto. 1987. Complete nucleotide sequence of cDNA and predicted sequence of rat acyl-CoA oxidase. *J. Biol. Chem.* **262:** 8131–8137.
39. Hua, X., A. Nohturfft, J. L. Goldstein, and M. S. Brown. 1996. Sterol resistance in CHO cells traced to point mutation in SREBP cleavage-activating protein. *Cell.* **87:** 415–426.
40. Seidah, N. G., S. J. Mowla, J. Hamelin, A. M. Mamarbachi, S. Benjannet, B. B. Toure, A. Basak, J. S. Munzer, J. Marcinkiewicz, M. Zhong, J. C. Barale, C. Lazure, R. A. Murphy, M. Chretien, and M. Marcinkiewicz. 1999. Mammalian subtilisin/kexin isozyme SKI-1. A widely expressed proprotein convertase with a unique cleavage specificity and cellular localization. *Proc. Natl. Acad. Sci. USA.* **96:** 1321–1326.
41. Rawson, R. B., N. G. Zelenski, D. Nijhawan, J. Ye, J. Sakai, M. T. Hasan, T. Y. Chang, M. S. Brown, and J. L. Goldstein. 1997. Complementation cloning of S2P, a gene encoding a putative metalloprotease required for intramembrane cleavage of SREBPs. *Mol. Cell.* **1:** 47–57.
42. Lincoln, A. J., S. C. Williams, and P. F. Johnson. 1994. A revised sequence of the rat C/EBP gene. *Genes Dev.* **8:** 1131–1132.
43. Descombes, P., M. Chojkier, S. Lichtsteiner, E. Falvey, and U. Schibler. 1990. LAP, a novel member of the C/EBP gene family, encodes a liver-enriched transcriptional activator protein. *Genes Dev.* **4:** 1541–1551.
44. Kageyama, R., Y. Sasai, and S. Nakanishi. 1991. Molecular characterization of transcription factors that bind to the cAMP responsive region of the substance P precursor gene. cDNA cloning of a novel C/EBP-related factor. *J. Biol. Chem.* **266:** 15525–15531.
45. Feng, L., W. Sun, Y. Xia, W. W. Tang, P. Chanmugam, E. Soyoola, C. B. Wilson, and D. Hwang. 1993. Cloning two isoforms of rat cyclooxygenase: differential regulation of their expression. *Arch. Biochem. Biophys.* **307:** 361–368.

46. Blanquicett, C., M. R. Johnson, M. Heslin, and R. B. Diasio. 2002. Housekeeping gene variability in normal and carcinomatous colorectal and liver tissues: applications in pharmacogenomic gene expression studies. *Anal. Biochem.* **303:** 209–214.
47. Xu, J., T. Nakamura, H. P. Cho, and S. D. Clarke. 1999. Sterol regulatory element binding protein-1 expression is suppressed by dietary polyunsaturated fatty acids. *J. Biol. Chem.* **274:** 23577–23583.
48. Demoz, A., A. Garras, D. K. Asiedu, B. Netteland, and R. K. Berge. 1995. Rapid method for the separation and detection of tissue short-chain coenzyme A esters by reversed-phase high-performance liquid chromatography. *J. Chromatogr.* **667:** 148–152.
49. Caivano, M., B. Gorgoni, P. Cohen, and V. Poli. 2001. The induction of cyclooxygenase-2 mRNA in macrophages is biphasic and requires both CCAAT enhancer-binding proteinβ (C/EBPβ) and C/EBPδ transcription factors. *J. Biol. Chem.* **276:** 48693–48701.
50. Casado, M., N. A. Callejas, J. Rodrigo, X. Zhao, S. K. Dey, L. Bosca, and P. Martin-Sanz. 2001. Contribution of cyclooxygenase 2 to liver regeneration after partial hepatectomy. *FASEB J.* **15:** 2016–2018.
51. Cao, Y., and S. M. Prescott. 2002. Many actions of cyclooxygenase-2 in cellular dynamics and in cancer. *J. Cell. Physiol.* **190:** 279–286.
52. Stephen, M., F. A. Perscott, and F. A. Fritzpatrick. 2000. Cyclooxygenase-2 and carcinogenesis. *Biochim. Biophys. Acta.* **1470:** M69–M78.
53. Woutersen, R. A., M. J. Appel, A. van Garderen-Hoetmer, and V. W. Wijnands. 1999. Dietary fat and carcinogenesis. *Mutat. Res.* **443:** 111–127.
54. Marnett, L. J. 1994. Generation of mutagens during arachidonic acid metabolism. *Cancer Metastasis Rev.* **13:** 303–308.
55. Hu, K. Q., C. H. Yu, Y. Mineyama, J. D. McCracken, D. J. Hillebrand, and M. Hasan. 2003. Inhibited proliferation of cyclooxygenase-2 expressing human hepatoma cells by NS-398, a selective COX-2 inhibitor. *Int. J. Oncol.* **22:** 757–763.
56. Shiota, G., M. Okubo, T. Noumi, N. Noguchi, K. Oyama, Y. Takano, K. Yashima, Y. Kishimoto, and H. Kawasaki. 1999. Cyclooxygenase-2 expression in hepatocellular carcinoma. *Hepatogastroenterology.* **46:** 407–412.
57. Qiu, D. K., X. Ma, Y. S. Peng, and X. Y. Chen. 2002. Significance of cyclooxygenase-2 expression in human primary hepatocellular carcinoma. *World J. Gastroenterol.* **8:** 815–817.
58. Koga, H., S. Sakisaka, M. Ohishi, T. Kawaguchi, E. Taniguchi, K. Sasatomi, M. Harada, T. Kusaba, M. Tanaka, R. Kimura, Y. Nakashima, O. Nakashima, M. Kojiro, T. Kurohiji, and M. Sata. 1999. Expression of cyclooxygenase-2 in human hepatocellular carcinoma: relevance to tumor dedifferentiation. *Hepatology.* **29:** 688–696.
59. Bae, S. H., E. S. Jung, Y. M. Park, B. S. Kim, B. K. Kim, D. G. Kim, and W. S. Tyu. 2001. Expression of cyclooxygenase-2 (COX-2) in hepatocellular carcinoma and growth inhibition of hepatoma cell lines by a COX-2 inhibitor, NS-398. *Clin. Cancer Res.* **7:** 1410–1418.
60. Tian, G., J. P. Yu, H. S. Luo, B. P. Yu, H. Yue, J. Y. Li, and Q. Mei. 2002. Effect of nimesulide on proliferation and apoptosis of human hepatoma SMMC-7721 cells. *World J. Gastroenterol.* **8:** 483–487.
61. Kern, M. A., D. Schubert, D. Sahi, M. M. Schoneweiss, I. Moll, A. M. Haugg, H. P. Dienes, K. Breuhahn, and P. Schirmacher. 2002. Proapoptotic and antiproliferative potential of selective cyclooxygenase-2 inhibitors in human liver tumor cells. *Hepatology.* **36:** 885–894.
62. Gorgoni, B., M. Caivano, C. Arizmendi, and V. Poli. 2001. The transcription factor C/EBPbeta is essential for inducible expression of the cox-2 gene in macrophages but not in fibroblasts. *J. Biol. Chem.* **276:** 40769–40777.
63. Ramji, D. P., and P. Foka. 2002. CCAAT/enhancer-binding proteins: structure, function and regulation. *Biochem. J.* **365:** 561–575.
64. Jelinska, M., A. Tokarz, R. Oledzka, and A. Czorniuk-Sliwa. 2003. Effects of dietary linseed, evening primrose or fish oils on fatty acid and prostaglandin E2 contents in the rat livers and 7,12-dimethylbenz[a]anthracene-induced tumours. *Biochim. Biophys. Acta.* **1637:** 193–199.