Unsaturated Fatty Acids Down-regulate SREBP Isoforms 1a and 1c by Two Mechanisms in HEK-293 Cells*

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Sterol regulatory element-binding proteins (SREBPs) are membrane-bound transcription factors that increase the synthesis of fatty acids as well as cholesterol in animal cells. All three SREBP isoforms (SREBP-1a, -1c, and -2) are subject to feedback regulation by cholesterol, which blocks their proteolytic release from membranes. Previous data indicate that the SREBPs are also negatively regulated by unsaturated fatty acids, but the mechanism is uncertain. In the current experiments, unsaturated fatty acids decreased the nuclear content of SREBP-1, but not SREBP-2, in cultured human embryonic kidney (HEK)-293 cells. The potency of unsaturated fatty acids increased with increasing chain length and degree of unsaturation. Oleate, linoleate, and arachidonate were all effective, but the saturated fatty acids palmitate and stearate were not effective. Down-regulation occurred at two levels. The mRNAs encoding SREBP-1a and SREBP-1c were markedly reduced, and the proteolytic processing of these SREBPs was inhibited. When SREBP-1a was produced by a cDNA expressed from an independent promoter, unsaturated fatty acids reduced nuclear SREBP-1a without affecting the mRNA level. There was no effect when the cDNA encoded a truncated version that was not membrane-bound. When administered together, sterols and unsaturated fatty acids potentiated each other in reducing nuclear SREBP-1. In the absence of fatty acids, sterols did not cause a sustained reduction of nuclear SREBP-1, but they did reduce nuclear SREBP-2. We conclude that unsaturated fatty acids, as well as sterols, can down-regulate nuclear SREBPs and that unsaturated fatty acids have their greatest inhibitory effects on SREBP-1a and SREBP-1c, whereas sterols have their greatest inhibitory effects on SREBP-2.

Ingestion of n-3 and n-6 polyunsaturated fatty acids in place of saturated fatty acids shifts the pattern of fat metabolism in liver from storage to oxidation (1). Genes involved in fatty acid oxidation are induced, and genes involved in fatty acid synthesis or lipogenesis are repressed (2, 3). The increase in genes involved in lipid synthesis and uptake. Target genes include the low density lipoprotein receptor and the cholesterol regulatory enzymes 3-hydroxy-3-methylglutaryl CoA synthase and reductase as well as the lipogenic enzymes acetyl-CoA carboxylase, fatty acid synthetase, and stearyl CoA desaturase (8, 12–16).

The proteolytic processing of SREBPs is under feedback control by cholesterol. Thus, when sterols accumulate in cells, the SCAP:SREBP complex fails to move to the Golgi, and SREBPs are not processed (17). The nuclear SREBPs are rapidly degraded by a proteasomal process, and the synthesis of sterols and fatty acids (primarily 18:1 unsaturates) declines.

Three isoforms of SREBP have been identified (8). Two of

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1 The abbreviations used are: SREBP, sterol regulatory element-binding protein; nSREBP, cleaved nuclear form of SREBP; ALLN, N-acetyl-Leu-Leu-norleucinal; BSA, bovine serum albumin; FCS, fetal calf serum; HEK-293 cells, human embryonic kidney-293 cells; HSV, herpes simplex virus; PBS, phosphate-buffered saline; SCAP, SREBP cleavage-activating protein; TK, thymidine kinase; PAGE, polyacrylamide gel electrophoresis.
these proteins, designated SREBP-1a and SREBP-1c, are derived from a single gene through use of alternate transcriptional start sites producing different forms of exon 1 that are spliced to a common exon 2. The SREBP-1a isoform has a relatively long NH2-terminal acidic activation domain, and it is a potent transcriptional activator. The SREBP-1c isoform has a short acidic activation domain, and it may require post-translational modification to activate transcription (18). The third isoform, SREBP-2, derived from a separate gene, is ~50% identical to the SREBP-1 isoforms, and it contains a long activation domain. Although the target genes for the SREBPs overlap, the two SREBP-1 isoforms are relatively more potent in stimulating fatty acid synthesis, whereas SREBP-2 acts primarily on the cholesterol biosynthetic pathway (8, 16, 18).

The ratio of SREBP-1a to -1c isoforms varies markedly among different cells. In most adult tissues, including the liver, SREBP-1c predominates (19), but the reverse is true in cultured cells (19). The amount of SREBP-2 is generally approximately equal to the combined total of SREBP-1a and -1c (8). SREBP-1c predominates (19), but the reverse is true in cultured cells (19). The amount of SREBP-2 is generally approximately equal to the combined total of SREBP-1a and -1c (8).

Inasmuch as unsaturated fatty acids are end-products of SREBP action, several laboratories have sought to determine whether fatty acids exert feedback effects on SREBP activity. In general, these studies have shown regulatory effects, but the details have been contradictory. In the first of these studies, Thewke et al. (20) showed that oleate synergized with sterols in reducing the amounts of nuclear SREBP-1 (nSREBP-1) and nSREBP-2 in Chinese hamster ovary cells. However, oleate without sterols had no effect. Worgall et al. (21) found that unsaturated fatty acids (but not saturated fatty acids) without sterols decreased the amount of nSREBP-1 in several types of cultured cells and lowered the mRNA levels for 3-hydroxy-3-methylglutaryl-CoA synthase, an SREBP target gene. Xu et al. (22) fed oils rich in n-6 or n-3 polyunsaturated fatty acids to rats and observed a decrease in nSREBP-1 in liver cells. Both n-6 and n-3 polyunsaturated fatty acids decreased the amount of mRNA for SREBP-1, but they did not appear to affect transcription of the SREBP-1 gene as determined by nuclear run-on assays. In contrast, Xu et al. (22) found no inhibitory effect on SREBP-1 expression with dietary monounsaturated triolein. Kim et al. (23) found that long-term feeding (5 months) of a fish oil diet enriched in n-3 polyunsaturated fatty acids decreased nSREBP-1 and nSREBP-2 levels in the livers of mice. The mRNA for SREBP-1c was reduced dramatically and that of SREBP-2 was reduced partially, but there was no effect on SREBP-1a. In agreement with the earlier results of Xu et al. (22), Kim et al. (23) found that oleic acid-enriched safflower oil had little effect in their animals.

The current studies were undertaken to provide a comprehensive analysis of the effects of various fatty acids on the levels of mRNA and protein for all three isoforms of SREBP in nonhepatic human cells (HEK-293 cells) maintained in tissue culture. The results show that unsaturated fatty acids lowered the levels of nSREBP-1, even in the absence of exogenous sterols. In general, the inhibitory effect of the fatty acids increased with increasing chain length and degree of unsaturation. The decrease in nSREBP-1 was accompanied by a decrease in the mRNAs encoding both SREBP-1a and SREBP-1c. However, even when the level of mRNA for SREBP-1a was held constant through transfection, arachidonic acid still reduced nSREBP-1a, indicating that fatty acids have actions at the level of SREBP-1a protein as well as at the level of mRNA. Arachidonic acid did not have an effect on cells that were engineered to express a truncated form of SREBP-1a that enters the nucleus without a requirement for proteolysis, suggesting that the post-transcriptional effect is exercised at the level of proteolytic processing of the SREBP-1a precursor. In the absence of sterols, unsaturated fatty acids had very little effect on the levels of SREBP-2 mRNA or the nSREBP-2 isoform.

**EXPERIMENTAL PROCEDURES**

**Materials**—We obtained affinity-purified donkey anti-mouse IgG from Jackson Immunoresearch Laboratories; anti-rabbit IgG from Sigma-Aldrich, Inc. and Nu-Chek-Prep, Inc. (Elysian, MN); N-acetyl-Leu-Leu-norleucinal (ALLN) from Calbiochem; defatted bovine serum albumin (BSA, catalog no. 100069) from Roche Molecular Biochemicals; and triacsin C from BioMol Research Laboratories, Inc. (Plymouth Meeting, PA; [6-3H]arachidonic acid [1800 Ci/mmol] and Rediprime II Random Primer Labeling kit from Amersham Pharmacia Biotech; and human β-actin cDNA probe control from CLONTECH Laboratories, Inc. Rabbit polyclonal antibodies against human SREBP-1 (amino acids 31–175 of the SREBP-1a isoform) and human SREBP-2 (amino acids 48–403) were prepared as previously described (24). Lipoprotein-deficient fetal calf serum (d < 1.215 g/ml) was prepared by ultracentrifugation (25). Other reagents were obtained from previously described sources (9, 26).

**Preparation of Delipidated Fetal Calf Serum**—Fetal calf serum (FCS, Life Technologies, Inc.) was delipidated by a modification of the method of Cham and Knowles (27). Briefly, 500 ml of serum was mixed with 400 ml of n-butanol and 600 ml of isopropanol ether at room temperature for 20 min, followed by a 20-min incubation on ice. After centrifugation, the aqueous phase was re-extracted with 200 ml of isopropanol ether, recentrifuged, subjected to evaporation under a stream of nitrogen gas, lyophilized, reconstituted with 200 ml of distilled water, and dialyzed against phosphate-buffered saline (PBS). Multiple aliquots were stored at −20 °C. The concentration of free fatty acids in the delipidated serum was measured with the Free Fatty Acid, Half-micro Test assay kit (Roche Molecular Biochemicals); the concentrations of cholesterol and triglycerides were measured as previously described (28, 29). In 12 preparations of delipidated fetal calf serum, the mean concentration of free fatty acids was reduced from 840 to 7.7 μM, the mean concentration of cholesterol was reduced from 280 to 7.5 μg/ml, and the mean concentration of triglycerides was reduced from 600 to 23 μg/ml.

**Preparation of Albumin-bound Fatty Acids**—A 5 or 10 ml stock solution of each fatty acid was prepared by diluting the free fatty acid in ethanol and precipitating it with the addition of NaOH (final concentration of 0.25 M). The precipitated sodium salt was then evaporated under nitrogen gas, reconstituted with 0.9% (w/v) NaCl, and stirred at room temperature for 10 min with defatted BSA (final concentration at 10 mg/ml; 0.15 μM NaCl). Each solution was adjusted to pH 7.4 with NaOH and stored in multiple aliquots at −20 °C protected from light in tubes evacuated under nitrogen gas.

**Expression Plasmids—pTK-HSV-BP1a is an expression vector that produces epitope-tagged human SREBP-1a under control of the HSV TK promoter (30).**

**pTK-HSV-BP1a-T7 encodes human SREBP-1a flanked at the NH2 terminus by two tandem copies of the HSV epitope tag and at the COOH terminus by three tandem copies of the T7 epitope tag sequence.** This TK-driven plasmid was constructed as follows: pTK-HSV-BP2-Ras-T7 (26) was digested with BspDI and NdeI to isolate a 6-kb fragment that contained the HSV and T7 epitope repeats but not the sequence of the SREBP-2/Ras fusion protein. A pair of primers, 5′-CAGCCTGAACTCTCGCCGGACG-3′, corresponding to amino acids 1–7 of the second HSV tag, and 5′-GCTAGGATATGGTCTAGGAAGTGCAAGGTTCCCAAGCT-3′, corresponding to amino acid residues 1140–1147 of human SREBP-1a, was used to amplify the coding region of SREBP-1a. The resulting polymerase chain reaction product was digested with BspDI and NdeI, and a 3.4-kb fragment encoding amino acids 1–1147 of SREBP-1a was isolated. The two isolated fragments were ligated to generate pTK-HSV-BP1a-T7, which was sequenced in its entirety.

**Site-directed Mutagenesis—A plasmid encoding the nuclear form of human SREBP-1a (amino acids 1–490), designated pTK-HSV-BP1a-T7, which was sequenced in its entirety.**

**Cell Culture and Fractionation of HEK-293 Cells**—Unless otherwise stated, monolayers of human embryonic kidney (HEK)-293 cells were set up on day 0 (7 × 105 cells/100-mm dish) and cultured in 5–9% CO2.
Regulation of SREBP-1a and -1c by Unsaturated Fatty Acids

**Fig. 1.** Immunoblot analysis of endogenous SREBP-1 (A) and SREBP-2 (B) in HEK-293 cells incubated with lipoprotein-deficient serum or delipidated serum: effects of sterols and fatty acids. On day 0, HEK-293 cells were set up for experiments as described under “Experimental Procedures” except that the cells were plated at a density of 4 × 10^5 cells/60-mm dish. On day 2, the cells were refed with medium A containing either 5% fetal calf lipoprotein-deficient serum (lanes A–D and I–L) or 5% delipidated FCS (lanes E–H and M–P) and one of the following additions as indicated: 100 μM sodium arachidonate, 1 μM/ml 25-hydroxycholesterol plus 10 μg/ml cholesterol (sterols), or sodium arachidonate plus sterols. All culture medium was adjusted to contain 0.1% BSA and 0.2% ethanol. After incubation for 16 h, the cells were harvested, and nuclear extracts and membrane fractions were prepared as described under “Experimental Procedures.” Aliquots of the membranes (40 μg) and nuclear extracts (40 μg) were subjected to SDS-PAGE. Immunoblot analysis was carried out with 5 μg/ml rabbit anti-SREBP-1 IgG (lanes A–H) or anti-SREBP-2 IgG (lanes I–P). The filters were exposed to film for 20 s (membranes) or 10 s (nuclear extracts). P and N denote the precursor and cleaved nuclear forms of SREBP-1 or SREBP-2, respectively. The intensity of the nuclear bands was quantified with the NIH IMAGE 1.61 software as described under “Experimental Procedures.” The values are expressed relative to the signal intensity in lane A (SREBP-1) or lane I (SREBP-2).

A. SREBP-1

| Serum | LP Deficient | Delipidated |
|-------|--------------|-------------|
|        |              |             |
| Arachidonate | - | + | - | + |
| Sterols | - | + | - | + |
| Lane | A | B | C | D | E | F | G | H | I | J | K | L | M | N | O | P |
| Membranes | 97 |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |

B. SREBP-2

| Serum | LP Deficient | Delipidated |
|-------|--------------|-------------|
|        |              |             |
|        |              |             |
| Arachidonate | - | + | - | + |
| Sterols | - | + | - | + |
| Lane | I | J | K | L | M | N | O | P |   |   |   |   |   |   |   |
| Nuclear Extract | 69 |   |   |   |   |   |   |   |

Relative Level of nSREBP

at 37 °C in medium A (Dulbecco’s modified Eagle’s medium (low glucose) containing 100 units/ml penicillin and 100 μg/ml streptomycin sulfate) supplemented with 10% (v/v) FCS. Unless otherwise stated, on day 2 the cells were washed once with PBS and refed with medium A supplemented with 5% (v/v) delipidated FCS in the absence or presence of BSA-bound fatty acids (100 μg/ml) and/or sterols (1 μg/ml 25-hydroxycholesterol plus 10 μg/ml cholesterol added in a final concentration of 0.2% (v/v) ethanol) as indicated in the legends. After incubation with fatty acids and/or sterols at 37 °C for 14 h, ALLN was added directly to the medium at a final concentration of 25 μg/ml unless otherwise stated. 2 h later, the cells were harvested by scraping in the medium, and the cell suspension from triplicate dishes was pooled and centrifuged at 10^3 × g for 5 min at 4 °C. The resulting cell pellet was washed by resuspension in PBS at 4 °C, after which the cell pellet was resuspended in buffer A (250 mM sucrose, 10 mM Hepes-KOH at pH 7.6, 10 mM KCl, 1.5 mM MgCl2, 1 mM sodium EDTA, 1 mM sodium EGTA, and a mixture of protease inhibitors that included 2.8 μg/ml aprotinin, 10 μg/ml leupeptin, 25 μg/ml ALLN, 5 μg/ml pepstatin A, and 0.5 mM Pefabloc). The cell suspension was passed through a 23-gauge needle 20 times and centrifuged at 10^3 × g at 4 °C for 5 min. The 10^3 × g pellet was resuspended in 0.1 ml of buffer B (20 mM Hepes-KOH at pH 7.6, 0.42 M NaCl, 2.5% (v/v) glycerol, 1.5 mM MgCl2, 1 mM sodium EDTA, 1 mM sodium EGTA, and the above mixture of protease inhibitors). This suspension was rotated at 4 °C for 1 h and centrifuged at 10^3 × g for 15 min at 4 °C in a Beckman TLA 100 rotor. The resulting supernatant is designated as the nuclear extract fraction. The supernatant of the original 10^3 × g spin was centrifuged at 10^6 × g for 15 min at 4 °C, after which the pellet was dissolved in 0.1 ml of SDS lysis buffer (10 mM Tris-HCl at pH 6.8, 100 mM NaCl, 1% (w/v) SDS, 1 mM sodium EDTA, 1 mM sodium EGTA, and the above mixture of protease inhibitors) and designated as the membrane fraction.

**Transfection—**HEK-293 cells were transfected for 3 h with the indicated plasmids using the MBS kit (Stratagene) as described previously (31).

**SDS-PAGE and Immunoblot Analysis—**Protein concentration of nuclear extract and membrane fractions was measured with a BCA kit (Pierce). A given amount of nuclear extract or membrane fraction was mixed with 5× SDS loading buffer (1× loading buffer contains 30 mM Tris-HCl at pH 7.4, 3% SDS, 5% (v/v) glycerol, 0.004% (w/v) bromphenol blue, 2.5% (v/v) β-mercaptoethanol). After boiling for 5 min, aliquots of the proteins (50 μg) were subjected to 5% SDS-PAGE and transferred to Hybond-C extra-nitrocellulose filters (Amersham Pharmacia Biotech). The filters were incubated with antibodies as described in the figure legends. Bound antibodies were visualized with peroxidase-conjugated donkey anti-rabbit IgG or donkey anti-mouse IgG using the SuperSignal CL-HRP substrate system (Pierce) according to the manufacturer’s instructions. Gels were calibrated with precasted molecular weight markers (New England BioLabs). Filters were exposed to Reflection NEF0496 film (PerkinElmer Life Sciences) at room temperature for the indicated time. The intensity of the nuclear bands was quantified with the National Institutes of Health IMAGE 1.61 software.

**Northern Blot Hybridization of mRNA—**Total RNA was prepared from triplicate dishes of monolayers of HEK-293 cells using the RNA-Stat 60 kit (Tel-test, Inc., Friendswood, TX), followed by phenol/chloroform extraction and ethanol precipitation. For Northern gel analysis, 30 μg of total RNA was mixed with RNA sample loading buffer (containing 50 μg/ml ethidium bromide (Sigma), denatured with formaldehyde and formamide, subjected to electrophoresis in a 1% agarose formaldehyde gel, and transferred to Hybond N membranes. The cDNA probes for human SREBP-1 and SREBP-2 were prepared by excising a 1.4-kb EcoRI/XbaI fragment from pIND-BP1c-FLAG (18) and a 1.2-kb HindIII fragment from pTK-HSV-BP2 (30). The cDNA probe for human β-actin was obtained from CLONTECH. cDNA probes were labeled with [α-32P]dCTP using a Rediprime II Random Labeling kit. The membranes were hybridized with the indicated labeled probes (2 × 10^6 cpm/ml) for 15 h at 65 °C using Rapid-hyb buffer (Amersham Pharmacia Biotech); washed twice with 0.1% (w/v) SDS/2% SSC at room temperature for 10 min followed by 0.1% (w/v) SDS/0.1% SSC at 65 °C for 15 min and exposed at −80 °C to film with intensifying screens for the indicated time. The amount of radioactivity was quantified using the NIH IMAGE 1.61 software.

**RNase Protection Assay—**The cDNA fragments for human SREBP-1a and SREBP-1c used as templates for cRNA probe synthesis were generated by polymerase chain reaction using the plasmids pIND-BP1a-FLAG and pIND-BP1c-FLAG (18). The primers used to amplify SREBP-1a were: 5′ primer, 5′-CGCTCCTAGGAGGGCGCTA-3′; and 3′ primer, 5′-ACTGTCATCTTGTTGGATAAGC-3′. The primers used to amplify SREBP-1c were: 5′ primer, 5′-AGGGGTAGGGCGCTGCT-3′ and 3′ primer, 5′-CAGCTGCTGCGGAGCTGTA-3′. The amplified human SREBP-1a fragment corresponds to 66 bp of 5′-untranslated region, exon 1a and 36 bp of exon 2. The amplified human SREBP-1c fragment corresponds to 54 bp of 5′-untranslated region, 15 bp of exon 1c, and 128 bp of exon 2. Note that exon 2 is common to SREBP-1a and SREBP-1c. The amplified fragments were subcloned into the pCRII vector (Invitrogen). After linearization of plasmid DNA with XhoI, antisense RNA was transcribed with [α-32P]CTP (20 mCi/ml) using bacteriophage SP6 RNA polymerase (Ambion, Austin, TX) as described previously (19). Aliquots of total RNA (50 μg) from each sample were incubated with the above SREBP-1 cRNA probe plus a cRNA probe for the mRNA of human β-actin (19) plus the reagents contained in the HybSpeed RPA kit (Ambion). In preparing the probes, we adjusted the specific activity of the [α-32P]CTP to give comparable signals for β-actin and SREBP-1. After digestion with RNase A/T1, protected fragments were separated on 8 × urea/5% polyacrylamide gels.
In previous studies from this laboratory, the sterol-mediated regulation of SREBP processing was measured in cultured cells that were grown in lipoprotein-deficient serum, which is essentially devoid of low density lipoprotein cholesterol, owing to the removal of low density lipoprotein by ultracentrifugal flotation (25). Ultracentrifugation does not remove free fatty acids, which are bound to albumin. To delipidate serum more completely, in the current studies we used a solvent extraction procedure that employs butanol and isopropyl ether (see "Experimental Procedures"). The concentrations of free fatty acids in medium containing 5% delipidated serum and 5% delipidated serum were 18 and 0.39 mM, respectively (45-fold reduction).

FIG. 2. Immunoblot analysis (A and B) and RNase protection assay (C) of endogenous SREBPs in HEK-293 cells treated with different fatty acids. On day 0, HEK-293 cells were set up as described in "Experimental Procedures." On day 2, the cells were refed with medium A containing 5% delipidated FCS and 100 μM of the indicated fatty acid added in a final concentration of 0.1% BSA. After incubation for 16 h, the cells were harvested in two groups. A and B, from one group, nuclear extract and membrane fractions were prepared. Aliquots of the membranes (50 μg) and nuclear extracts (50 μg) were subjected to SDS-PAGE. Immunoblot analysis was carried out with 5 μg/ml rabbit anti-SREBP-1 IgG (lanes A–J) or anti-SREBP-2 IgG (lanes K–T). The filters were exposed to film for 5 s (membranes) or 1 s (nuclear extracts). Quantification of bands was done as described in Fig. 1, and the values are expressed relative to the signal intensity for cells incubated in the absence of fatty acids (average of lanes A and J for SREBP-1 and lanes K and T for SREBP-2). C, from the second group, total RNA was isolated and aliquots of total RNA (50 μg) from pooled dishes were hybridized for 10 min at 68 °C to 32P-labeled cRNA probes. Protected fragments were separated by gel electrophoresis and exposed to film for 48 h (SREBP-1c), 16 h (SREBP-1a), or 6 h (β-actin) at −80 °C. The amount of radioactivity in each band was quantified as described in Fig. 1 and calculated after correction for loading differences with β-actin. The values are expressed relative to the signal intensity in lane A.

**RESULTS**

In previous studies from this laboratory, the sterol-mediated regulation of SREBP processing was measured in cultured cells that were grown in lipoprotein-deficient serum, which is essentially devoid of low density lipoprotein cholesterol, owing to the removal of low density lipoprotein by ultracentrifugation flotation (25). Ultracentrifugation does not remove free fatty acids, which are bound to albumin. To delipidate serum more completely, in the current studies we used a solvent extraction procedure that employs butanol and isopropyl ether (see "Experimental Procedures"). The concentrations of free fatty acids in medium containing 5% delipidated serum and 5% delipidated serum were 18 and 0.39 μM, respectively (45-fold reduction).

Fig. 1 shows the content of SREBPs in membrane fractions and nuclear extracts of HEK-293 cells that were incubated for 16 h in medium containing either lipoprotein-deficient serum or delipidated serum with or without added sterols or arachidonate. Inasmuch as our antibody does not differentiate between SREBP-1a and -1c, we use the general term SREBP-1 to refer to the results of immunoblotting. When the cells were incubated in lipoprotein-deficient serum, we observed the full-length precursor form of SREBP-1 in cell membranes and the cleaved mature form in nuclear extracts (Fig. 1A, lane A). Addition of sterols (mixture of 25-hydroxycholesterol plus cholesterol) led to an increase in the 120-kDa precursor form of SREBP-1 in cell membranes and the cleaved mature form in nuclear extracts (Fig. 1C, lane C). When sterols were added in delipidated serum, nSREBP-1 (lane D) was in undetectable, but the precursor accumulated (lane G). When arachidonate (100 μM) was added, the nuclear form of SREBP-1 (designated nSREBP-1) also disappeared, but there was no buildup of the precursor (lane C). In the presence of sterols plus arachidonate, the nuclear form remained low, but the precursor again accumulated (lane D). When the experiments were conducted in delipidated serum, nSREBP-1 (lane E) was increased as compared with that observed in lipoprotein-deficient serum (lane A). When sterols were added in delipidated serum, the precursor accumulated, but there was only a slight decline in nSREBP-1 (lane F). The addition of arachidonate abolished nSREBP-1, again without a buildup of the precursor (lane G). When arachidonate and sterols were present, nSREBP-1 was also undetectable, but the precursor accumulated (lane H).

In lipoprotein-deficient serum, sterols regulated SREBP-2 processing in a manner that was similar to that of SREBP-1 (Fig. 1B, lanes I and J). However, in contrast to the findings with SREBP-1, arachidonate did not effect the nuclear form of SREBP-2 (lane K). Sterols plus arachidonate gave a result that...
was similar to that of sterols alone (lane L). In delipidated serum, sterols alone abolished nSREBP-2 (lanes M and N).

The data of Fig. 1 indicate a difference in the ability of sterols and arachidonate to regulate SREBP-1 and SREBP-2. Sterols were unable to abolish nSREBP-1 unless a fatty acid such as arachidonate was present. In contrast, sterols eliminated nSREBP-2 even in the absence of fatty acids. Moreover, arachidonate alone reduced the amount of nSREBP-1, but it did not reduce the content of nSREBP-2. The data further suggest that the previously observed ability of sterols to suppress nSREBP-1 in lipoprotein-deficient serum is dependent on the presence of fatty acids in the lipoprotein-deficient serum.

To study the effects of sterols and fatty acids independently, all further experiments in this paper were performed with cells that were incubated in delipidated serum plus and minus added sterols or fatty acids. Fig. 2A shows that the ability of various fatty acids to reduce nSREBP-1 increased in proportion to chain length and degree of unsaturation. Thus, palmitate (16:0) had no effect, but palmitoleate (16:1) caused a 50% reduction. Stearate (18:0) reduced nSREBP-1 only slightly, but its monounsaturated derivative, oleate (18:1), had a pronounced effect (40% of control). Polyunsaturated linoleate (18:2) and linolenate (18:3) were even more effective. Arachidonate (20:4) and docosatetraenoate (22:4) were also potent. None of these fatty acids had a significant effect on nSREBP-2 (Fig. 2B) with the possible exception of arachidonate, which produced a slight reduction.

To measure the effects of these fatty acids on the mRNA for SREBP-1a and -1c, we used an RNase protection assay (Fig. 4).
We adjusted the exposure times to compensate partially for the fact that the absolute level of SREBP-1α mRNA was 10-fold greater than that of SREBP-1c. Unsaturated fatty acids reduced both the SREBP-1α and -1c mRNAs. The effectiveness of individual fatty acids increased with increasing chain length and degree of unsaturation in parallel with the effects on the nuclear protein. In experiments not shown, we found that saturated and monounsaturated fatty acids up to 14 carbons in length failed to lower the SREBP-1 mRNA or the levels of the nuclear protein.

To determine the amount of time required for unsaturated fatty acids to decrease the SREBP-1 mRNA and the nuclear protein, we measured the time course of the response to arachidonate (Fig. 3). At zero time, the cells were switched from medium containing 10% FCS to medium containing 5% delipidated serum, and the cells were harvested 20 h later. Arachidonate (100 μM) was added to the medium at the indicated times before harvest. A decline in the amount of nSREBP-1 was detectable when arachidonate was added 4 h before harvest (lane C), and the level declined by 70% at 6 h (lane D) and 90% at 16 h. There was little fall in nSREBP-2 even after 16 h (lane K). The precursor form of SREBP-2, but not SREBP-1, increased with time after addition of arachidonate. We observed a parallel decrease in the mRNAs for both SREBP-1α and SREBP-1c that was detectable within 2–4 h after arachidonate addition (Fig. 3C, lanes N and O) and reached 90% in 6 h (lane P). The 90% decline persisted for the 16 h duration of the experiment (lane Q). We observed no change in the SREBP-2 mRNA, which was measured by Northern blotting (Fig. 3D).

As noted in Fig. 1, in the absence of fatty acids, sterols alone cause only a slight decrease in nSREBP-1 when measured at 16 h. To determine whether this sterol resistance was constant with time, we performed a time course experiment (Fig. 4). At
293 cells were set up as described under “Experimental Procedures.” On day 0, HEK-293 cells treated with arachidonate. COOH-terminal domains of epitope-tagged SREBP-1a in trans-

were harvested, and membrane fractions and nuclear extracts were all cultures contained 0.1% BSA. After incubation for 16 h, the cells with 1 μg/ml IgG-TT-Tag (membranes) and 0.5 μg/ml IgG-HSV-Tagm (nuclear extracts). The top filter (P, precursor of SREBP-1) and the middle filter (C, cleaved COOH-terminal fragment) was exposed to film for 5 s. The lower filter (N, cleaved NH2-terminal nuclear fragment) was exposed to film for 3 s. Quantification of bands was done as described in Fig. 1. This experiment was repeated once with similar results.

zero time, the cells were switched from medium containing FCS to medium containing delipidated serum plus sterols in the absence or presence of arachidonate. Cells were harvested at different intervals, and SREBPs were measured by immunoblotting (panels A and B). At zero time, nSREBP-1 and nSREBP-2 were both present (lanes A, G, H, and N). At the 4- and 8-h time points after addition of sterols in delipidated serum, both nuclear proteins were markedly reduced (lanes B, C, I, and J). The nSREBP-2 remained low throughout the experiment (lanes K and L) and remained high at 16 h (lane F). The reappearance of nSREBP-1 was not detectable at 20 μm arachidonate. As expected, the TK-driven HSV-SREBP-1a(Stop 490) exhibited the properly sized nuclear form of SREBP-1a as visualized by blotting with an antibody against the epitope tag (Fig. 6A, lane G). The amount of this nuclear protein did not change when increasing amounts of arachidonate were added (lanes H and I). As expected, the TK-driven HSV-SREBP-1a(Stop 490) mRNA was not down-regulated by arachidonate (Fig. 6B, lanes P–R), even though the endogenous SREBP-1a was decreased by arachidonate under the same conditions.

As an additional test of the hypothesis that arachidonate inhibits the proteolytic processing of SREBP-1, we tested its effect on the amount of the COOH-terminal fragment that is generated when SREBP-1 is cleaved by Site-1 protease. For this purpose, we transfected HEK-293 cells with a cDNA encoding full-length SREBP-1a with a T7 epitope tag at the COOH terminus and an HSV epitope tag at the NH2 terminus. Membranes and nuclear extracts were subjected to electrophoresis and immunoblotted with antibodies against the T7 and HSV epitope tags, respectively (Fig. 7). When the cells were incubated in delipidated serum, the membranes contain-
Regulation of SREBP-1a and -1c by Unsaturated Fatty Acids

The mechanism by which unsaturated fatty acids lower SREBP-1a and -1c mRNA levels roughly in parallel in HEK-293 cells remains to be explored. These two mRNAs use different promoters that are separated by at least 10 kb (19, 32). The two transcripts contain different first exons with different 5’-untranslated regions and translation start sites. These transcripts splice into a common second exon, and thereafter they are identical. The fatty acids might act on a single transcription factor that regulates both promoters. Alternately, fatty acids might affect transcription elongation through an action on a sequence that is common to both transcripts. They might also affect the stability of the two mRNAs, as suggested for liver by Xu et al. (22). Fatty acids did not affect the level of mRNA encoded by a transfected cDNA. This transcript was driven by a different promoter than the endogenous SREBPs. It contained neither of the two 5’-untranslated regions, and it lacked all of the introns. However, it did contain nearly all of the 3’-untranslated region of the SREBP-1a and -1c transcripts (549 bp).

The likely mechanism for the protein level regulation of SREBP-1a and -1c lies in proteolytic processing. This follows from the observation that fatty acids down-regulate nSREBP-1a when it is produced from a transgene encoding the full-length precursor, but not when it encodes the truncated nuclear form of SREBP-1a. This conclusion is supported strongly by the observation that the level of the other product of SREBP cleavage, i.e. the membrane-bound COOH-terminal fragment is also decreased by arachidonic acid (Fig. 7). We do not know whether this apparent regulation of SREBP-1 cleavage by fatty acids is mediated by SCAP, which mediates the suppressive effects of sterols on SREBP cleavage. Under some conditions fatty acids have a synergistic effect with sterols (see Fig. 5), but whether this synergy is exerted by a combined effect on a single regulatory machinery remains to be determined.

The question of whether fatty acids inhibit SREBP-2 processing is not directly addressed by these studies. In sharp contrast to the reduction in mRNA levels for SREBP-1a and SREBP-1c, fatty acids did not reduce the mRNA for SREBP-2. Moreover, the content of nSREBP-2 did not decline. Yet, we consistently observed an increase in the precursor form of SREBP-2 in the cell membranes when arachidonate was added. These findings raise the possibility that unsaturated fatty acids partially inhibit SREBP-2 processing, but the reduction in nSREBP-2 is minor because the SREBP-2 mRNA remains high and the cells continue to produce relatively large amounts of the precursor form.

Whether the results in HEK-293 cells extend to other cells, most notably hepatocytes, is unknown. As detailed in the introduction, several laboratories have noted a decrease in SREBP-1 mRNA and nSREBP-1 protein when hepatocytes are incubated with various fatty acids, or when animals are fed diets rich in polyunsaturated fatty acids, but there is disagreement over which fatty acids are most effective, and the mechanism has not been explored in detail. Inasmuch as the liver is the major site of synthesis of fatty acids and inasmuch as diets rich in polyunsaturated fatty acids reduce plasma levels of cholesterol and triglycerides (1–3), the feedback regulation of SREBPs by fatty acids in liver merits further study.

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