

# Oleic acid is a potent inhibitor of fatty acid and cholesterol synthesis in C6 glioma cells

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**Abstract** Glial cells play a pivotal role in brain fatty acid metabolism and membrane biogenesis. However, the potential regulation of lipogenesis and cholesterologenesi s by fatty acids in glial cells has been barely investigated. Here, we show that physiologically relevant concentrations of various saturated, monounsaturated, and polyunsaturated fatty acids significantly reduce [<sup>1-14</sup>C]acetate incorporation into fatty acids and cholesterol in C6 cells. Oleic acid was the most effective at depressing lipogenesis and cholesterologenesi s; a decreased label incorporation into cellular palmitic, stearic, and oleic acids was detected, suggesting that an enzymatic step(s) of de novo fatty acid biosynthesis was affected. To clarify this issue, the activities of acetyl-coenzyme A carboxylase (ACC) and FAS were determined with an in situ digitonin-permeabilized cell assay after incubation of C6 cells with fatty acids. ACC activity was strongly reduced (~80%) by oleic acid, whereas no significant change in FAS activity was observed. Oleic acid also reduced the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR). The inhibition of ACC and HMGCR activities is corroborated by the decreases in ACC and HMGCR mRNA abundance and protein levels. **■** The down-regulation of ACC and HMGCR activities and expression by oleic acid could contribute to the reduced lipogenesis and cholesterologenesi s.—Natali, F., L. Siculella, S. Salvati, and G. V. Gnoni. Oleic acid is a potent inhibitor of fatty acid and cholesterol synthesis in C6 glioma cells. *J. Lipid Res.* 2007. 48: 1966–1975.

**Supplementary key words** acetyl-coenzyme A carboxylase • cholesterologenesi s • fatty acid synthase • 3-hydroxy-3-methylglutaryl coenzyme A reductase • lipogenesi s

After white adipose tissue, the brain is the organ with the highest lipid content of the body. The biosynthesis and deposition of lipids play an important role in maintaining brain structure and function, for example, during development-associated biogenesis of neural cell membranes. It is well established that alterations in lipid me-

tabolism are the cause of or are associated with many neurological diseases (1–3).

Astrocytes, the major class of glial cells in the mammalian brain, play an active role in brain metabolism. These cells surround intraparenchymal blood capillaries so that they represent the first cellular barrier for nutrients and other substances entering the brain system. A metabolic coupling between astrocytes and neurons to maintain energy metabolism homeostasis has been described (4, 5). Metabolic regulation in the brain has been investigated extensively, and those studies focused mostly on carbohydrate and amino acid metabolism (for review, see Ref. 6). During neuronal activity, glucose taken up by astrocytes is converted into lactate, which is then released into the extracellular space to be used by neurons (6). Regarding lipid metabolism, astroglial ketone body synthesis, showing characteristics strikingly similar to those of hepatic ketogenesis (7), may represent an important pathway for brain energy production and/or biosynthetic processes. The involvement of fatty acids in cell death pathways, particularly in the context of lipid-mediated apoptotic signaling, has also been described (8, 9). It has been shown that exogenous fatty acids may influence the fatty acid composition of neuronal and glial membranes (10–14), and these changes in turn can affect cellular metabolism and regulatory (15) and inflammatory processes (16). Furthermore, in primary cultures of rat astroglia, it has been shown that the addition of oleic and linoleic acids to the medium reduces several aminopeptidase activities (17).

Despite the great impact of lipid-metabolizing processes in brain development and homeostasis, the potential regulation of lipogenesis and cholesterologenesi s by fatty acids has not been studied in glial cells. Moreover, to our knowledge, only a few studies (10, 18–20) have been reported regarding lipid synthesis in brain cells. There-

Abbreviations: ACC, acetyl-coenzyme A carboxylase; HMGCR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide.

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fore, the aim of this work was to study fatty acid and cholesterol biosynthesis and their regulation by different exogenous fatty acids in glial cells. For this purpose, we used the rat C6 glioma cell line, which expresses a large repertoire of astrocyte-expressing enzymatic activities (21, 22) and exhibits a prevalent astrocyte-like phenotype when cultured in serum-rich medium (23). We found that oleic acid greatly inhibits fatty acid and cholesterol synthesis by a mechanism that involves, at least in part, the downregulation of either activity and the expression of acetyl-coenzyme A carboxylase (ACC; EC 6.4.1.2) and 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR; EC 1.1.1.34), key regulatory enzymes of lipogenesis and cholesterologenesis, respectively.

## MATERIALS AND METHODS

### Materials

Rat C6 glioma cells were from the American Type Culture Collection. DMEM, FBS, penicillin/streptomycin, PBS, and pCR 2.1 TOPO vector were from Gibco-Invitrogen, Ltd. (Paisley, UK); [ $^{14}\text{C}$ ]acetate was from GE Healthcare (Little Chalfont, UK); [ $^{14}\text{C}$ ]acetyl-CoA, [ $^3\text{H}$ ]water, [ $^{14}\text{C}$ ]HMG-CoA, and [ $^{32}\text{P}$ ]UTP were from Perkin-Elmer (Boston, MA). Brij<sub>97</sub> detergent, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), fatty acid sodium salts, and peroxidase-conjugated streptavidin were purchased from Sigma-Aldrich (St. Louis, MO). Primary antibodies for HMGCR,  $\alpha$ -tubulin, and horseradish peroxidase-conjugated IgGs were from Santa Cruz Biotechnology (Santa Cruz, CA). All other reagents (from Sigma-Aldrich) were of analytical grade.

### Cell culture

C6 cells were grown in DMEM supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. C6 cells were seeded at a density of  $5 \times 10^5$  cells per 35 mm diameter Petri dishes; 24 h after plating, the medium was changed and, after another 24 h, sodium salts of different fatty acids at 99% of purity (C18:0, stearic acid; C18:1 *cis*, oleic acid, *cis*- $\Delta^9$ -octadecaenoic acid; C18:1 *trans*, elaidic acid, *trans*- $\Delta^9$ -octadecaenoic acid; C18:2, linoleic acid, *all-cis*- $\Delta^{9,12}$ -octadecadienoic acid; C20:4, arachidonic acid, *all-cis*- $\Delta^{5,8,11,14}$ -eicosatetraenoic acid; C20:5, *all-cis*- $\Delta^{5,8,11,14,17}$ -eicosapentaenoic acid; C22:6, *all-cis*- $\Delta^{4,7,10,13,16,19}$ -docosahexaenoic acid) were added to the serum-rich (10% FBS) medium, obtaining 100  $\mu\text{M}$  final concentration, as reported by Salvati et al. (24). Unless specified otherwise, cells were in contact with the exogenous fatty acid for a total period of 4 h. In each experiment and for each determination, control dishes without any fatty acid addition were used.

### MTT assay

An MTT assay was used for the quantification of metabolically active, living cells. C6 cells were plated at a density of  $0.5 \times 10^4$  cells/well in a 96-well culture dish. After 24 h, the serum-rich medium was refreshed, and after another 24 h, cells were incubated for 4 h with the indicated fatty acid sodium salts. Then, cell monolayers were incubated for 3 h with 1 mg/ml MTT. Mitochondria of living cells convert the yellow tetrazolium compound to its purple formazan derivative. After removal of the unconverted MTT, the formazan product was dissolved in isopropanol and the absorbance of formazan dye was measured at

450 nm. Viability was calculated as percentage of absorbance relative to control cells.

### Determination of the rate of fatty acid and cholesterol synthesis

Lipogenic activity was determined by monitoring the incorporation of [ $^{14}\text{C}$ ]acetate (16 mM, 0.96 mCi/mol) or [ $^3\text{H}$ ]water (5 mCi/ml) into fatty acids and cholesterol essentially as reported (25). Labeled substrate was added 1 h before ending the experiment.

To terminate the lipogenic assay, the medium was aspirated, cells were washed three times with ice-cold 0.14 M KCl to remove unreacted labeled substrate, and the reaction was stopped with 1.5 ml of 0.5 M NaOH. The cells were scraped off with a rubber policeman, transferred to a test tube, saving 100  $\mu\text{l}$  for protein assay (26), and saponified with 4 ml of ethanol and 2 ml of double-distilled water for 90 min at 90°C. Unsaponifiable sterols and, after acidification with 1 ml of 7 M HCl, fatty acids were extracted with  $3 \times 5$  ml of petroleum ether. The extracts were collected, dried under a stream of nitrogen, and counted for radioactivity.

### Incorporation of radiolabeled acetate into lipid fractions

Because newly synthesized labeled fatty acids are incorporated mainly into complex lipids, phospholipid analysis was carried out. Experimental conditions were the same as those reported for fatty acid and cholesterol synthesis assays. At the end of the incubation period, the reaction was blocked by washing the cells three times with ice-cold 0.14 M KCl and treated with 2 ml of KCl/CH<sub>3</sub>OH (1:2, v/v); total lipids were extracted according to Giudetti et al. (27).

Phospholipids were resolved by TLC on silica gel plates using CHCl<sub>3</sub>/CH<sub>3</sub>OH/28% NH<sub>4</sub>OH (65:25:4) as a developing system (28). Lipid spots were visualized by placing the plate in a tank saturated with iodine vapor. The areas corresponding to the individual lipid classes were marked and scraped individually into counting vials for radioactivity measurement.

### HPLC analysis of newly synthesized radiolabeled fatty acids

Total extracted fatty acids were separated by HPLC using a modification of the method described by Mehta, Oeser, and Carlson (29). Briefly, fatty acid extract, obtained from six Petri dishes as described above, was resuspended in 100  $\mu\text{l}$  of  $\alpha$ -bromoacetophenone (15 mg/ml in acetone) and 100  $\mu\text{l}$  of triethylamine (25 mg/ml in acetone). Samples were put into a boiling-water bath for 15 min and then cooled at room temperature. A total of 150  $\mu\text{l}$  of acetic acid (10 mg/ml in acetone) was added to the sample, which was heated again for 5 min, dried under a stream of nitrogen, resuspended in 40  $\mu\text{l}$  of acetonitrile, and centrifuged for a few seconds. For HPLC analysis, 20  $\mu\text{l}$  of each sample was injected into a Beckman System Gold chromatograph equipped with a C18 ODS column (4.6  $\times$  250 mm). The chromatographic system was programmed for elution using two mobile phases: solvent A, acetonitrile-water (4:1) and solvent B, acetonitrile. Solvent A ran for 45 min, and then solvent B ran for 15 min. The flow rate was 2 ml/min, and detection was at 242 nm. Eluted fractions, corresponding to the different fatty acids, were collected for radioactivity measurement.

### Assay of de novo fatty acid synthesis enzymatic activities

A procedure that allows one to assay directly in situ the activities of the lipogenic enzymes, ACC and FAS (EC 2.3.1.85),

was set up. To this end, after incubation with exogenous fatty acids, culture medium was removed and C6 glioma cells were permeabilized using 400  $\mu$ l of assay mixture containing digitonin (400  $\mu$ g/ml). The reaction mixture was prepared within 15 min before use by mixing a known amount of digitonin, dissolved in an EGTA stock solution by heating in a boiling-water bath, with the other components of the assay mixture. The assay mixture did not contain any exogenous fatty acid.

Because ACC and FAS are cytosolic enzymes and they leak from permeabilized cells at the digitonin concentration used, cell permeabilization and ACC and FAS assays were carried out simultaneously (30). ACC activity was determined as the incorporation of radiolabeled acetyl-CoA into fatty acids in a reaction coupled with that catalyzed by FAS, essentially as described by Bijleveld and Geelen (31) in isolated rat hepatocytes. This method avoids a number of interferences associated with the classical bicarbonate fixation assay of ACC activity (30).

The ACC assay reaction mixture contained 100 mM HEPES (pH 7.9), 4.2 mM  $MgCl_2$ , 1 mM citrate, 5 mM EGTA, 20 mM  $KHCO_3$ , 20.5 mM NaCl, 4 mM ATP, 1 mM NADPH, 0.44 mM dithioerythritol, 0.85% (w/v) BSA, 800  $\mu$ g/ml digitonin, 0.12 mM [ $^{14}C$ ]acetyl-CoA (0.5  $\mu$ Ci/ml), 0.12 mM butyryl-CoA, and 3 mU of purified FAS (before use, FAS was preincubated for 30 min at room temperature with 12.5 mM dithioerythritol). FAS was purified from rat liver according to Linn (32) and stored at  $-80^\circ C$ . The assay mixture was diluted 1:1 in culture medium, and 400  $\mu$ l of this solution was added to the plates that were incubated at  $37^\circ C$  for 8 min.

FAS activity was assayed in permeabilized cells essentially as reported (30). The incubation time was 10 min at  $37^\circ C$ . The lipogenic assays were stopped by the addition of 100  $\mu$ l of 10 M NaOH.

Thereafter, cells were scraped off with a rubber policeman and transferred to a test tube. Plates were washed twice with 450  $\mu$ l of 0.5 M NaOH, and these washing solutions were collected into the same tubes. One drop of phenol red and 5 ml of  $CH_3OH$  were added, and the samples were saponified by boiling for 45–60 min in capped tubes. After cooling and acidification with 200  $\mu$ l of 12 M HCl, fatty acids were extracted three times with 4 ml of petroleum ether each time. The combined petroleum ether extracts were evaporated to dryness. Residua were dissolved in scintillation fluid and counted for radioactivity.

The activities of ACC and FAS are expressed as nanomoles of [ $^{14}C$ ]acetyl-CoA incorporated into fatty acids per minute per milligram of protein.

### HMGCR activity assay

The HMGCR activity assay was performed essentially as described by Volpe and Hennessy (20). Briefly, C6 cells were seeded at a density of  $2 \times 10^6$  cells per 100 mm diameter Petri dish. At 24 h after plating, medium was changed and, after another 24 h, exogenous fatty acid sodium salt was added to the serum-rich (10% FBS) medium for 4 h. Afterward, the medium from each Petri dish was discarded and the cells were washed twice with 4 ml of ice-cold PBS. Cells were scraped with a rubber policeman into 1 ml of buffer containing 0.05 M Tris-HCl (pH 7.4) and 0.15 M NaCl. After centrifugation (900 g, 3 min, room temperature), the pellet was frozen once in liquid nitrogen and kept at  $-80^\circ C$  until use.

Cell extracts were prepared by dissolving the thawed pellet of C6 cells in 0.2 ml of buffer containing 50 mM  $K_2HPO_4$  (pH 7.5), 5 mM DTT, 1 mM EDTA, and 0.25% Brij<sub>97</sub>. A total of 100–250  $\mu$ g of protein from cell extract was preincubated for 10 min at  $37^\circ C$  in a total volume of 0.2 ml containing 0.1 M  $K_2HPO_4$  (pH 7.5), 5 mM DTT, and 2.5 mM NADPH. The reaction was started by

the addition of [ $^{14}C$ ]HMG-CoA (75  $\mu$ M, 1.8 Ci/mol). After incubation at  $37^\circ C$  for 120 min, the reaction was stopped by the addition of 20  $\mu$ l of 7 M HCl. Conversion to mevalonolactone was carried out with an additional 60 min incubation at  $37^\circ C$ , and the radioactive product was isolated by TLC using toluene-acetone (1:1) as the mobile phase. Silica spots were recovered and subjected to scintillation counting.

### Probe design for the RNase protection assay

Three fragments of ACC, FAS, and HMGCR cDNA were amplified by reverse transcriptase polymerase chain reaction, as reported by Siculella et al. (33), using rat liver total RNA as the template and the following primers: F1, 5'-GTCATGCCTC-CGAGAACC-3', and R1, 5'-GCCAATCCACTCGAAGACC-3' (National Center for Biotechnology Information accession number J03808), for the ACC probe; F2, 5'-TTGCCCGAGTCAGAGAAC-3', and R2, 5'-CGTCCACAATAGCTTCATAGC-3' (accession number M76767), for the FAS probe; and F3, 5'-CTCACAGGAT-GAAGTAAGGG-3', and R3, 5'-CTGAGCTGCCAAATTGGACG-3' (accession number NM\_013134), for the HMGCR probe.

The amplified products (180, 197, and 244 bp for the ACC, FAS, and HMGCR probes, respectively) were subcloned into pCR 2.1 TOPO vector, and their identities were verified by sequence analysis. After linearization, the recombinant plasmids were used in the in vitro transcription reactions.

### RNase protection assay

Antisense RNAs were synthesized by an in vitro transcription reaction as reported (33). Nuclear RNA (25  $\mu$ g) isolated from  $\sim 5 \times 10^6$  C6 cells, as described by Chomczynski and Sacchi (34), was hybridized with  $2 \times 10^5$  cpm of  $^{32}P$ -labeled specific antisense probe in 20  $\mu$ l of hybridization reaction at  $50^\circ C$  for 16 h. For the normalization, a  $\beta$ -actin antisense  $^{32}P$ -labeled RNA probe was added in each hybridization reaction. Probes were also hybridized with 10  $\mu$ g of yeast RNA used as a control to test the RNase activity (data not shown). After digestion with RNase A/T1, the protected fragments were separated onto a 6% denaturing polyacrylamide gel. Gels were dried and exposed for radiography, and the intensity of the bands was evaluated by densitometry with Molecular Analyst software.

### Western blot analysis

Cells grown in six-well dishes were treated with C18:1 *cis* as indicated above and lysed with a pH 7.5 buffer containing 50 mM HEPES, 250 mM mannitol, 10 mM citrate, 4 mM  $MgCl_2$ , 20 mM Tris-HCl, 500 mM NaCl, 0.5  $\mu$ M PMSF, 0.05% Tween 20, 0.5%  $\beta$ -mercaptoethanol, and protease inhibitors. The extracts were heat-denatured for 5 min, and samples containing an equal amount of total protein (25  $\mu$ g) were loaded on 7% SDS-polyacrylamide gels. After electrophoresis, the proteins were transferred onto a nitrocellulose membrane (35). To detect biotinylated ACC, the blot was incubated with peroxidase-conjugated streptavidin at a dilution of 1:4,000 at room temperature for 2 h. To detect HMGCR, the blot was first incubated with HMGCR antibody (dilution, 1:400) for 1 h at room temperature and then for 1 h with donkey anti-goat horseradish peroxidase-conjugated IgG (dilution, 1:5,000). Signals were detected by enhanced chemiluminescence. For signal normalization,  $\alpha$ -tubulin detection was used (9).

### Statistical analysis

Results shown represent means  $\pm$  SD of the number of experiments indicated in every case. In each experiment, determinations were carried out in triplicate. Statistical analysis was

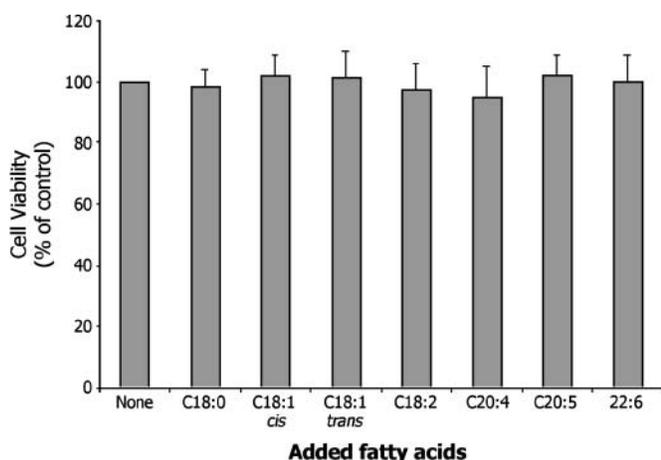
performed with Student's *t*-test. Differences were considered statistically significant at  $P < 0.05$ .

## RESULTS

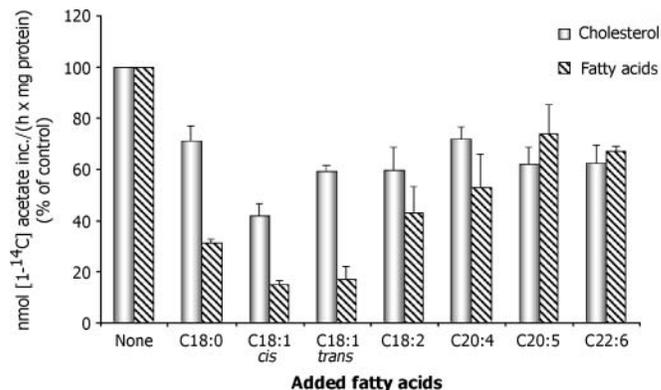
### Effect of exogenously added fatty acids on fatty acids and cholesterol synthesis

After plating, exogenous fatty acids (100  $\mu\text{M}$ ) were added to C6 glioma cells and the cultures were incubated, unless specified otherwise, for 4 h. MTT test (Fig. 1), morphological observation, protein assay, and Trypan Blue exclusion showed that treated cells had the same viability as control cells during the experimental period, thus excluding a nonspecific toxic cellular effect of the added fatty acids.

Radiolabeled acetate was added 1 h before ending the experiment, and its incorporation into fatty acids and cholesterol was monitored. Because acetyl-CoA is a precursor for both fatty acid and cholesterol synthesis, the capability of C6 glioma cells to incorporate acetate into these lipid fractions was measured. In the absence of exogenous fatty acids, a significant activity of cholesterologenesis ( $1.01 \pm 0.04$  nmol [ $1\text{-}^{14}\text{C}$ ]acetate incorporated/h/mg protein) and fatty acid biosynthesis ( $8.36 \pm 0.43$  nmol [ $1\text{-}^{14}\text{C}$ ]acetate incorporated/h/mg protein) was observed. After fatty acid addition to the cells, a general decrease in [ $1\text{-}^{14}\text{C}$ ]acetate incorporation into total fatty acids as well as into cholesterol was detected (Fig. 2). Both C18:1 *cis* and *trans* isomers showed the greatest inhibitory effect (80%) of [ $1\text{-}^{14}\text{C}$ ]acetate incorporation into fatty acids, whereas cholesterol synthesis was inhibited by  $\sim 60\%$ , mainly by the C18:1 *cis* isomer. A smaller reduction of labeled acetate incorporation into both fatty acids and cholesterol was observed by incubating C6 cells with 100  $\mu\text{M}$  PUFAs (i.e., fatty acids from C18:2 to C22:6). The



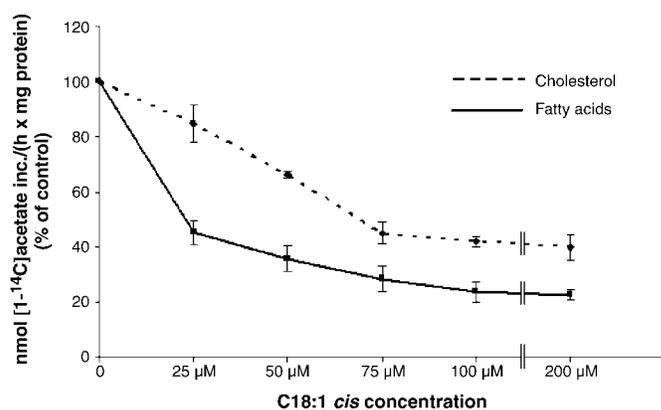
**Fig. 1.** Effects of exogenous fatty acids on C6 cell viability. C6 cells were incubated for 4 h with 100  $\mu\text{M}$  of different exogenous fatty acids in serum-rich medium. Cell viability was estimated by a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay. Values, expressed as percentage of control, are means  $\pm$  SD of five experiments.



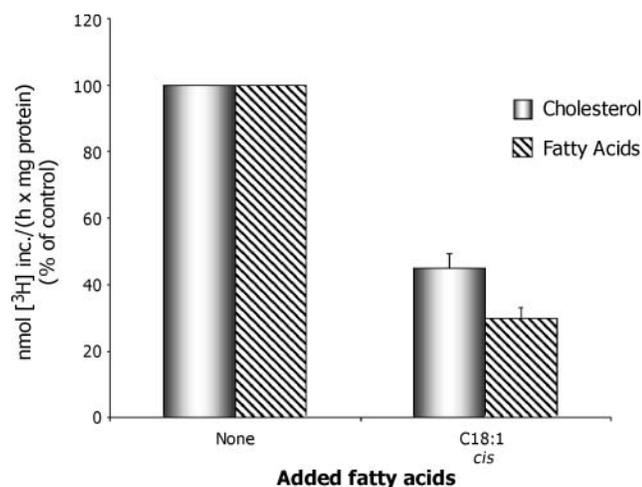
**Fig. 2.** Effects of exogenous fatty acids on cholesterol and fatty acid synthesis in C6 glioma cells. After an initial 48 h plating period, C6 glioma cells, growing in serum-rich medium, were incubated for 4 h with 100  $\mu\text{M}$  of different exogenous fatty acids. At 3 h, labeled acetate was added and its incorporation into cholesterol and fatty acids was followed. Values, expressed as percentage of control (no addition), are means  $\pm$  SD of six independent experiments. In each experiment, determinations were carried out in triplicate. Control rates of cholesterol and fatty acid synthesis were  $1.01 \pm 0.04$  and  $8.36 \pm 0.44$  nmol [ $1\text{-}^{14}\text{C}$ ]acetate incorporated/h/mg protein, respectively.

saturated fatty acid C18:0 showed a reducing effect more pronounced on fatty acid synthesis than on cholesterologenesis. As the C18:1 *cis* isomer showed the greatest inhibitory effect, only this fatty acid was tested in the next experiments.

The effect exerted by C18:1 *cis* was dose-dependent (Fig. 3). A gradual decrease of lipogenic activity was found. The reduction of fatty acid and cholesterol synthesis was already evident at 25  $\mu\text{M}$  C18:1 *cis*. At this concentration, labeled acetate incorporation into cholesterol and total



**Fig. 3.** Dose-dependent effects of C18:1 *cis* on cholesterol and fatty acid synthesis. C6 cells were incubated with increasing oleic acid concentrations for 4 h in serum-rich medium. After 3 h, labeled acetate was added, and 1 h later its incorporation into cholesterol and fatty acids was stopped. Values, expressed as percentage of control, are means  $\pm$  SD of six experiments. For control rates of cholesterol and fatty acid synthesis, see the legend to Fig. 2.



**Fig. 4.** Effects of C18:1 *cis* on  $^3\text{H}$  incorporation into cholesterol and fatty acids in C6 cells. After an initial 48 h plating period, C6 glioma cells, growing in serum-rich medium, were incubated for 4 h with 100  $\mu\text{M}$  C18:1 *cis* fatty acids. At 3 h, [ $^3\text{H}$ ]water was added and its incorporation into cholesterol and fatty acids was followed. Values, expressed as percentage of control (no addition), are means  $\pm$  SD of three independent experiments. In each experiment, determinations were carried out in triplicate. Control rates of cholesterol and fatty acid synthesis were  $3.29 \pm 0.30$  and  $9.78 \pm 0.78$  nmol incorporated/h/mg protein, respectively.

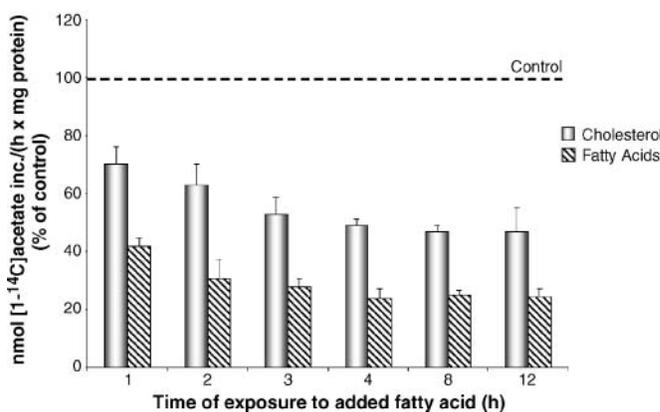
fatty acids was reduced by  $\sim 15\%$  and  $55\%$ , respectively. Maximum inhibitory effect was observed at 100  $\mu\text{M}$  C18:1 *cis*, at which cholesterol and fatty acid biosynthesis were reduced by  $\sim 60\%$  and  $80\%$ , respectively. No significant changes were observed at higher C18:1 *cis* concentrations.

Similar behavior was shown when [ $^3\text{H}$ ]water, instead of labeled acetate, was used as an independent index of lipogenic activity (36) (**Fig. 4**). At 100  $\mu\text{M}$  C18:1 *cis* concentration, tritium incorporation into cholesterol was reduced by  $\sim 55\%$ , whereas label incorporation into fatty acids was inhibited by  $\sim 70\%$ .

The time-dependent effect reported in **Fig. 5** shows that 100  $\mu\text{M}$  oleic acid addition inhibited lipogenesis already after 1 h of C6 cell incubation, reducing the incorporation of labeled acetate into fatty acids and into cholesterol by  $\sim 60\%$  and  $30\%$ , respectively, compared with the control. Maximum reduction of fatty acid ( $\sim 75\%$ ) and cholesterol ( $\sim 50\%$ ) synthesis was observed at 4 h of C18:1 *cis* addition. Incubation for longer than 4 h did not produce any further decrease.

#### Effect of C18:1 *cis* on radiolabeled acetate incorporation into phospholipids

The effect of C18:1 *cis* addition to C6 cells on [ $^{14}\text{C}$ ]acetate incorporation into phospholipid fractions and neutral lipids was tested (**Fig. 6**). A general decrease of labeled precursor incorporation into all phospholipids, and in particular into phosphatidylcholine, the most abundant phospholipid in C6 glioma cells, was observed. Interestingly, no significant change in the incorporation of labeled acetate into neutral lipids was detected after C18:1 *cis* addition to the cells.



**Fig. 5.** Time-dependent effects of C18:1 *cis* on cholesterol and fatty acid synthesis. [ $^{14}\text{C}$ ]acetate was added at 0, 1, 2, 3, 7, or 11 h, and 1 h later the lipogenic assay was terminated. Cells, growing in serum-rich medium, were in contact with 100  $\mu\text{M}$  C18:1 *cis* for the indicated times. Rates of cholesterol and fatty acid synthesis in the presence of C18:1 *cis* are expressed as percentage of control. Each result is the mean of six experiments  $\pm$  SD. For control rates of cholesterol and fatty acid synthesis, see the legend to **Fig. 2**.

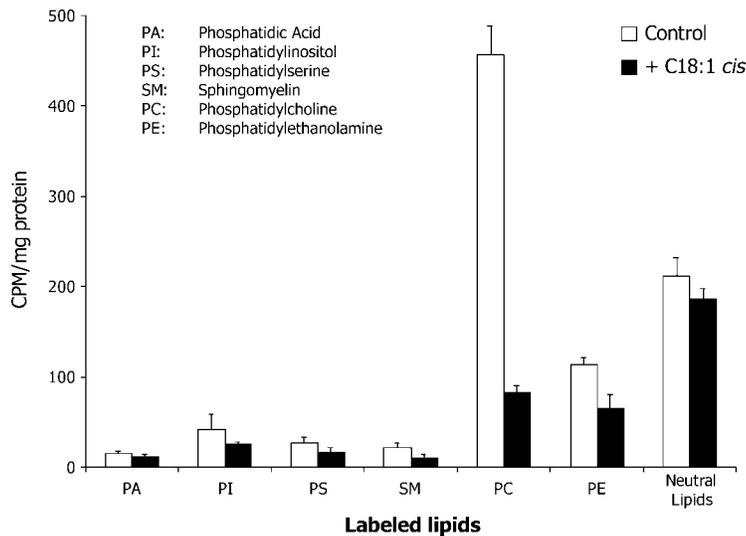
#### Analysis of newly synthesized radiolabeled fatty acids

To investigate the effect of C18:1 *cis* addition to C6 cells on the individual fatty acids synthesized from labeled acetate, an HPLC analysis of a total fatty acid extract was carried out. Moreover, in these experiments, a comparison was also made with a saturated fatty acid (C18:0) or a PUFA (C20:4) added to the cells.

The results in **Fig. 7** show that in the control dishes, palmitic acid (C16:0) was the most prominent newly synthesized fatty acid; a noticeable label incorporation into stearic acid and oleic acid was also observed. A relatively small incorporation of label into PUFAs was found, in accordance with the fact that these fatty acids are hardly present in C6 cells (37). However, exogenous C18:1 *cis* showed the greatest inhibitory effect on [ $^{14}\text{C}$ ]acetate incorporation into individual fatty acids. The decrease of radiolabel incorporation was particularly evident with regard to C16:0, which in the cell is the main product of de novo fatty acid biosynthesis, as well as C18:0 and C18:1 *cis*.

#### ACC, FAS, and HMGCR activity modulation by saturated, monounsaturated, and polyunsaturated fatty acids

On the basis of the data reported in **Fig. 7**, the enzymatic activities of de novo fatty acid synthesis (i.e., ACC and FAS) were investigated with an in situ assay using digitonin-permeabilized C6 cells. This tool offers the advantage of a rapid measurement of intracellular enzyme activities in a more or less natural environment, thus reducing the necessity of preparing cellular fractions for enzyme assays (30). In a first series of experiments (data not shown), the optimal concentration of digitonin and the time of exposure to digitonin necessary to permeabilize the C6 plasma membrane, without affecting subcellular organelle integrity, were determined. HMGCR activity was investigated by following labeled mevalonolactone synthesis (20).



**Fig. 6.** Effects of C18:1 *cis* on [ $^{14}\text{C}$ ]acetate incorporation into various lipid fractions in C6 cells. C6 cells, growing in serum-rich medium, were incubated with 100  $\mu\text{M}$  oleic acid for a total period of 4 h, and labeled acetate was added 1 h before ending the incubation. Then, cells were washed and total lipids were extracted. Phospholipids were resolved by TLC, and the radioactivity associated with the different lipid fractions was counted. Values are means  $\pm$  SD of five experiments.

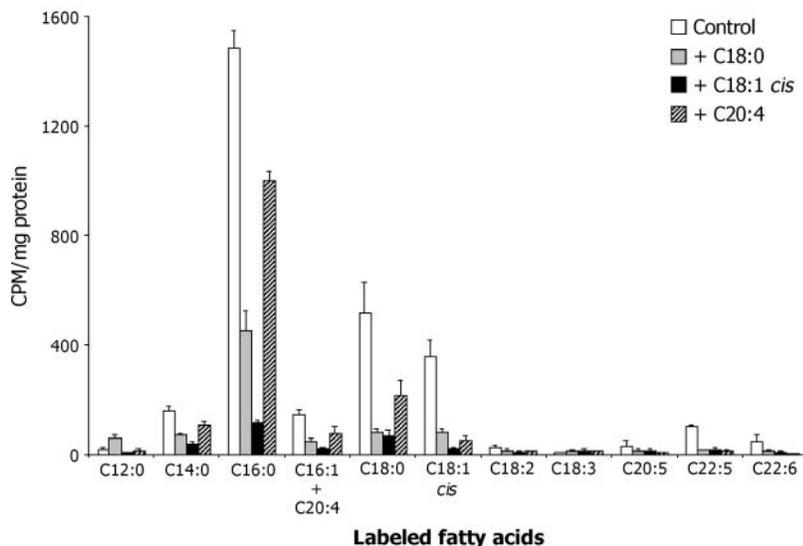
The comparative effects of 100  $\mu\text{M}$  C18:0, C18:1 *cis*, or C20:4 on ACC, FAS, and HMGCR activities were then investigated in C6 glioma cells. FAS activity was not affected by any fatty acid addition, whereas ACC activity was reduced ( $\sim 50\%$ ) by C20:4 and ( $\sim 80\%$ ) by C18:0 and C18:1 *cis* addition to the cells (**Fig. 8A**). Moreover, stearic and arachidonic acids similarly decreased HMGCR activity ( $\sim 20\%$ ), whereas oleic acid showed the strongest inhibitory effect on this enzyme ( $\sim 45\%$ ) (Fig. 8A).

To deepen the oleic acid effect, we next tested two oleic acid concentrations on ACC, FAS, and HMGCR activities. We found that the addition of either 25 or 100  $\mu\text{M}$  C18:1 *cis* to C6 cells for 4 h decreased ACC activity by  $\sim 40\%$  and 70%, respectively (Fig. 8B). Again, FAS activity was not affected by any tested C18:1 *cis* concentration. Finally, in good agreement with the data reported in Figs. 2 and 3 on [ $^{14}\text{C}$ ]acetate incorporation into cholesterol, we found that the addition of either 25 or 100  $\mu\text{M}$  C18:1 *cis* to C6 cells decreased HMGCR activity by  $\sim 20\%$  and 45%, respectively.

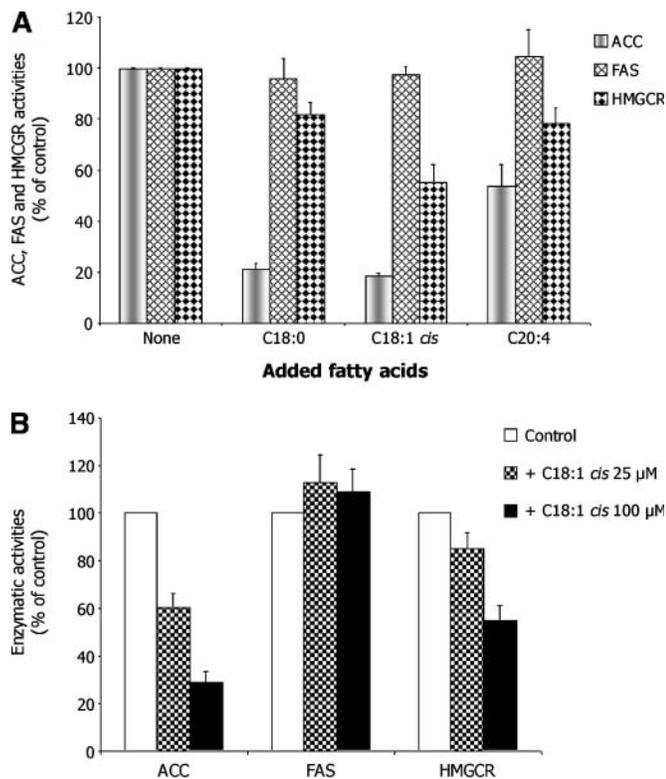
### Effects of C18:1 *cis* on mRNA abundance and protein levels of ACC, FAS, and HMGCR

To investigate the molecular mechanism responsible for the regulation of lipogenic activity by C18:1 *cis*, the levels of mRNA encoding for ACC and FAS were measured by RNase protection assay. Using ACC and HMGCR probes, we found that the amount of protected ACC and HMGCR RNAs was decreased by  $\sim 55\%$  and 35%, respectively, in C6 cells upon 100  $\mu\text{M}$  C18:1 *cis* treatment (**Fig. 9A**). The reduction of both ACC and HMGCR mRNA abundances was already significant at 25  $\mu\text{M}$  C18:1 *cis*. By contrast, no significant change in the abundance of FAS mRNA was observed. The amount of  $\beta$ -actin mRNA, used for normalization, was unmodified.

Furthermore, after 4 h of 100  $\mu\text{M}$  C18:1 *cis* supplementation, the ACC and HMGCR protein contents, quantified by Western blot analysis, decreased ( $\sim 50\%$  and 40%, respectively) in treated cells compared with controls (Fig. 9B). Significant reductions of ACC and HMGCR protein levels were observed also at 25  $\mu\text{M}$  C18:1 *cis*.



**Fig. 7.** Effects of different exogenous fatty acids on [ $^{14}\text{C}$ ]acetate incorporation into individual fatty acids. The effects of 100  $\mu\text{M}$  C18:0, C18:1 *cis*, and C20:4 on the incorporation of labeled acetate into different fatty acids were assayed. The radiolabeled neosynthesized fatty acids were separated by HPLC. Eluted fractions, corresponding to the different fatty acids, were collected for radioactivity measurement. Values are means  $\pm$  SD of six experiments.



**Fig. 8.** Effects of saturated, monounsaturated, and polyunsaturated fatty acids on acetyl-coenzyme A carboxylase (ACC), FAS, and 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) activities. Acetyl-CoA carboxylase and fatty acid synthase activities were tested with an *in situ* assay in digitonin-permeabilized C6 cells. HMGCR activity was studied by following labeled mevalonolactone synthesis. **A:** C6 cells were incubated with 100  $\mu$ M C18:0, C18:1 *cis*, and C20:4 for a total period of 4 h in serum-rich medium. After the incubation with exogenous fatty acids, culture medium was removed and, for ACC and FAS assay, C6 glioma cells were permeabilized using 400  $\mu$ l of assay mixture containing digitonin (400  $\mu$ g/ml) and no exogenous fatty acid. Values, expressed as percentage of the control C6 cells, are means  $\pm$  SD of five experiments. In each experiment, determinations were carried out in triplicate. **B:** C6 cells, growing in serum-rich medium, were incubated with either 25 or 100  $\mu$ M oleic acid for a total period of 4 h, as above. Values, expressed as percentage of control, are means  $\pm$  SD of four independent experiments. Control specific activities were as follows: for ACC,  $0.152 \pm 0.005$  nmol [ $^{14}$ C]acetyl-CoA incorporated/min/mg protein; for FAS,  $0.041 \pm 0.002$  nmol [ $^{14}$ C]acetyl-CoA incorporated/min/mg protein; for HMGCR,  $38.6 \pm 2.4$  pmol mevalonolactone formed/min/mg protein.

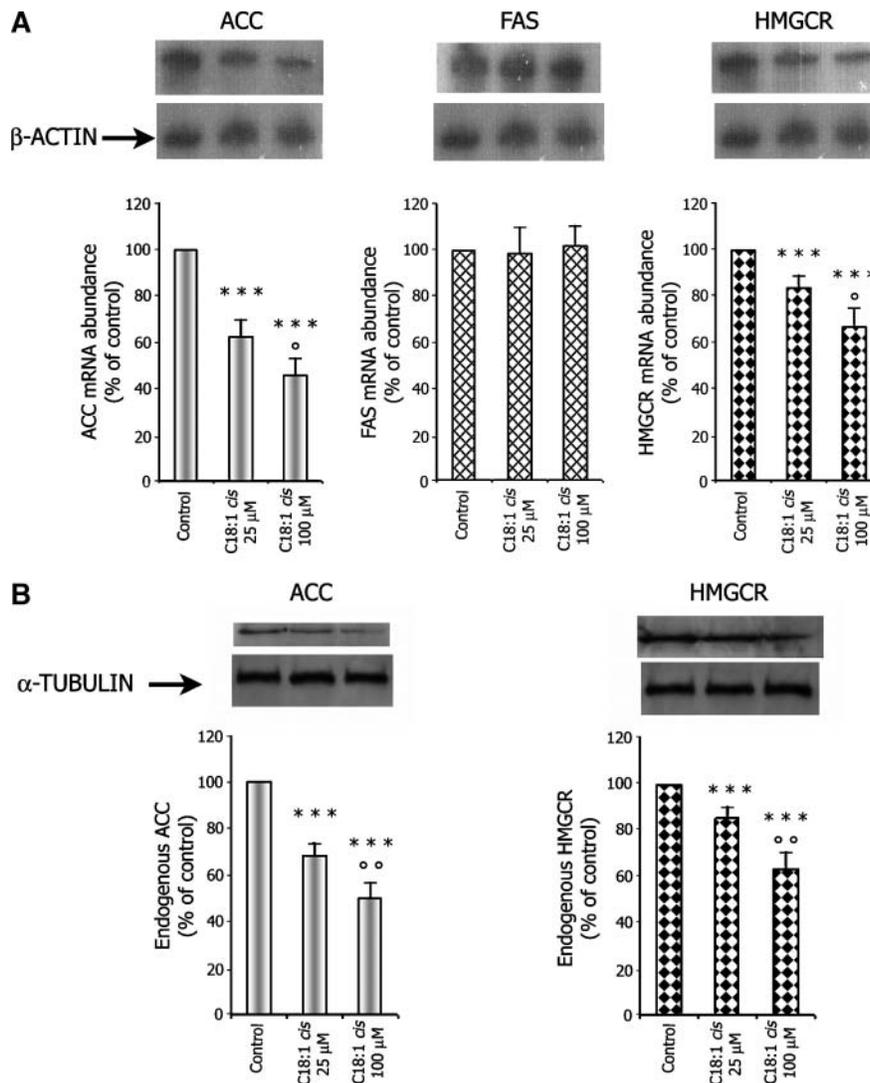
## DISCUSSION

Studies from a number of research groups have established that variations in dietary fatty acid levels are able to change the pattern of fatty acyl moieties as well as the cholesterol content of neuronal and glial membranes. These changes, in turn, can influence cellular metabolism and regulatory processes (38–40). It has also been reported that cultured astrocytes take up exogenous linoleic acid (C18:2) and incorporate its metabolites into phospholipids, modifying their membrane fatty acid compo-

sition and certain specific cell properties (38). These biochemical changes can be accompanied by alterations in the physical state of the membrane, for example in membrane fluidity (39). Moreover, the addition of C18:1 *cis* and C18:2 to cultured astrocytes decreases several aminopeptidase activities (17). Furthermore, it has been shown that C18:1 *cis* addition determines a dose-dependent inhibition of GAP junction permeability in cultured rat astrocytes. The authors suggested that C18:1 *cis* may play an important role in the transduction pathway leading to the inhibition of intracellular communication (41).

However, despite the crucial role of fatty acids in brain function and metabolism, little is known about the effect of exogenous fatty acids on lipogenesis in brain cells (18, 19). Therefore, the two major aims in this study were as follows: *i*) to determine the capability of glioma cells to synthesize different lipid fractions, starting from labeled acetate; and *ii*) to investigate the effect of exogenous fatty acids on fatty acid and cholesterol biosynthesis in these cells. We show that both fatty acid and cholesterol biosynthesis are rather active in cultured glioma cells when [ $^{14}$ C]acetate is used as the common precursor for both metabolic pathways. In fact, in control cells (i.e., in the absence of any addition to the culture medium), specific activities of  $8.36 \pm 0.43$  nmol [ $^{14}$ C]acetate incorporated into fatty acids/h/mg protein and  $1.01 \pm 0.04$  nmol [ $^{14}$ C]acetate incorporated into cholesterol/h/mg protein were found. It must be emphasized that these values are much higher than those observed previously, under similar experimental conditions, in cultured hepatocytes with regard to both fatty acid synthesis and cholesterologenesis (25, 42). Our findings on lipid biosynthesis add further support to previous studies showing very active *de novo* fatty acid and cholesterol synthesis in human malignant glial cells compared with their normal counterparts (43–45).

A general decrease of fatty acid synthesis by exogenous fatty acids was observed; the reduction was particularly pronounced when the C18:1 *cis* or C18:1 *trans* isomer was added to the culture medium. Overall, the reduction of cholesterologenesis by exogenous fatty acids was often less pronounced, compared with fatty acid synthesis, especially in the case of C18:1 *trans*. It might be argued that the observed reduction of label incorporation into fatty acids and cholesterol after fatty acid addition could be attributable to the precursor-dilution effect by exogenous fatty acids.  $\beta$ -Oxidation of fatty acids and consequently the generation of acetyl-CoA might dilute the [ $^{14}$ C]acetyl-CoA pool derived from [ $^{14}$ C]acetate and may lead to a lower apparent synthesis rate of  $^{14}$ C-labeled fatty acids and [ $^{14}$ C]cholesterol. To circumvent this problem, experiments were carried out using [ $^3$ H]water incorporation into lipids as an independent index of lipogenic activity (36). The rate of tritium incorporation into fatty acids and cholesterol was reduced by exogenous fatty acids in a manner similar to that measured by the incorporation of [ $^{14}$ C]acetate. Thus, the occurrence of a dilution effect is unlikely.



**Fig. 9.** Effects of C18:1 *cis* on ACC, FAS, and HMGCR mRNA accumulation and protein content in C6 glioma cells. C6 cells were incubated with either 25 or 100  $\mu$ M oleic acid for a total period of 4 h in serum-rich medium. **A:** Approximately 25  $\mu$ g of nuclear RNA from  $\sim 5 \times 10^6$  C6 cells was analyzed by RNase Protection Assay and hybridized with  $^{32}$ P-labeled specific antisense probes for ACC, FAS, and HMGCR. For normalization, a  $\beta$ -actin antisense  $^{32}$ P-labeled riboprobe was added in each hybridization reaction. The protected fragments were separated onto a denaturing polyacrylamide gel. The radiolabeled gel was dried and exposed to X-ray film, and the intensity of the resulting bands was evaluated by densitometry with Molecular Analyst software. **B:** ACC from control C6 cells or from C18:1 *cis*-treated C6 cells was immunodecorated with peroxidase-conjugated streptavidin and revealed by enhanced chemiluminescence. HMGCR was immunodecorated with specific primary antibody and horseradish peroxidase-conjugated IgG and revealed by enhanced chemiluminescence. The contents of ACC, HMGCR, and  $\alpha$ -tubulin, used as a control, were quantified by photodensitometric analysis. Control, control cells; C18:1 25  $\mu$ M, C6 cells incubated for 4 h with 25  $\mu$ M C18:1 *cis*; C18:1 100  $\mu$ M, C6 cells incubated for 4 h with 100  $\mu$ M C18:1 *cis*. For treatment with 25 or 100  $\mu$ M C18:1 *cis* versus control, \*\*\*  $P < 0.001$ ; for treatment with 100  $\mu$ M C18:1 *cis* versus 25  $\mu$ M C18:1 *cis*, °  $P < 0.01$ , °°  $P < 0.001$ .

Of importance, the reduction of fatty acid synthesis by C18:1 *cis* addition to C6 glioma cells reached a maximum (80%) at 4 h, but it was already evident within 1 h of incubation, thus indicating that short-term regulation may cooperate with long-term mechanisms in the reduction of lipogenesis by exogenous fatty acids in C6 cells (18).

De novo fatty acid synthesis is catalyzed by two enzymatic systems functioning in sequence: ACC (generally consid-

ered the rate-determining step of this metabolic pathway), which leads to the production of malonyl-CoA, and FAS, leading to the formation of the end product C16:0. Interestingly, the results in Fig. 8A showing the inhibition of ACC activity by exogenous fatty acids are in good agreement with those reported in Fig. 2 regarding total fatty acid synthesis. Therefore, the global inhibitory effect we observed most likely can be ascribed to the rapid inhibi-

tion of ACC activity (in which allosteric regulation and phosphorylation-dephosphorylation of the enzyme could participate) and the downregulation of ACC expression exerted by C18:1 *cis* treatment. Volpe and Marasa (21) also found that in C6 cells, ACC activity was downregulated (within seconds to minutes) by exogenous long-chain acyl-CoAs, whereas no significant change of FAS activity was observed. However, unlike us, they did not find any changes in either RNA and protein synthesis involved in the mechanism of ACC regulation. The very short incubation time they used to investigate the molecular mechanism underlying ACC regulation may explain this discrepancy.

Lastly, because ACC is not required for cholesterol synthesis, the possibility that supplemented fatty acids could impair some enzymatic step(s) directly connected with this pathway, such as HMGCR, was considered. To this end, HMGCR activity, as well as the corresponding mRNA and protein levels, were investigated. Our data suggest that the inhibition of cholesterol biosynthesis in C18:1 *cis*-treated cells might be ascribed, at least in part, to a reduced activity and expression of HMGCR. Therefore, these findings could have potential implications in some brain dysfunction in which alteration of lipid metabolism occurs. Several investigations have shown an abnormally active synthesis of cholesterol from acetate in malignant glial cells compared with their normal counterparts (43, 44). The inhibition of HMGCR activity and expression by exogenous fatty acids reported in this study adds further support to previous findings (46, 47), indicating that fatty acids exogenously added to C6 cells may represent specific means of controlling gliomatous growth.

Overall, this study clearly shows rather active lipogenic and cholesterologenic activities of C6 glioma cells and a downregulation of fatty acid and cholesterol synthesis by exogenous fatty acids, mainly C18:1 *cis*. The reduced ACC and HMGCR expression may be considered important factors in this regulation. Experiments are in progress in our laboratory to ascertain the possible level of this regulation (i.e., transcriptional and/or posttranscriptional).

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