Impact of mitochondrial β-oxidation in fatty acid-mediated inhibition of glioma cell proliferation

Kjetil Berge[1,*], Karl Johan Tronstad,* Pavol Bohov,*,† Lise Madsen,* and Rolf K. Berge*

Department of Clinical Biochemistry,* Haukeland Hospital, University of Bergen, N-5021 Bergen, Norway; and Institute of Experimental Endocrinology,† Slovak Academy of Sciences, SK-83101 Bratislava, Slovak Republic

Abstract **Tetradecylthioacetic acid (TTA), which cannot be β-oxidized, exerts growth-limiting properties in glioma cells. In order to investigate the importance of modulated lipid metabolism and alterations in mitochondrial properties in this cell death process, we incubated glioma cells both with TTA and the oxidizable fatty acid palmitic acid (PA), in the presence of L-carnitine and the carnitine palmitoyltransferase inhibitors etomoxir and aminocarnitine. L-carnitine partly abolished the PA-mediated growth reduction of glioma cells, whereas etomoxir and aminocarnitine enhanced the antiproliferative effect of PA. The production of acid-soluble products increased and the incorporation of PA into glycerolipids decreased after L-carnitine supplementation. L-carnitine was found to enhance the antiproliferative effect of TTA, but did not affect the incorporation of TTA into glycerolipids, or ceramide. PDMP, sphingosine 1-phosphate, desipramine, fumonisin B_1, and L-cycloserine were able not to rescue the glioma cells from PA and TTA-induced growth inhibition, suggesting that increased ceramide production is not important in the growth reduction. TTA-mediated growth inhibition was accompanied with an increased uptake of PA and increased incorporation of PA into triacylglycerol (TG). Our data suggest that mitochondrial functions are involved in fatty acid-mediated growth inhibition. Whether there is a causal relationship between TG accumulation and the apoptotic process remains to be determined.**—Berge, K., K. J. Tronstad, P. Bohov, L. Madsen, and R. K. Berge. **Impact of mitochondrial β-oxidation in fatty acid-mediated inhibition of glioma cell proliferation.** ***J. Lipid Res.*** **2003.** 44: **118–127.**

Supplementary key words triacylglycerol • phospholipids • fatty acid oxidation • growth regulation • apoptosis • tetradecylthioacetic acid • palmitic acid • ceramide

Since the early 1980s, it has been firmly established that fatty acids can be selectively cytotoxic to tumor cells but not normal cells (1–3). Numerous studies have been carried out and several reports have revealed that fatty acids and compounds modulating the fatty acid metabolism exert growth-limiting properties in a variety of cancer cells (4, 5). One of these compounds is tetradecylthioacetic acid (TTA) [CH_3-$(CH_2)_{13}$-S-CH_2-COOH], which is a sulfur-substituted fatty acid that has been shown to reduce proliferation of tumor cells (6–9).

Cell death induced by long-chain fatty acids in pancreatic islets has been explained by the involvement of a lipoapoptotic pathway and nitric oxide overproduction (10, 11). It has recently been proposed that a change in cellular triacylglycerol (TG) levels has implications for the regulation of cell growth (12). Furthermore, there was an inverse relationship between cytotoxicity of free fatty acids in pancreatic islet cells and TG accumulation (13). These data indicate that TG accumulation may play a central role in the regulation of cellular proliferation.

During the past two decades there has been growing evidence that sphingolipids play a central role in growth regulation (14), and many of the enzymes participating in the sphingolipid metabolism are involved in signal transduction and cell growth regulation. One of these sphingolipids, ceramide, is a lipid second messenger involved in the apoptotic response induced by TNFα, ionizing radiation, and heat shock (15). An increased ceramide level can be induced by at least two alternative biochemical pathways: the hydrolysis of sphingomyelin by sphingomyelinases (16, 17), or elevated de novo synthesis, where serine palmitoyltransferase is the rate-limiting enzyme (18, 19). It has been suggested that a reduced fatty acid oxidation, accompanied by TG accumulation, at least partly contributes to high ceramide level in pancreatic islets (11, 20).

Thus, factors influencing the balance between TG biosynthesis and mitochondrial fatty acid oxidation may ultimately influence the regulation of cell growth. To contribute to the understanding of relations between mitochondrial β-oxidation, TG accumulation, and ceramide synthesis in growth regulation and apoptosis, the metabolism and the

Manuscript received 7 August 2002.

Published, JLR Papers in Press, October 1, 2002.
DOI 10.1194/jlr.M200312-JLR200

Abbreviations: CPT, carnitine palmitoyltransferase; $\Delta\psi_m$, mitochondrial membrane potential; PA, palmitic acid; PL, phospholipid; TG, triacylglycerol; TTA, tetradecylthioacetic acid.

[1] To whom correspondence should be addressed.
e-mail: kjetil.berge@ikb.uib.no

capacity of a natural utilizable fatty acid and TTA to reduce the growth of glioma cells were compared. The experiments demonstrated that a low fatty acid oxidation capacity results in excess TG accumulation when the cells are exposed to saturated fatty acids. TG accumulation seems to correlate with the growth limiting properties of palmitic acid (PA) and TTA, and is independent of ceramide synthesis. Furthermore, a stimulation of fatty acid oxidation retarded PA-mediated growth inhibition, whereas inhibition enhanced the growth-limiting properties of both PA and TTA.

EXPERIMENTAL PROCEDURES

Chemicals

Palmitic acid, L-carnitine, C_2-ceramide, L-erythro-sphingosine, fumonisin B_1, L-cycloserine, etomoxir, PDMP (1-phenyl-2-decanoylamino-3-morpholino-1-propanol hydrochloride), sphingosine 1-phosphate, desipramine, and fatty acid-free BSA (BSA) were obtained from Sigma Chemical Co, St. Louis, MO. [^{3}H]thymidine and [1-^{14}C]palmitic acid were obtained from Amersham Pharmacia Biotech, Piscataway, NJ. TTA and [1-^{14}C]TTA were prepared at the Department of Chemistry, University of Bergen, Norway, as described previously (21, 22). L-Aminocarnitine (L-3-amino-4-trimethyl-aminobutyrate) was a kind gift of A. C. Rustan, University of Oslo, Norway. All other chemicals and solvents were obtained from common commercial sources.

Cell cultures

The human glioma cell line D54Mg (23) and the rat glioma cell line BT4Cn (24) were grown in DMEM supplemented with 10% new-born calf serum, streptomycin (100 μg/ml), penicillin (100 U/ml), and three times the prescribed concentration of non-essential amino acids (all from Sigma Chemical Co.). For metabolism experiments, cells were seeded in 3.5 cm or 6 cm culture dishes. All cells were cultured under humidified conditions of 95% air and 5% CO_2 at 37°C. In all experiments, the number of cells seeded was controlled to avoid confluence during the experiment period. The cells were allowed to settle overnight before the treatments were started. Fatty acid solutions were prepared as described earlier (25).

Growth assays

Cells were grown in 96-well tissue culture plates, and [^{3}H]thymidine incorporation was measured after 4 h of incubation with 1.0 μCi/well of [^{3}H]thymidine diluted in 0.9% NaCl. After incubation, the cellular DNA was transferred to a UniFilter™-96 GF/C™ using a Filtermate Harvester (Packard Instruments, Meriden, CT). Radioactivity was measured using a TopCount NXT™ Microplate Scintillation Counter (Packard Instruments).

Measurement of acid-soluble products

The glioma cells were cultivated in medium containing [1-^{14}C]PA or [1-^{14}C]TTA. Final specific activity in the medium was in the range 1,000–3,000 dpm/nmol PA/TTA. After 4 h, 9 h, or 24 h incubation, the medium was removed, cells kept on ice, and then washed once with PBS followed by scraping in PBS. Acid-soluble products were extracted by the following procedure: at the termination of the experiment, 250 μl medium was transferred to a new glass tube, and 100 μl 6% fatty acid free BSA was added. The mixture was shaken for 15 s followed by the addition of 1 ml ice cold 1 M $HClO_4$ and 150 μl 0.1 M KOH. The mixture was shaken for 30 s, centrifuged (10 min, 1,800 *g*), and the radioactivity was measured in the supernatant.

Extraction and quantitation of lipids

Lipids were extracted from pelleted cells according to Folch et al. (26). Twenty volumes of chloroform-methanol (2:1, v/v) and 4 vol of 0.9% NaCl (pH = 2) were added to the cell suspension, and the mixture was allowed to separate into two phases. After evaporating the organic phase under N_2, the extracted lipids were dissolved in chloroform-methanol (2:1, v/v) and separated by TLC on silica gel plates developed in hexane-diethyl ether-acetic acid (80:20:1, v/v/v) as described previously (27). The lipid bands were visualized by iodine vapor, cut into pieces and the radioactivity was measured by scintillation counting.

GC/MS analysis

Polar lipids were separated by TLC as described previously (28). Lipid spots were scraped, transferred to a test tube, and transesterified with 14% BF_3-methanol (29).

Fatty acids were determined on a GC 8,000 Top gas chromatograph (CE Instruments, Milano, Italy), equipped with a flame ionization detector, programmable temperature of vaporization injector, and AS 800 autosampler using a fused silica capillary column coated with SP 2340 stationary phase (60 m × 0.25 mm × 0.20 μm) (Supelco, Bellefonte, PA). Chromatographic conditions were similar as referred (30). Identification of chromatographic peaks were performed by means of known standards (Larodan Fine Chemicals, Malmo, Sweden) and confirmed by GC/MS analysis (GCQ, Finnigan MAT, Austin, TX) on the same column. Quantification was made with Chrom-Card A/D 1.0 chromatography station (CE Instruments, Milano, Italy) based on heneicosanoic acid as an internal standard.

Triacylglycerol and phospholipid measurements

5–7 × 10^6 glioma cells were kept on ice, the medium was removed, and the cells were washed once with PBS followed by scraping in PBS. Pelleted cells were lysed in 150 μl distilled H_2O and the cellular levels of TG and phospholipid were measured enzymatically (TRINDER reaction kits from bioMèrieux, MO) with an AXON Byer spectrophotometer (31, 32).

Oxidation

Glioma cells were grown in 25 cm^2 flasks. After 24 h of treatment, the medium was removed, followed by addition of 5 ml medium containing [1-^{14}C]PA (200 μM, 2,775 dpm/nmol) complexed to BSA (molar ratio 2.5:1). Each flask was sealed with a rubber stopper containing a filter paper in a holder. Following incubation for 4 h, the flasks were kept on ice, 0.75 ml cold 1 M $HClO_4$ was added, and the filter paper was moistened by addition of 0.3 ml phenyl ethylamine-methanol (1:1, v/v). The cultures were left for 1 h in room temperature for [^{14}C]CO_2 trapping. The filter papers (containing trapped [^{14}C]CO_2) were then transferred to vials for scintillation counting. Acid-soluble products were extracted by addition of 5 ml of cold 1 M $HClO_4$ into the flask. After centrifugation (10 min, 1,800 *g*), radioactivity in the supernatant was measured by scintillation counting.

Apoptosis

The level of apoptosis was determined by microscopic cell surface examination and fluorescence analysis after staining with Hoechst 33342 (10 μg/ml) in 4% paraformaldehyde. Apoptotic cells were discriminated from non-apoptotic cells by the appear-

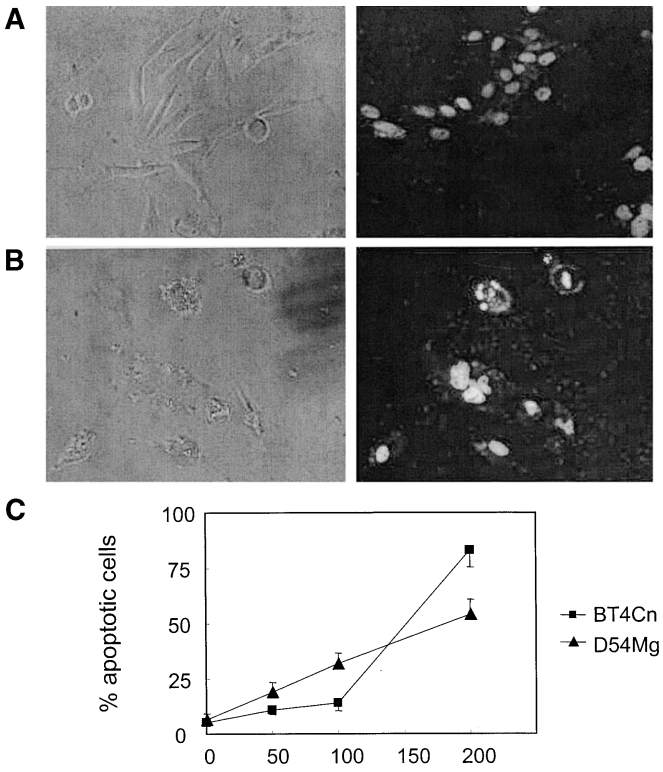

Fig. 1. Induction of apoptosis in glioma cells. D54Mg cells were treated with control medium (A) or 200 μM tetradecylthioacetic acid (TTA) (B) for 24 h. The cells were incubated with Hoechst 33342 and condensed chromatin was detected by fluorescence microscopy. Intense white color appears in condensed chromatin in apoptotic cells. Cell morphology was also investigated by microscopy (C) and the amount of apoptotic D54Mg or BT4Cn cells was determined after cultivation in control medium or medium containing 50, 100, or 200 μM TTA for 48 h (n = 3).

ance of multiple surface buds and intense color associated with chromatin condensation and fragmentation.

Staining of lipid droplets

Glioma cell cultures were washed twice with PBS, and then fixed for 1 h in a 10% formaldehyde solution (diluted in PBS) at room temperature. After removing the fix solution, the cells were stained using a 0.5% Oil Red O in isopropanol for 1 h at room temperature. Following staining, cells were rinsed several times using distilled water to remove any non-dissolved stain. The cells were then observed under a Leica DM IRB inverted microscope and photographs of representative cells were taken for documentation.

Mitochondrial membrane potential and glutathione distribution

After treatment, glioma cells were exposed to 6 μM CellTracker green 5-chloromethylfluorescein diacetate (CMFDA) and 0.6 μM Mitotracker Red CM-H_2Xros (MTR) (Molecular Probes, Inc., Eugene, OR), according the manufacturer's directions. Following incubation, medium was removed, cover slips were mounted with fresh tissue culture medium, and the cells were immediately studied with a BIO-RAD MRC-1000 Laser Scanning Confocal imaging System connected to a Zeiss Axiovert 100 microscope. MTR and CMFDA staining were identified by independent simultaneous scans of red (ex. 568 nm, em. 585 nm) and green (ex. 488 nm, em. 522/35 nm) fluorescence, respectively.

Statistical analysis

The data are presented as mean ± SD. The results were evaluated by a two-sample variance Student's *t*-test (two-tailed distribution). Results were considered as statistically significant when $P < 0.05$.

RESULTS

TTA induces apoptosis in glioma cells

Fatty acids are known to induce apoptosis in several different cells lines. To decide whether apoptosis contributes to the retarded proliferation reported in glioma cells after treatment with TTA (8), we performed morphological studies of D54Mg cells treated with control medium or medium containing a high dose of TTA for 24 h (**Fig. 1A, B**). The amount of apoptotic cells grown in control medium was less than 5% (data not shown). After TTA exposure, more than 50% of the cells showed characteristic apoptotic morphology. Further, massive apoptotic nuclear condensation and fragmentation was also observed after staining with Hoechst 33342. Figure 1C shows that the induction of apoptosis after TTA exposure was dose dependent both in D54Mg cells and BT4Cn cells. The apoptogenic properties of PA have previously been described (33–35), and characteristic apoptosis was also observed after PA exposure in our experiments (data not shown).

Relation between fatty acid oxidation and cell growth

The capacity of TTA to inhibit cancer cell growth is pronounced when compared with normal fatty acids such as PA (6, 7). This might be related to their capacity to modulate mitochondrial oxidation and thereby lipid accumulation. Therefore, D54Mg cells were incubated with 150 μM TTA or PA, which are reported to be the approximate IC_{50} concentration of TTA after 48 h of cultivation (25), in the presence of L-carnitine and inhibitors of mitochondrial fatty acid oxidation. L-carnitine, which is essential for mitochondrial transmembrane transport [reviewed in ref. (36)], partly abolished the PA-mediated reduction in [^{3}H]thymidine incorporation in D54Mg cells (**Fig. 2**). In contrast,

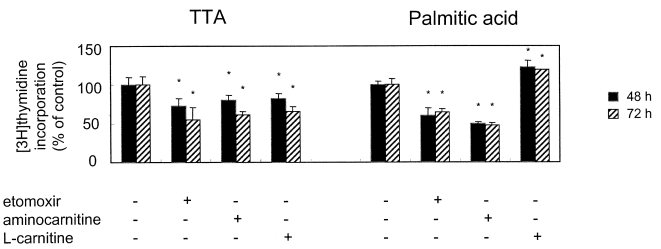

Fig. 2. Effects of palmitic acid (PA) and TTA on the growth of glioma cells in the absence and presence of L-carnitine amino carnatine, and etomoxir. Bars represent incorporation of [^{3}H]thymidine in human glioma cells (D54Mg), calculated as a percentage of control (fatty acids alone). Cells were treated with 150 μM palmitic acid or TTA ± 50 μM etomoxir, 50 μM aminocarnitine, or 500 μM L-carnitine for 48 h or 72 h. Data shown are mean values ± SD (n = 3). * Significantly different from control cells (fatty acids alone), $P < 0.05$.

TABLE 1. Uptake and oxidation of radiolabeled fatty acids in glioma cells

[^{14}C]Fatty Acid	L-Carnitine	Hours	[^{14}C]Fatty Acid Uptake	[^{14}C]Fatty Acid Oxidation
			nmol/mg protein	
Palmitic acid	−	4	157.3 ± 2.0	5.2 ± 0.6
		24	284.0 ± 31.3	39.9 ± 2.7
Palmitic acid	+	4	157.6 ± 24.2	17.6 ± 4.1[a]
		24	245.2 ± 60.2	143.3 ± 34.0[a]
TTA	−	4	53.8 ± 1.5	nd
		24	96.7 ± 3.5	nd
TTA	+	4	53.3 ± 3.4	nd
		24	104.1 ± 22.7	nd

BT4Cn cells were incubated with 100 μM [1-^{14}C]TTA or [1-^{14}C]PA ± 500 μM L-carnitine. The amount of acid-soluble products and the uptake of labeled fatty acids were measured after 4 h and 24 h of cultivation. The values represent mean values (nmol/mg protein) ± SD (n = 3). nd, not detected.

[a] Significantly different from cells grown without supplementary L-carnitine, $P < 0.05$.

L-carnitine enhanced the antiproliferative effect of TTA. On the other hand, inhibition of either mitochondrial carnitine palmitoyltransferase (CPT) I or II by etomoxir or aminocarnitine, respectively, enhanced the antiproliferative effect of both TTA and PA. Similar results were obtained in BT4Cn cells (data not shown). At the concentrations used, etomoxir, aminocarnitine, and L-carnitine did not significantly affect cell growth when used alone (data not shown).

Relation between fatty acid oxidation and glycerolipid synthesis

Since stimulation of the mitochondrial fatty acid transport system by L-carnitine partly abolished the antiproliferative effect of PA, but not TTA, we sought to determine how L-carnitine influenced the metabolism of these individual fatty acids in glioma cells. L-carnitine stimulated the production of acid-soluble products from [1-^{14}C]PA ~3-fold (**Table 1**). This is probably due to increased fatty acid oxidation capacity, as L-carnitine did not influence the uptake of [1-^{14}C]PA. Moreover, the incorporation of [1-^{14}C]PA into glycerolipids in BT4Cn cells was reduced after L-carnitine supplementation (**Table 2**). As expected, there were no detectable levels of acid-soluble products from [1-^{14}C]TTA in the presence of additional L-carnitine (Table 1). Furthermore, L-carnitine did not affect the incorporation of [1-^{14}C]TTA into glycerolipids. The uptake of [1-^{14}C]PA was 2–3-fold higher when compared with [1-^{14}C]TTA (Table 1), which is in accordance with a higher incorporation of [1-^{14}C]PA into glycerolipids than [1-^{14}C]TTA (Table 2).

TABLE 2. Effect of L-carnitine on the incorporation of palmitic acid and TTA into glycerolipids in glioma cells

[^{14}C]Fatty Acid	L-Carnitine	Hours	Incorporation into PL	Incorporation into TG
			nmol/mg protein	
Palmitic acid	−	24	123.5 ± 17.5	87.5 ± 19.5
Palmitic acid	+	24	87.1 ± 35.8	48.6 ± 17.0
TTA	−	24	52.6 ± 10.3	13.8 ± 2.1
TTA	+	24	60.4 ± 24.7	12.3 ± 5.4

BT4Cn cells were incubated with 100 μM [1-^{14}C]TTA or [1-^{14}C]PA ± 500 μM L-carnitine. The amount of labeled fatty acids incorporated into phospholipids (PL) and triacylglycerol (TG) after 24 h of cultivation were measured by TLC as described in Experimental Procedures. The values represent mean values (nmol/mg protein) ± SD (n = 3).

Incorporation of TTA and palmitic acid into glycerolipids

Fatty acid metabolism is balanced between glycerolipid biosynthesis and fatty acid oxidation, and, independent of the uptake of these fatty acids and the supplementation of L-carnitine, TTA seemed to be preferentially incorporated into phospholipid (PL) (Table 2). In order to investigate this in more details, glioma cells were cultured at different concentrations of TTA and PA. **Figure 3** shows that there was a dose-dependent increase in the incorporation of labeled PA and TTA into glycerolipids. At a concentration of 200 μM, there was a more rapid increase of [1-^{14}C]PA into TG than PL (Fig. 3A), whereas the rate of TTA incorporation into these lipid classes was almost equal (Fig. 3B).

As a function of dose, the amount of PA-labeled TG increased several-fold, whereas the increase in [1-^{14}C]PA-labeled PL was less significant (Fig. 3C). This was not observed after TTA treatment, where [1-^{14}C]TTA was predominantly found in PL with all doses (Fig. 3D). In all cases, the amount of labeled PA incorporated into PL and TG was significantly higher than the corresponding incorporation of TTA. The quantity of cell-associated [1-^{14}C]PA and [1-^{14}C] TTA corresponded to the disappearance of the respective fatty acids from the medium (data not shown).

Effect of TTA, etomoxir, and L-carnitine on PA metabolism

Since TTA may modulate cellular fatty acid metabolism, it was of interest to examine how TTA affects the incorporation of normal fatty acids, such as PA, into glycerolipids. TTA increased both the cellular uptake (**Fig. 4A**) and oxidation (Fig. 4E) of [1-^{14}C]PA in D54Mg cells. The increased amount of [1-^{14}C]PA in these cells were reflected in an increased esterification of [1-^{14}C]PA into TG (Fig. 4D). The amount of [1-^{14}C]PA incorporated into PLs was unaffected by TTA treatment (Fig. 4C), whereas the amount of [1-^{14}C]PA in cholesterol ester was significantly decreased upon TTA exposure at different doses (Fig. 4B).

Since TTA appeared to stimulate [1-^{14}C]PA uptake and [1-^{14}C]PA incorporation into TG, selectively, it is likely

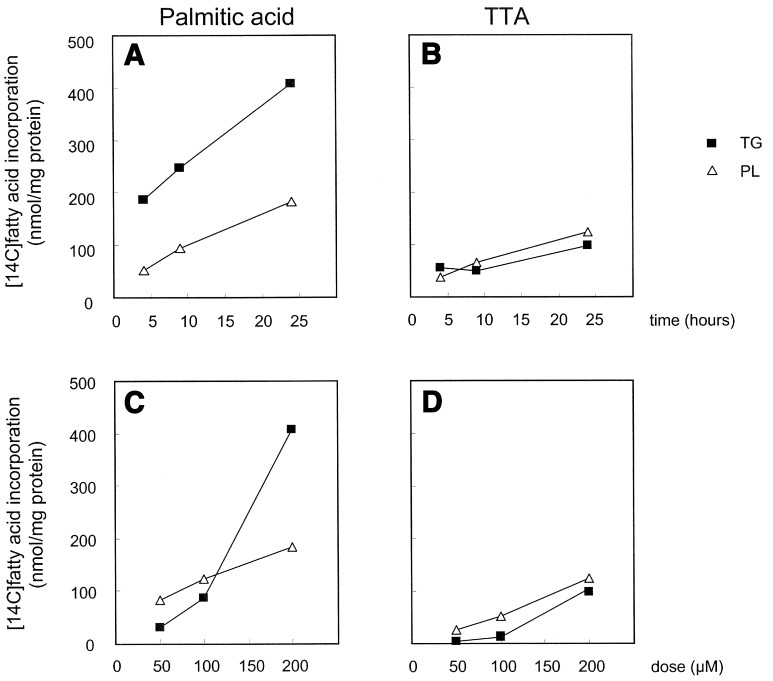

Fig. 3. Incorporation of PA and TTA into PL and TG in glioma cells. BT4Cn cells were incubated with (A) 200 μM [1-^{14}C]PA or (B) 200 μM [1-^{14}C]TTA for 4 h, 9 h, or 24 h. BT4Cn cells were incubated with 50 μM, 100 μM, or 200 μM (C) [1-^{14}C]PA or (D) [1-^{14}C]TTA for 24 h. All values represent the amount of PA or TTA (nmol/mg protein) incorporated into phospholipid (PL) and triacylglycerol (TG) (n = 3).

that TG synthesis was stimulated. Staining of the cells with Oil Red O confirmed lipid accumulation in D54Mg cells treated with TTA (**Fig. 5A**) as more clusters of larger lipid droplets in D54Mg were observed in these cells when compared with control. Lipid accumulation was, however, also seen after PA supplementation.

Increased accumulation of TG in cells treated with saturated fatty acids might be related to poor capacity of the cells to oxidize fatty acids. Inhibition of mitochondrial β-oxidation by etomoxir indeed amplified the TTA- and PA-mediated stimulation of TG accumulation (Fig. 5B), whereas no significant change in cellular PL levels could be observed after etomoxir supplementation (Fig. 5C). On the other hand, L-carnitine significantly reduced the amount of TG in PA-treated cells, but not cells exposed to TTA. In contrast, no increase in cellular PL

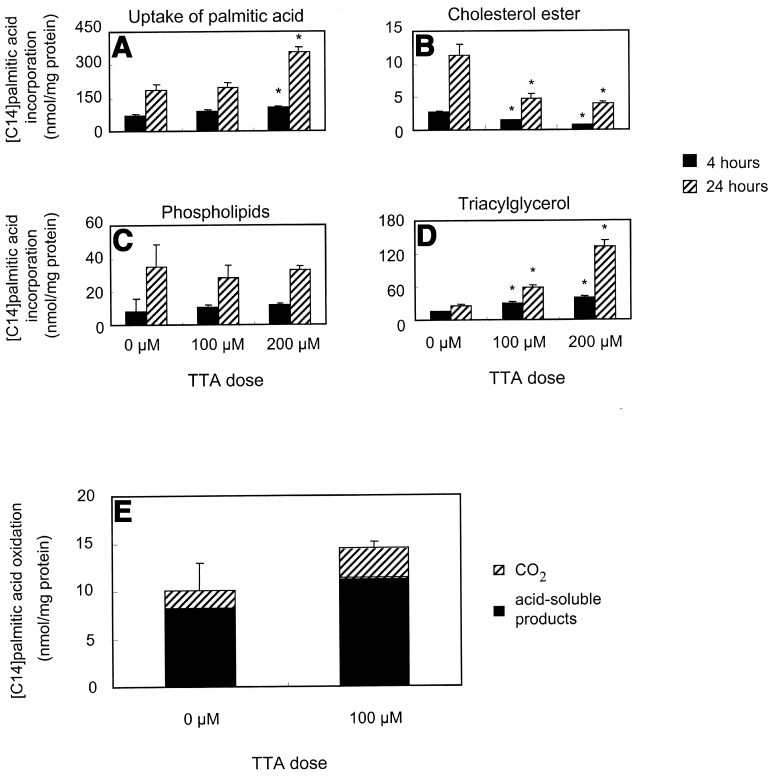

Fig. 4. Effect of TTA on the metabolism and oxidation of PA and in glioma cells. Bars represent (A) the uptake of [1-^{14}C]PA or the incorporation of [1-^{14}C]PA into (B) cholesterol ester, (C) phospholipids, or (D) triacylglycerol D54Mg cells after incubation with 50 μM [1-^{14}C]PA ± 100 or 200 μM TTA for 4 h or 24 h. E: Bars represent CO_2 and acid-soluble products in D54Mg cells. The oxidation was measured subsequently to cultivation with 50 μM [1-^{14}C]PA ± 100 μM TTA for 24 h. All data shown are mean values (nmol/mg protein) ± SD (n = 3). * Significantly different from cells grown with PA alone, $P < 0.05$.

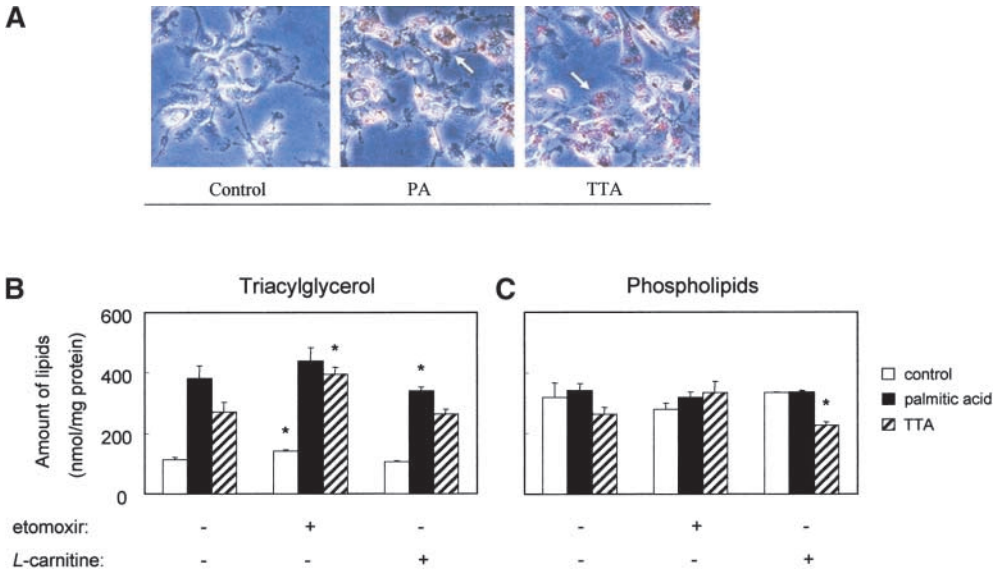

Fig. 5. Triacylglycerol accumulation in glioma cells treated with fatty acids. A: D54Mg cells were cultured in the presence of control medium, 150 μM PA, or TTA for 48 h and stained with Oil Red O as described in Experimental Procedures. Areas with elevated level of lipids are indicated with white arrows. Magnification: 20×. D54Mg cells were incubated for 48 h in control medium, or in medium containing 150 μM PA or TTA as indicated ± 50 μM etomoxir or 500 μM L-carnitine. Values represent total cellular amount of (B) TG and (C) PL (nmol/mg protein), and the values represent mean ± SD (n = 3). * Significantly different from cells incubated without etomoxir or L-carnitine, $P < 0.05$.

levels could be observed after L-carnitine supplementation.

Effect of PA, TTA, and L-carnitine on sphingolipid metabolism

Inhibition of mitochondrial β-oxidation by etomoxir has been shown to stimulate incorporation of fatty acids into ceramides (34). Increased ceramide production has been reported to induce apoptosis in several different systems (37), both via elevated de novo synthesis and degradation of sphingomyelin. Since stimulation of β-oxidation by L-carnitine partly abolished the antiproliferative effect of PA but potentiated the growth-limiting effect of TTA (Fig. 2), we measured the incorporation of labeled TTA and PA into sphingolipids in the absence or presence of supplementary L-carnitine. The level of PA-labeled ceramide, gangliosides, cerebrosides, and sphingomyelin decreased after L-carnitine supplementation (**Fig. 6**). In contrast, the incorporation of [1-^{14}C]TTA into these sphingolipids was at least not reduced by stimulation with L-carnitine. Moreover, the amount of amide-linked fatty acids in ceramides after PA and TTA supplementation was marginally influenced when compared with untreated control cells (**Table 3**).

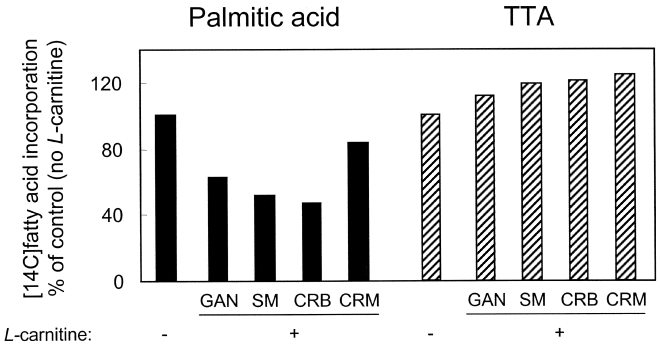

Fig. 6. Effect of L-carnitine on the incorporation of PA and TTA into sphingolipids. BT4Cn cells were incubated with 200 μM [1-^{14}C]PA (black bars) or [1-^{14}C]TTA (striped bars) in the presence or absence of 500 μM L-carnitine. Sphingolipids were separated by TLC and the radioactivity was measured with liquid scintillation counting. Bars represents mean incorporation of [1-^{14}C]fatty acids into sphingolipids calculated as percent of incorporation in cells cultivated with no L-carnitine supplementation (n = 3). GAN, gangliosides; SM, sphingomyelin; CRB, cerebrosides; and CRM, ceramide.

Sensitivity to sphingolipids and the effect of inhibitors of sphingolipid metabolism

Based on the possible involvement of ceramide in PA and/or TTA-mediated growth arrest, we sought to determine if D54Mg and BT4Cn cells were sensitive to C_2-ceramide, a

TABLE 3. Effect of palmitic acid and TTA on ceramide synthesis in glioma cells

Treatment	Amount of Fatty Acids in Ceramides
	nmol/mg protein
Control	14.7
PA	17.0
TTA	17.1

D54Mg cells were incubated with 100 μM palmitic acid or 100 μM TTA for 24 h, and cells grown in medium without additional fatty acids were used as control. Cells were pooled from 2–4 dishes, ceramide was separated by TLC, and fatty acids were measured by GC/MS analysis as described in Experimental Procedures.

cell-permeable ceramide analog previously shown to cause apoptosis (38), and sphingosine, an intermediate in sphingolipid metabolism which has been reported to induce apoptosis in different cell systems (39). Indeed, glioma cells were sensitive to both C_2-ceramide (**Fig. 7A**) and sphingosine (Fig. 7B), but not C_2-dihydroceramide (Fig. 7A). The IC_{50} values for C_2-ceramide and sphingosine were ~30 μM and 6 μM, respectively, which is in a similar range to that observed in other cell lines (34, 39–41).

To further evaluate if ceramide synthesis could be involved in the growth-limiting properties of PA and TTA, we cultivated PA and TTA-treated D54Mg (Fig. 7C) and BT4Cn (Fig. 7D) cells in the absence or presence of sphingosine 1-phosphate, a compound that is known to prevent ceramide-mediated apoptosis (42), and four different inhibitors of enzymes involved in sphingolipid metabolism. Sphingosine 1-phosphate, PDMP (UDP-glucose:ceramide glucosyltransferase inhibitor; inhibits GD3 synthesis), desipramine (acidic sphingomyelinase inhibitor), fumonisin B_1 (ceramide synthase inhibitor), and L-cycloserine (serine palmitoyltransferase inhibitor) were not able to rescue the cells from PA and TTA-induced growth inhibition.

Effects on mitochondria and glutathione

The results indicate that the mitochondria may be of importance in TTA-induced apoptosis. In order to investigate this, BT4Cn cells were dually stained with the glutathione-reactive dye, CMFDA, and MTR, which is a mitochondrial membrane potential ($\Delta\psi_m$)-dependent probe (**Fig. 8**). The intensity of MTR was modestly decreased in TTA-treated cells, indicating that TTA-mediated apoptosis is associated with a decrease in $\Delta\psi_m$. Glutathione, as assessed by CMFDA-staining, was ubiquitously present in the cytoplasm in healthy control cells, but the concentration was evidently higher near the nuclei. TTA dose-dependently induced a redistribution of glutathione from the nuclei and mitochondria toward cellular bodies appearing as highly intensified particles. The decreased level of mitochondrial glutathione was also supported by the loss of yellow staining in the merged pictures.

DISCUSSION

This investigation shows that TTA and PA reduced the growth of glioma cells and that the modified 3-thia fatty acid induced apoptosis. The main finding was that the mitochondrial functions are involved in this fatty acid-mediated growth inhibition. This conclusion is evident from the data on mitochondrial β-oxidation, the use of L-carnitine, etomoxir, and aminocarnitine, incorporation of fatty acids into glycerolipids and ceramide and the mitochondrial membrane potential $\Delta\psi_m$.

L-carnitine was found to enhance the antiproliferative effect of TTA, but partly abolished the effect of PA (Fig.

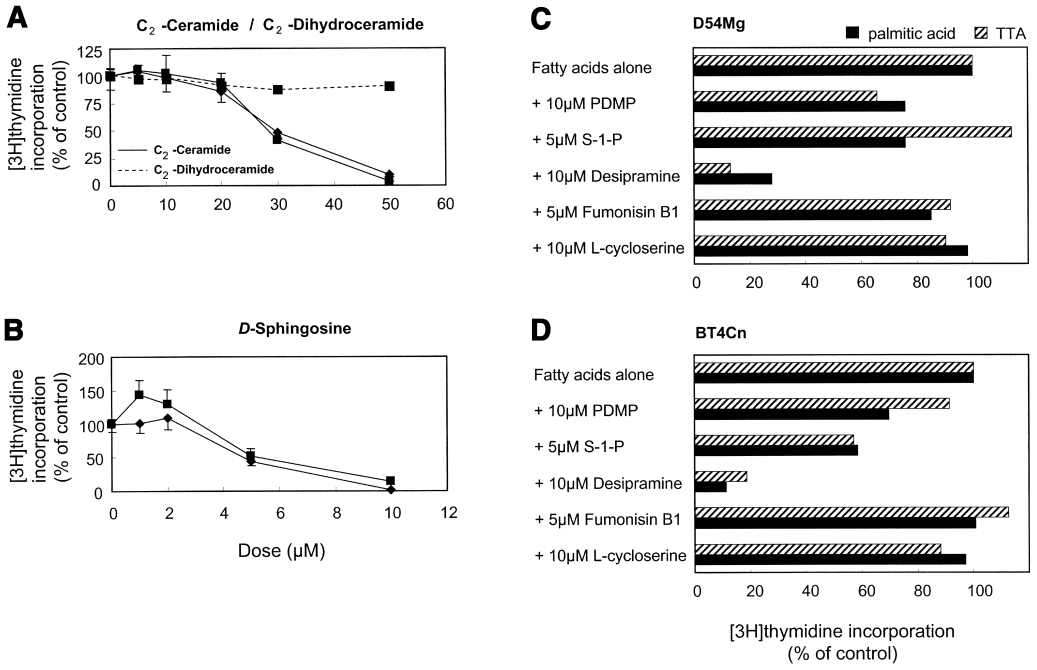

Fig. 7. Effect of sphingolipids and inhibitors of sphingolipid metabolism on the growth of glioma cells. BT4Cn (diamond) and D54Mg (square) cells were grown in the presence of increasing doses of (A) C_2-ceramide and C_2-dihydroceramide (as indicated) or (B) D-sphingosine, for 48 h. All values represent incorporation of [^{3}H]thymidine, calculated as a percentage of control (no sphingolipids added). Data shown are mean values ± SD (n = 3). D54Mg (C) and BT4Cn (D) cells were grown in the presence of 150 μM TTA or PA alone, or in the presence of 10 μM PDMP, 5 μM sphingosine 1-phosphate, 10 μM desipramine, 5 μM fumonisin B_1, or 10 μM L-cycloserine, for 48 h. Bars represent incorporation of [^{3}H]thymidine, calculated as percentage of control (fatty acids alone) (n = 3).

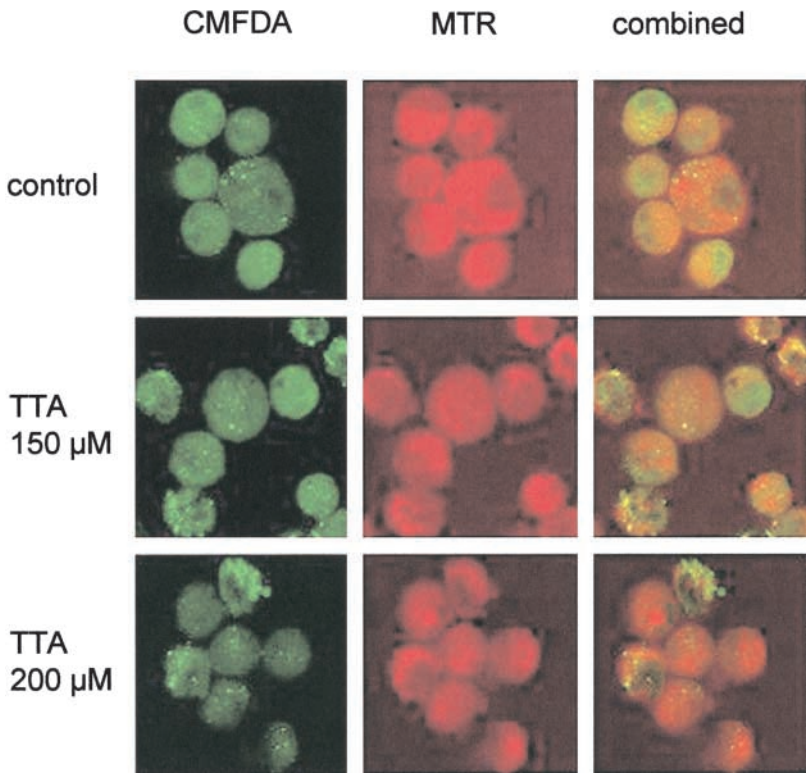

Fig. 8. Cell micrographs: mitochondrial membrane potential and glutathione distribution in cells exposed to TTA. BT4Cn cells were grown in control medium or treated with 150 μM or 200 μM TTA for 21 h, followed by dual staining with the glutathione-reactive dye CMFDA (green) and the mitochondrial dye Mitotracker red (MTR). The samples were studied by the use of laser scanning confocal microscopy. Red (585 nm) and green (522/35 nm) fluorescence indicates mitochondria and glutathione, respectively.

2). In the presence of L-carnitine the oxidation of labeled PA was elevated, independent of the fatty acid uptake (Table 1). Furthermore, addition of L-carnitine decreased the incorporation of [1-^{14}C]PA into TG and PL (Table 2), suggesting that less palmitoyl-CoA was available for glycerolipid synthesis after stimulation of the mitochondrial β-oxidation. In support, L-carnitine reduced the cellular TG concentration in PA-treated cells, which was not observed for cells exposed to TTA (Fig. 5B). The enhanced effect of L-carnitine on TTA-mediated growth suppression suggests that a direct effect on the mitochondria might be involved. It has been demonstrated that CPT-I might be involved in the regulation of apoptosis through regulation of the carnitine/palmitoylcarnitine ratio (43). An elevated level of palmitoylcarnitine activated caspases, and carnitine-coupled TTA may have similar functions. We have recently detected formation of TTA-carnitine in plasma of rats treated with TTA (Berge et al., unpublished observations). Furthermore, it has been demonstrated that CPT-I directly interacts with the antiapoptotic protein Bcl-2 (44), but the importance of this interaction in the apoptotic process is a matter of dispute (33).

TTA also stimulated the oxidation of PA (Fig. 4E), which is in agreement with previous findings (8). Compared with L-carnitine stimulation, which induced oxidation of PA ~3-fold (Table 1), the stimulating effect of TTA was very low. Hence, it is likely that the increased mitochondrial oxidation observed after TTA treatment is not sufficient to prevent increased TG synthesis, as observed in D54Mg cells (Fig. 5). It is noteworthy that in hepatocytes, an increased oxidation of fatty acids was accompanied by a reduced synthesis of PA-labeled TG after TTA treatment (45), indicating that the mitochondrial β-oxidation capacity is higher or more inducible in rat hepatocytes than in glioma cells. One important factor involved in the stimulation of fatty acid oxidation is the activation of peroxisome proliferator-activated receptor (PPAR)α (46). However, the level of PPARα in rat glioma cells is very low (7), supporting the hypothesis that the oxidative capacity is restricted.

It is unclear whether it is the amount of fatty acids taken up by the cells, or the specific effects of the fatty acids, that gives rise to growth suppression. The uptake of TTA was low when compared with PA, suggesting that TTA has a more specific effect. However, TTA increased the uptake of PA (Fig. 4A), and might therefore induce indirect growth-limiting signals, as well as direct effects. The mechanism behind the increased uptake of PA in the presence of 200 μM TTA is currently not known, but might be related to a passive uptake of fatty acids due to the high total fatty acid concentration in the medium. With 100 μM TTA, however, we did not observe any increased uptake of PA, but still found a stimulation of TG synthesis. Thus, TTA specifically stimulates TG synthesis. Since TTA is

more favored for incorporation into PL than TG (Fig. 3B, D), it might therefore occupy positions in PL and, consequently, direct PA (and other fatty acids) to TG synthesis.

The cellular TG concentration increased in the presence of etomoxir in control cells, as well as in cells treated with fatty acids (Fig. 5B). These results demonstrate that an inhibition of the mitochondrial β-oxidation leads to an elevated intracellular level of TG. Retarded cell growth after etomoxir treatment has also been described in a hematopoietic cell line (34), where PA was found to enhance the generation of the pro-apoptotic second messenger ceramide after inhibition of CPT1. The mechanism accounting for enhanced growth inhibition by etomoxir treatment is elusive and requires further investigation, but decreased mitochondrial β-oxidation might lead to a channeling of acyl-CoA toward ceramide synthesis. Moreover, increased TG accumulation might be considered as a contributing factor for an elevated ceramide synthesis due to an expanded source of acyl-CoA (11, 20). Our experiments, however, indicates that neither PA nor TTA mediate growth inhibition via increased ceramide synthesis. Even if increased β-oxidation was accompanied by a decreased rate of de novo ceramide synthesis after PA exposure (Fig. 6), the amount of ceramides was not significantly elevated after treatment with saturated fatty acids (Table 3). Moreover, ceramide synthesis does not seem to be essential, since an inhibition of both de novo ceramide synthesis, sphingomyelinase, and GD3 synthesis could not rescue the cells from PA and TTA-mediated growth suppression (Fig. 7C, D).

It is now well established that the mitochondrion plays a crucial role in the regulation of growth and apoptosis (47, 48). This report shows that modulation of the mitochondrial β-oxidation significantly affects both PA and TTA-mediated growth suppression. Second, TTA induced alterations in $\Delta\psi_m$ and mitochondrial glutathione content (Fig. 8). Recently, we have found that TTA induced an early depletion of mitochondrial glutathione and a reduction in the percentage of non-oxidized glutathione (GSH) in leukemia cells (unpublished observations). In these IPC-81 leukemia cells, we also saw that the apoptotic morphology and cytochrome c release were partly blocked by over-expression of Bcl-2. As TTA-induced apoptosis was associated by depolarization of $\Delta\psi_m$ in both glioma cells and in IPC-81 cells (unpublished observations), the potential of PA and TTA to inhibit growth of glioma cells seems to be related to mitochondrial functions (33).

Lipid droplet formation and increased cellular TG level has been suggested to enhance the apoptotic process (12, 49). Thus, our data support that apoptosis at least could be associated with TG accumulation. Whether there is a causal relationship between lipid droplets and TG accumulation and/or the apoptotic process remains to be determined.

The authors thank Svein Kruger for his technical contribution. This work was supported by the University of Bergen and Connie Gulborg Jansens legat.

REFERENCES

1. Begin, M. E., G. Ells, U. N. Das, and D. F. Horrobin. 1986. Differential killing of human carcinoma cells supplemented with n-3 and n-6 polyunsaturated fatty acids. *J. Natl. Cancer Inst.* **77:** 1053–1062.
2. Begin, M. E., U. N. Das, G. Ells, and D. F. Horrobin. 1985. Selective killing of human cancer cells by polyunsaturated fatty acids. *Prostaglandins Leukot. Med.* **19:** 177–186.
3. Maehle, L., E. Eilertsen, S. Mollerup, S. Schonberg, H. E. Krokan, and A. Haugen. 1995. Effects of n-3 fatty acids during neoplastic progression and comparison of in vitro and in vivo sensitivity of two human tumour cell lines. *Br. J. Cancer.* **71:** 691–696.
4. Pineau, T., W. R. Hudgins, L. Liu, L. C. Chen, T. Sher, F. J. Gonzalez, and D. Samid. 1996. Activation of a human peroxisome proliferator-activated receptor by the antitumor agent phenylacetate and its analogs. *Biochem. Pharmacol.* **52:** 659–667.
5. Samid, D., Z. Ram, W. R. Hudgins, S. Shack, L. Liu, S. Walbridge, E. H. Oldfield, and C. E. Myers. 1994. Selective activity of phenylacetate against malignant gliomas: resemblance to fetal brain damage in phenylketonuria. *Cancer Res.* **54:** 891–895.
6. Abdi-Dezfuli, F., L. Froyland, T. Thorsen, A. Aakvaag, and R. K. Berge. 1997. Eicosapentaenoic acid and sulphur substituted fatty acid analogues inhibit the proliferation of human breast cancer cells in culture. *Breast Cancer Res. Treat.* **45:** 229–239.
7. Berge, K., K. J. Tronstad, E. N. Flindt, T. H. Rasmussen, L. Madsen, K. Kristiansen, and R. K. Berge. 2001. Tetradecylthioacetic acid inhibits growth of rat glioma cells ex vivo and in vivo via PPAR-dependent and PPAR-independent pathways. *Carcinogenesis.* **22:** 1747–1755.
8. Tronstad, K. J., K. Berge, E. Dyroy, L. Madsen, and R. K. Berge. 2001. Growth reduction in glioma cells after treatment with tetradecylthioacetic acid: changes in fatty acid metabolism and oxidative status. *Biochem. Pharmacol.* **61:** 639–649.
9. Tronstad, K. J., O. Bruserud, K. Berge, and R. K. Berge. 2002. Antiproliferative effects of a non-beta-oxidizable fatty acid, tetradecylthioacetic acid, in native human Acute Myelogen Leukemia blast cultures. *Leukemia.* **16:** 2292–2301.
10. Shimabukuro, M., M. Ohneda, Y. Lee, and R. H. Unger. 1997. Role of nitric oxide in obesity-induced beta cell disease. *J. Clin. Invest.* **100:** 290–295.
11. Shimabukuro, M., Y. T. Zhou, M. Levi, and R. H. Unger. 1998. Fatty acid-induced beta cell apoptosis: a link between obesity and diabetes. *Proc. Natl. Acad. Sci. USA.* **95:** 2498–2502.
12. Finstad, H. S., H. Dyrendal, M. C. Myhrstad, H. Heimli, and C. A. Drevon. 2000. Uptake and activation of eicosapentaenoic acid are related to accumulation of triacylglycerol in Ramos cells dying from apoptosis. *J. Lipid Res.* **41:** 554–563.
13. Cnop, M., J. C. Hannaert, A. Hoorens, D. L. Eizirik, and D. G. Pipeleers. 2001. Inverse relationship between cytotoxicity of free fatty acids in pancreatic islet cells and cellular triglyceride accumulation. *Diabetes.* **50:** 1771–1777.
14. Hannun, Y. A. 1994. The sphingomyelin cycle and the second messenger function of ceramide. *J. Biol. Chem.* **269:** 3125–3128.
15. Kolesnick, R., and Y. A. Hannun. 1999. Ceramide and apoptosis. *Trends Biochem. Sci.* **24:** 224–225.
16. Okazaki, T., R. M. Bell, and Y. A. Hannun. 1989. Sphingomyelin turnover induced by vitamin D3 in HL-60 cells. Role in cell differentiation. *J. Biol. Chem.* **264:** 19076–19080.
17. Kim, M. Y., C. Linardic, L. Obeid, and Y. Hannun. 1991. Identification of sphingomyelin turnover as an effector mechanism for the action of tumor necrosis factor alpha and gamma-interferon. Specific role in cell differentiation. *J. Biol. Chem.* **266:** 484–489.
18. Williams, R. D., E. Wang, and A. H. Merrill, Jr. 1984. Enzymology of long-chain base synthesis by liver: characterization of serine palmitoyltransferase in rat liver microsomes. *Arch. Biochem. Biophys.* **228:** 282–291.
19. Perry, D. K., J. Carton, A. K. Shah, F. Meredith, D. J. Uhlinger, and Y. A. Hannun. 2000. Serine palmitoyltransferase regulates de novo ceramide generation during etoposide-induced apoptosis. *J. Biol. Chem.* **275:** 9078–9084.
20. Shimabukuro, M., M. Higa, Y. T. Zhou, M. Y. Wang, C. B. Newgard, and R. H. Unger. 1998. Lipoapoptosis in beta-cells of obese prediabetic fa/fa rats. Role Of serine palmitoyltransferase overexpression. *J. Biol. Chem.* **273:** 32487–32490.
21. Spydevold, O., and J. Bremer. 1989. Induction of peroxisomal beta-oxidation in 7800 C1 Morris hepatoma cells in steady state by fatty acids and fatty acid analogues. *Biochim. Biophys. Acta.* **1003:** 72–79.

22. Muna, Z. A., K. Doudin, J. Songstad, R. J. Ulvik, and R. K. Berge. 1997. Tetradecylthioacetic acid inhibits the oxidative modification of low density lipoprotein and 8-hydroxydeoxyguanosine formation in vitro. *Arterioscler. Thromb. Vasc. Biol.* **17:** 3255–3262.
23. Akslen, L. A., K. J. Andersen, and R. Bjerkvig. 1988. Characteristics of human and rat glioma cells grown in a defined medium. *Anticancer Res.* **8:** 797–803.
24. Laerum, O. D., M. F. Rajewsky, M. Schachner, D. Stavrou, K. G. Haglid, and A. Haugen. 1977. Phenotypic properties of neoplastic cell lines developed from fetal rat brain cells in culture after exposure to ethylnitrosourea in vivo. *Z. Krebsforsch. Klin. Onkol. Cancer Res. Clin. Oncol.* **89:** 273–295.
25. Tronstad, K. J., K. Berge, E. N. Flindt, K. Kristiansen, and R. K. Berge. 2001. Optimization of methods and treatment conditions for studying effects of fatty acids on cell growth. *Lipids.* **36:** 305–313.
26. Folch, J., M. Lees, and G. H. Sloane-Stanley. 1957. Method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226:** 497–509.
27. Mangold, H. K. 1969. Aliphatic lipids. *In* Thin-Layer chromatography: a Laboratory Handbook. 2nd ed. E. Stahl, editor. Springer, Berlin. 363–421.
28. Bohov, P., E. Sebokova, D. Gasperikova, P. Langer, and I. Klimes. 1997. Fatty acid composition in fractions of structural and storage lipids in liver and skeletal muscle of hereditary hypertriglyceridemic rats. *Ann. N. Y. Acad. Sci.* **827:** 494–509.
29. Morrison, W. R., and L. M. Smith. 1964. Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride-methanol. *J. Lipid Res.* **5:** 600–608.
30. Bohov, P., V. Balaz, and J. Hrivnak. 1984. Analysis of fatty acid methyl esters on an SP 2340 glass capillary column. *J. Chromatogr.* **286:** 247–252.
31. Takayama, M., S. Itoh, T. Nagasaki, and I. Tanimizu. 1977. A new enzymatic method for determination of serum choline-containing phospholipids. *Clin. Chim. Acta.* **79:** 93–98.
32. Fossati, P., and L. Prencipe. 1982. Serum triglycerides determined colorimetrically with an enzyme that produces hydrogen peroxide. *Clin. Chem.* **28:** 2077–2080.
33. de Pablo, M. A., S. A. Susin, E. Jacocot, N. Larochette, P. Costantini, L. Ravagnan, N. Zamzami, and G. Kroemer. 1999. Palmitate induces apoptosis via a direct effect on mitochondria. *Apoptosis.* **4:** 81–97.
34. Paumen, M. B., Y. Ishida, M. Muramatsu, M. Yamamoto, and T. Honjo. 1997. Inhibition of carnitine palmitoyltransferase I augments sphingolipid synthesis and palmitate-induced apoptosis. *J. Biol. Chem.* **272:** 3324–3329.
35. Mu, Y. M., T. Yanase, Y. Nishi, A. Tanaka, M. Saito, C. H. Jin, C. Mukasa, T. Okabe, M. Nomura, K. Goto, and H. Nawata. 2001. Saturated FFAs, palmitic acid and stearic acid, induce apoptosis in human granulosa cells. *Endocrinology.* **142:** 3590–3597.
36. Bremer, J. 1983. Carnitine-metabolism and functions. *Physiol. Rev.* **63:** 1420–1480.
37. Gomez-Munoz, A. 1998. Modulation of cell signalling by ceramides. *Biochim. Biophys. Acta.* **1391:** 92–109.
38. Obeid, L. M., C. M. Linardic, L. A. Karolak, and Y. A. Hannun. 1993. Programmed cell death induced by ceramide. *Science.* **259:** 1769–1771.
39. Yoshimura, S., H. Sakai, K. Ohguchi, S. Nakashima, Y. Banno, Y. Nishimura, N. Sakai, and Y. Nozawa. 1997. Changes in the activity and mRNA levels of phospholipase D during ceramide-induced apoptosis in rat C6 glial cells. *J. Neurochem.* **69:** 713–720.
40. Nakamura, S., Y. Kozutsumi, Y. Sun, Y. Miyake, T. Fujita, and T. Kawasaki. 1996. Dual roles of sphingolipids in signaling of the escape from and onset of apoptosis in a mouse cytotoxic T-cell line, CTLL-2. *J. Biol. Chem.* **271:** 1255–1257.
41. Wagenknecht, B., W. Roth, E. Gulbins, H. Wolburg, and M. Weller. 2001. C2-ceramide signaling in glioma cells: synergistic enhancement of CD95- mediated, caspase-dependent apoptosis. *Cell Death Differ.* **8:** 595–602.
42. Cuvillier, O., G. Pirianov, B. Kleuser, P. G. Vanek, O. A. Coso, S. Gutkind, and S. Spiegel. 1996. Suppression of ceramide-mediated programmed cell death by sphingosine-1-phosphate. *Nature.* **381:** 800–803.
43. Mutomba, M. C., H. Yuan, M. Konyavko, S. Adachi, C. B. Yokoyama, V. Esser, J. D. McGarry, B. M. Babior, and R. A. Gottlieb. 2000. Regulation of the activity of caspases by L-carnitine and palmitoylcarnitine. *FEBS Lett.* **478:** 19–25.
44. Paumen, M. B., Y. Ishida, H. Han, M. Muramatsu, Y. Eguchi, Y. Tsujimoto, and T. Honjo. 1997. Direct interaction of the mitochondrial membrane protein carnitine palmitoyltransferase I with Bcl-2. *Biochem. Biophys. Res. Commun.* **231:** 523–525.
45. Berge, R. K., L. Madsen, H. Vaagenes, K. J. Tronstad, M. Gottlicher, and A. C. Rustan. 1999. In contrast with docosahexaenoic acid, eicosapentaenoic acid and hypolipidaemic derivatives decrease hepatic synthesis and secretion of triacylglycerol by decreased diacylglycerol acyltransferase activity and stimulation of fatty acid oxidation. *Biochem. J.* **343:** 191–197.
46. Schoonjans, K., B. Staels, and J. Auwerx. 1996. Role of the peroxisome proliferator-activated receptor (PPAR) in mediating the effects of fibrates and fatty acids on gene expression. *J. Lipid Res.* **37:** 907–925.
47. Costantini, P., E. Jacotot, D. Decaudin, and G. Kroemer. 2000. Mitochondrion as a novel target of anticancer chemotherapy. *J. Natl. Cancer Inst.* **92:** 1042–1053.
48. Kroemer, G., N. Zamzami, and S. A. Susin. 1997. Mitochondrial control of apoptosis. *Immunol. Today.* **18:** 44–51.
49. Al-Saffar, N. M., J. C. Titley, D. Robertson, P. A. Clarke, L. E. Jackson, M. O. Leach, and S. M. Ronen. 2002. Apoptosis is associated with triacylglycerol accumulation in Jurkat T-cells. *Br. J. Cancer.* **86:** 963–970.