Polyunsaturated Fatty Acids Decrease Expression of Promoters with Sterol Regulatory Elements by Decreasing Levels of Mature Sterol Regulatory Element-binding Protein*

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Membrane physiology, plasma lipid levels, and intracellular sterol homeostasis are regulated by both fatty acids and cholesterol. Sterols regulate gene expression of key enzymes of cholesterol and fatty acid metabolism through proteolysis of the sterol regulatory element-binding protein (SREBP), which binds to sterol regulatory elements (SRE) contained in promoters of these genes. We investigated the effect of fatty acids on SRE-dependent gene expression and SREBP. Consistent results were obtained in three different cell lines (HepG2, Chinese hamster ovary, and CV-1) transfected with SRE-containing promoters linked to the luciferase expression vector. We show that micromolar concentrations of oleate and other polyunsaturated fatty acids (C18:2, C22:6) dose-dependently (0.075–0.6 mmol) decreased transcription of SRE-regulated genes by 20–75%. Few or no effects were seen with saturated free fatty acids. Fatty acid effects on SRE-dependent gene expression were independent and additive to those of exogenous sterols. Oleate decreased levels of the mature sterol regulatory element-binding proteins SREBP-1 and -2 and HMG-CoA synthase mRNA. Oleate had no effect in sterol regulation defective Chinese hamster ovary cells or in cells transfected with mutant SRE-containing promoters. We hypothesize that unsaturated fatty acids increase intracellular regulatory pools of cholesterol and thus affect mature SREBP levels and expression of SRE-dependent genes.

Cholesterol and fatty acids interact in cholesterol homeosta-

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Oleate Inhibits the Transcription of SRE-containing Promoters—To determine effects of oleate on SRE-dependent gene expression, HepG2, CV-1, and CHO cells, cells differing in cell cholesterol homeostasis, were transiently transfected with a plasmid originating from hamster HMG-CoA synthase, containing three SRE linked to the luciferase reporter gene (pSyn SRE). HepG2 cells secrete lipoproteins and show high levels of cholesterol esterification through ACAT (21), CHO cells do not secrete lipoproteins but display substantial ACAT activity and storage of cholesteryl esters (22, 23). CV-1 cells do not secrete lipoproteins and display low levels of ACAT activity.2

As reported (24, 25) all three cell lines responded to incubation with sterols by repression of SRE transcription (Fig. 1). In each cell line, addition of increasing amounts of oleate caused progressive and significant \((p < 0.005)\) decreases of pSyn SRE expression (Fig. 1). Adding increasing amounts of sterols to oleate further decreased pSyn SRE expression. The effect was most marked in CV-1 cells compared with CHO and HepG2 cells. In CV-1 cells the combination of cholesterol and oleate was additive and resulted in almost complete inhibition of pSyn SRE expression (Fig. 1C).

Similar effects of oleate on SRE-mediated gene expression were observed with the rat fatty acid synthase promoter, FAS-150 (data not shown). Importantly, oleate had no detectable effect on cell growth, DNA synthesis, \(\beta\)-gal expression, or luciferase expression in luciferase control vectors (data not shown).

Oleate Decreases HMG-CoA Synthase mRNA Levels and the Mature SREBP Protein—We tested whether effects of oleate on SRE-dependent gene expression correspond to decreased mRNA levels of SRE-regulated genes and decreased levels of mature SREBP. After 24 h of incubation with oleate, HMG-CoA synthase mRNA levels in CV-1 cells were reduced to 57\%, compared with a 61\% decrease when incubated with sterols

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2 T. Seo, T. S. Worgall, and R. J. Deckelbaum, unpublished data.
FIG. 2. Oleate and cholesterol reduce HMG-CoA synthase mRNA. CV-1 cells were incubated with control medium (DMEM, 1% BSA), cholesterol (Chol, 10 μg/ml cholesterol plus 1 μg/ml 25-OH cholesterol), 0.15 mM oleate (OA), or 0.3 mM oleate for 24 h. 30 μg of total RNA were loaded per lane and electrophoresed on a 1.2% agarose/formaldehyde gel and transferred to a nylon membrane. The membrane was hybridized with a 32P-labeled probe for HMG-CoA synthase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as outlined under "Experimental Procedures." Lane 1, control conditions with medium alone; lane 2, 10 μg/ml cholesterol, 1 μg/ml 25-OH cholesterol; lane 3, 0.15 mM oleate; lane 4, 0.3 mM oleate.

(Fig. 2). Using sterols as a control, Western analysis of CV-1 cells incubated with oleate alone showed a substantial decrease in the amount of mature SREBP-1 (Fig. 3). In five separate experiments, oleate alone (0.15–0.3 mM) markedly decreased levels of SREBP-2, as did cholesterol (data not shown).

Effect of Fatty Acid Chain Length and Saturation on SRE-dependent Gene Expression—pSyn SRE transfected CV-1 cells were incubated with fatty acids of increasing chain length and unsaturation (Fig. 4). The results demonstrate that saturated fatty acids had no significant effect on reporter gene expression. However, unsaturated fatty acids caused significant (p < 0.005) down-regulation of reporter gene expression (Fig. 4), and inhibition increased with fatty acid length and number of double bonds. Because linoleate had a wider range of inhibitory effects on SRE-dependent gene expression than did oleate, we assessed effects of increasing free fatty acid (FFA) concentrations using linoleate. Fig. 4 ( inset ) shows that the effect of increases in FFA levels occurs in a graded, concentration-dependent manner.

Effects of Oleate on SRE-dependent Gene Regulation Are Not Dependent on ACAT Activity—To characterize potential interactions of ACAT and oleate with SRE-dependent gene expression, pSyn SRE-transfected CHO cells were incubated with two ACAT inhibitors, SANDOZ 58–035 and DUP 128 (gift of Dr. J. Billheimer, DuPont Merck Pharmaceutical Co.). In the absence of fatty acids ACAT inhibitors alone caused a ~40% decrease of SRE-dependent gene expression, likely reflecting higher levels of free cholesterol in cellular regulatory pools. However, in the presence or absence of ACAT inhibitors, fatty acids and cholesterol resulted in very similar decreases of pSyn SRE gene expression (data not shown). Thus, effects of oleate on SRE-dependent gene expression are not linked to different levels of ACAT activity.

Effect of Oleate in Cells with Defects in Sterol Regulation—Different SRD cells, transfected with pSyn SRE were used to determine whether the effects of fatty acids on SRE-dependent gene expression are dependent on changes in the production of mature SREBP. SRD-2 cells produce a truncated, transcriptionally active form of SREBP-2 that is not suppressed by sterols (7, 25). If oleate would inhibit binding of mature SREBPs to SRE, effects of oleate would occur in a SREBP-independent manner, reporter gene expression would diminish with oleate in these cells. Oleate had no effect on pSyn SRE expression in SRD-2 cells (Table I), suggesting that in the presence of excess mature SREBP, oleate does not inhibit SRE-dependent gene expression.

SRD-4 cells are characterized by unregulated high levels of proteolysis of precursor SREBP due to a dominant activating mutation of SREBP cleavage-activating protein and show no ACAT activity (7, 26). If oleate decreased the effects of SREBP cleavage-activating protein on SREBP cleavage, luciferase expression should decrease when incubated with oleate. Incubation with oleate with and without sterols did not affect SRE-dependent gene expression in SRD-4 cells (Table I).

A second line of evidence that the fatty acid effect is mediated by SREBP interacting with a sterol-specific binding site is provided by experiments in which CHO cells were transiently transfected with JS-15, a pSyn SRE plasmid mutated in the SRE regions resulting in insensitivity to sterols (18). Incubation of these cells with fatty acids did not down-regulate SRE expression (Table I).

**DISCUSSION**

We studied the effects of fatty acids on SREBP-dependent gene expression. The regulatory effects of sterols on genes of fatty acid metabolism via SREBPs have been shown (8, 9, 27–29). We now demonstrate that the effects of mono- and polyunsaturated fatty acids are also SREBP-mediated and result in decreased transcription of at least two SRE regulated...
promoters. Oleate alone decreases SRE-dependent gene expression; also it substantially enhances the suppressive effects of sterols. The decreased SRE expression is reflected in a decreased amount of HMG-CoA synthase mRNA as well as decreased levels of mature SREBP protein. Our experiments with mutant cell lines provide strong evidence that the effects of FFA are mediated through SREBP. FFA could not decrease SRE expression in two cell lines (SRD-2 and SRD-4) that cannot down-regulate SREBP expression as well as CHO cells transfected with a mutated SRE promoter (Table I). Thus, the effect of oleate is independent of both additive to exogenous sterols and is mediated by SREBPs.

The promoter constructs used (10, 18) differed with regard to the number of SRE elements and the coregulatory transcription factor that is required for SREBP to activate transcription (10). Thus, effects of fatty acids are not dependent on the type of coregulator or the number of SRE elements.

What are potential mechanisms whereby fatty acids modulate SREBP cleavage? Changes in membrane fluidity are not likely to play a major role because both cholesterol and FFA decrease SRE expression but have opposite effects on fluidity (14, 15). Cholesterol increases and polyunsaturated fatty acids decrease membrane fluidity (14, 15). Moreover, not only do fatty acids and cholesterol have similar effects, but they also act additively in our study.

Another potential model is that the effects of fatty acids on SRE expression are linked to increasing the intracellular cholesterol pool. The effects of oleate in the presence of cholesterol were most marked in CV-1 cells. The threshold for SREBP regulation might be reached faster in CV-1 cells by the additive effects of cholesterol and oleate due to their low capacity to esterify cholesterol. Effects of oleate were not linked to its ability to enhance ACAT activity. Studies in animal and cell models suggest that long chain fatty acids influence the distribution of sterols in regulatory intracellular cholesterol pools (2, 3). Most cell cholesterol is located in the plasma membrane (31), where free cholesterol binds to sphingomyelin with higher affinity than to other membrane phospholipids. Depletion of sphingomyelin in cultured cells blocks proteolysis of SREBP at site 1 (30). Of interest, unsaturated FFAs are activators of neutral sphingomyelinases, which decrease membrane sphingomyelin (32, 33). Thus, by decreasing sphingomyelin, fatty acids may displace membrane free cholesterol, making it available as a signal to decrease SREBP maturation.

A number of genes affecting cholesterol homeostasis and fatty acid metabolism contain the SREBP sensitive SREs in their promoter regions. Although the effects of increasing FFA on inhibiting SRE-dependent gene expression are in line with their ability to decrease fatty acid synthase enzyme activity, polyunsaturated FFAs up-regulate LDL receptor activity both in cultured cells (4) and in vivo (13). Results of Rumsey et al. (3) suggest that oleate and polyunsaturated fatty acids do not alter levels of LDL receptor mRNA. Because the promoter region of the LDL receptor gene also contains an SRE, it is likely that FFA can change LDL receptor activity through mechanisms that do not involve SREBPs, perhaps at post-translational or post-transcriptional levels.

In summary, our data showing FFA-mediated effects on SREBP and SRE-dependent gene expression provide another link between fatty acid and cholesterol metabolism.

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TABLE I

| Cell type | Control Cholesterol (10 μg/ml) plus 25-OH cholesterol (1 μg/ml) | Oleate (0.3 μm) % |
|-----------|---------------------------------------------------------------|------------------|
| SRD-2 (n = 6) | 100 ± 5 | 112 ± 18 | 126 ± 32 |
| SRD-4 (n = 6) | 100 ± 2 | 82 ± 12 | 95 ± 16 |
| CHO-K1 (n = 3) | 100 ± 14 | 140 ± 8 | 140 ± 12 |

Effect of Fatty Acids on SRE and SREBP

Oleate does not inhibit the expression of SRE-Luciferase in SRD-2 and SRD-4 cells or in CHO-K1 cells transfected with mutated pSYN-SRE.