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Growth reduction in glioma cells after treatment with tetradecylthioacetic acid: changes in fatty acid metabolism and oxidative status

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Abstract

During aerobic metabolism, a small amount of partially reduced oxygen is produced, yielding reactive oxygen species (ROS). Peroxisomes and mitochondria are major contributors to cellular ROS production, which is normally balanced by consumption by antioxidants. The fatty acid analogue tetradecylthioacetic acid (TTA) promotes mitochondrial and peroxisomal proliferation, and may induce oxidative stress and change the growth potential of cancer cells. In the present study, we found that TTA reduced [^{3}H]thymidine incorporation in the glioma cell lines BT4Cn (rat), D54Mg (human), and GaMg (human) in a dose- and time-dependent manner. The 50% inhibitory TTA doses were approximately 125 μM for BT4Cn and D54Mg cells and 40 μM for GaMg cells after 4 days. α-Tochopherol counteracted this inhibition in GaMg cells. TTA enhanced the oxidation of [1-^{14}C]palmitic acid, which could be explained by stimulation of enzymes involved in peroxisomal (fatty acyl-CoA oxidase) and/or mitochondrial (carnitine palmitoyltransferase) fatty acid oxidation. The glutathione content and the activities of glutathione peroxidase, glutathione reductase, and glutathione *S*-transferase were differentially affected. Increased malondialdehyde (MDA) production was seen in TTA-treated GaMg and D54Mg cells, but not in BT4Cn cells, *in vitro*. In BT4Cn tumor tissue from TTA-treated rats, MDA was increased while the α-tocopherol content tended to decrease. TTA increased the level of cytosolic cytochrome *c* in BT4Cn cells, which suggests induction of apoptotic cascades. Although several mechanisms are likely to be involved in the TTA-mediated effects on growth, we propose that modulation of cellular redox conditions caused by changes in fatty acid metabolism may be of vital importance.

Keywords: Glioma; Tetradecylthioacetic acid; Fatty acid oxidation; Oxidative status; Glutathione; Cytosolic cytochrome *c*

1. Introduction

Several compounds modulating cellular lipid metabolism also affect the growth of cancer cells. Such compounds include fatty acids such as EFAs [1–8] as well as more complex compounds such as fibrates and aromatic fatty acids [9,10]. The cytotoxic potential of the EFAs depends on the number and localization of double bonds and the chain length [11,12]. A commonly suggested mechanism behind the EFA-mediated antitumoral influence is generation of oxidative stress and production of lipid peroxides. Reduced growth, or loss in cell viability, may result from the production of peroxides or other ROS that affect specific cellular pathways or irreversibly injure the cell.

For several years, we have investigated the metabolic effects of a novel sulfur-substituted fatty acid analogue, tetradecylthioacetic acid (CH_3-$(CH_2)_{13}$-S-CH_2-COOH), that has major impacts on cellular metabolism [13,14]. This analogue has many chemical and physical properties in common with normal saturated fatty acids, but it cannot undergo β-oxidation [15]. It is established that TTA is a ligand for nuclear receptors in the PPAR family [16,17]. The PPARs are reported to contribute to the regulation of differentiation, proliferation, and apoptosis [18]. In rat liver, TTA leads to increased amounts of mitochondria and peroxisomes [19,20], and the oxidation of fatty acids are enhanced [21–23].

In rats, TTA has been found to increase the hepatic activity of the peroxisomal H_2O_2-producing fatty acyl-CoA oxidase (FAO), and this was followed by increased lipid

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Abbreviations: CPT, carnitine palmitoyltransferase; EFA, essential fatty acid; FAO, fatty acyl-CoA oxidase; MDA, malondialdehyde; PPAR, peroxisome proliferator-activated receptor; PUFA, polyunsaturated fatty acid; ROS, reactive oxygen species; and TTA, tetradecylthioacetic acid.

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peroxidation [24]. The hepatic content of glutathione in TTA-treated rats increased, while the activities of glutathione peroxidase, glutathione *S*-transferase, and glutathione reductase decreased [24,25]. Our work was previously largely focused on physiologically normal cells such as hepatocytes, but it was recently found that TTA has antiproliferative properties in human breast cancer cells (MCF-7) [4,26]. In the present work, we expanded our research on cancer cells and studied the effects of TTA in three glioma cell lines.

It is well documented that mitochondria are major producers of ROS [27], and this may affect their pivotal role in the regulation of apoptosis [28,29]. The mitochondrial production of ROS is closely related to energy metabolism, associated as it is with respiratory activity. It is likely that TTA affects ROS generation by modulating the mitochondrial and/or peroxisomal oxidative function. TTA itself is reported to possess antioxidant properties *in vitro* due to the sulfur atom [30]. In this study, we investigated the effects of TTA on cell growth, fatty acid metabolism, and the redox situation in cancer cells.

2. Materials and Methods

2.1. Chemicals

L-[*methyl*-^{14}C] Carnitine hydrochloride and [^{3}H]thymidine (TRA 310) were purchased from Amersham International. [1-^{14}C]Palmitic acid (50 mCi/mmol) was obtained from New England Nuclear. TTA was prepared at the Department of Chemistry, University of Bergen, as previously described [31]. All other chemicals and solvents were of reagent grade from common commercial sources.

2.2. Cells

The rat glioma cell line BT4Cn [32] and the human glioma cell lines D54Mg and GaMg [33] were routinely kept in a standard culture incubator (37°, 5% CO_2 and 95% air). The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum (NBCS), L-glutamine (0.58 mg/mL), streptomycin (100 μg/mL), penicillin (100 IU/mL), and three times the prescribed concentration of non-essential amino acids (all from Sigma Chemical Co.). For experimental purposes, cells were seeded at a density of 4000 cells per cm^2 and 10,000 cells/mL in tissue culture flasks. The cells were allowed to settle overnight before the fatty acid treatments were started.

TTA (25 mM) was prepared in 0.1 M NaOH at 80°. The 25-mM TTA stock solution was diluted in NBCS (40°) to a concentration of 2 mM. This TTA-supplemented NBCS was used in the preparation of growth medium. The control medium was prepared in the same way, i.e. by replacing 25 mM TTA with 0.1 M NaOH. α-Tochopherol was dissolved in NBCS to a stock solution of 0.8 mM.

2.3. Cell growth

Cell growth was assayed by measuring the incorporation of [^{3}H]thymidine. One thousand cells per well were seeded in flat-bottomed 96-well plates (Costar). At the end of the growth period, [^{3}H]thymidine, 1.0 μCi per well, was added. Following 4 hr of incubation, the cells were harvested, and the nuclear radioactivity was measured by liquid scintillation counting. Cell number was determined in trypsinized cell suspensions using a Coulter Z1 Counter (Coulter Electronics).

2.4. Acid-soluble products and CO_2

The glioma cells were grown in 25-cm^2 flasks. After 6 days with treatment, the medium was removed, followed by addition of 5 mL medium containing [1-^{14}C]palmitic acid (200 μM, 0.25 μCi/mL) 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, the monolayers were placed on ice, 0.75 mL cold 1 M $HC1O_4$ was added to the medium, and the filter paper was moistened by 0.3 mL DL($\pm$)-α-methylbenzylamine ($\pm$)-1-phenyl-ethylamin and methanol mixed 1:1. The cultures were left for 1 hr at room temperature for [^{14}C]CO_2 trapping. The filter papers (containing trapped [^{14}C]CO_2) were then transferred to vials for scintillation measurements. Acid-soluble products were extracted by addition of 5 mL of cold 1 M $HC1O_4$ into the flask. Following centrifugation (10 min, 1800 $\times$ *g*), radioactivity was measured in 1 mL of the supernatant.

2.5. Enzyme activities

The cell cultures were washed with PBS before they were harvested by scraping. After centrifugation (5 min, 200 $\times$ *g*), the cells were suspended in PBS before they again were centrifuged. For CPT I measurements, the pellet was suspended in H-buffer (0.25 M sucrose, 2 mM HEPES, 0.2 mM EGTA, pH 7.40) followed by homogenization by repeatedly forcing the cell suspensions through a ball-bearing homogenizer [34] or by using a Dounce hand homogenizer. For measurements of enzyme activities not depending on intact mitochondrial membrane integrity, the cells were suspended in distilled H_2O after harvesting, and then stored at $-80°$. After thawing, nuclei and cell debris were removed by centrifugation for 15 min at 750 $\times$ *g* in a table-top centrifuge. CPT I and CPT II activities were determined in total homogenates essentially as described by Bremer [35] and modified as described in [36]. FAO activity was assayed in total homogenates as described in [37]. Glutathione peroxidase activity was measured as described by Flohè and Gunzler [38], with *t*-butyl hydroperoxide as substrate. Glutathione *S*-transferase activity was measured according to the method of Habig *et al*. [39], while the activity of glu-

tathione reductase was measured as described by Eklöw *et al*. [40].

2.6. Glutathione

Cells suspended in distilled H_2O were extracted with equal volumes of cold 5% sulfosalicylic acid with 50 μM dithioerythritol before they were maintained at −80°. Sample preparation and HPLC analysis were performed according to the method described by Svardal *et al*. [41].

2.7. In vivo experiments

Animal experiments were conducted in accordance with institutional guidelines. BT4Cn cells were intracranially implanted into male BD-IX rats (Gades Institute, Haukeland Hospital, Norway) using a stereotactical technique [42]. The rats were treated daily with palmitic acid or TTA (300 mg/kg body weight per day) using oro-gastric administration. Near the time of death, the rats were killed and the tumors collected and frozen in liquid nitrogen.

2.8. Lipid peroxidation

MDA in tumor tissue was determined according to the method described in [43].

2.9. α-Tocopherol

The α-tocopherol content was measured by HPLC as described in [43].

2.10. Cytochrome c in cytosol

Cells were homogenized as described in "Enzyme activities" and the suspension was centrifuged at 1930 × *g* for 15 min. The supernatant (total homogenate) was centrifuged at 111,000 × *g* for 90 min for preparation of cytosolic (supernatant) fraction. Cytochrome *c* was determined using a rat/mouse cytochrome *c* immunoassay (MCTC0) provided by R&D Systems.

2.11. Statistical analysis

The data are presented as means ± SD. The results were evaluated by a two-sample variance Student's *t*-test (two-tailed distribution). The level of significance was set at $P < 0.05$.

3. Results

3.1. Cell growth

The presented results demonstrate that TTA reduced [^{3}H]thymidine incorporation in the two human glioma lines (GaMg and D54Mg) and in the rat glioma line (BT4Cn) (Fig. 1). GaMg cells appeared to be most sensitive, showing 50% inhibition in [^{3}H]thymidine incorporation at 40 μM TTA after 4 days of treatment (Fig. 1A), while a 50% reduction in [^{3}H]thymidine incorporation was found at approximately 125 μM TTA in both D54Mg and BT4Cn (Fig. 1, B and C, respectively). The TTA concentration giving a 50% reduction in [^{3}H]thymidine incorporation decreased after 6 days to 30 μM and to near 100 μM in GaMg and D54Mg, respectively (Fig. 1, A and B). A dose-dependent reduction in cell number was found in BT4Cn cultures that were treated with TTA (Fig. 1D), showing that reduced proliferation is associated with decreased incorporation of [^{3}H]thymidine (Fig. 1C).

Addition of α-tocopherol to the growth medium counteracted the effect of TTA on [^{3}H]thymidine incorporation in GaMg cells (Fig. 2). Increased [^{3}H]thymidine incorporation was also found in α-tocopherol-supplemented D54Mg cultures, but in contrast to what was seen in GaMg cells, the stimulation was evident both in control and TTA-treated cultures. α-Tocopherol had a concentration-dependent influence on BT4Cn cells. α-Tocopherol (40 μM) stimulated whereas 80 μM α-tocopherol decreased [^{3}H]thymidine incorporation. Supplementation of BT4Cn cultures with α-tocopherol did not improve [^{3}H]thymidine incorporation in TTA-treated cells (Fig. 2).

3.2. Fatty acid oxidation

TTA is a potent mitochondrial and peroxisomal proliferator in tissues exhibiting PPARα-regulated catabolism of fatty acids [16]. In our experiments, TTA increased the total oxidation of [1-^{14}C]palmitic acid in both human glioma lines (Fig. 3C). In D54Mg cells, total [1-^{14}C]palmitic acid oxidation increased dose-dependently, exceeding 3-fold stimulation in 150 μM TTA (Fig. 3C). In contrast, total [1-^{14}C]palmitic acid oxidation in GaMg cells was not significantly increased until the TTA concentration reached 150 μM (Fig. 3C). A moderate dose-dependent stimulation of total [1-^{14}C]palmitic acid oxidation, with a maximum at 150 μM, was found in the rat BT4Cn line (Fig. 3C). Generally, since the production of acid-soluble products is the major contributor to the calculated total [1-^{14}C]palmitic acid oxidation, the effects on the production of acid-soluble products closely resemble those seen on total [1-^{14}C]palmitic acid oxidation (Fig. 3, A and C). Acid-soluble products are produced both in mitochondria and in peroxisomes, but complete oxidation to CO_2 only occurs in mitochondria. Although the production of acid-soluble products was higher in untreated GaMg cells than in D54Mg cells, CO_2 production was higher in the latter (Fig. 3, A and B). Both cell lines responded to TTA by increasing their CO_2 production (Fig. 3B). This effect was most significant in D54Mg cells, in which CO_2 production increased to a plateau at nearly 3-fold in 50 μM TTA, without showing a further increase at higher concentrations (Fig. 3B).

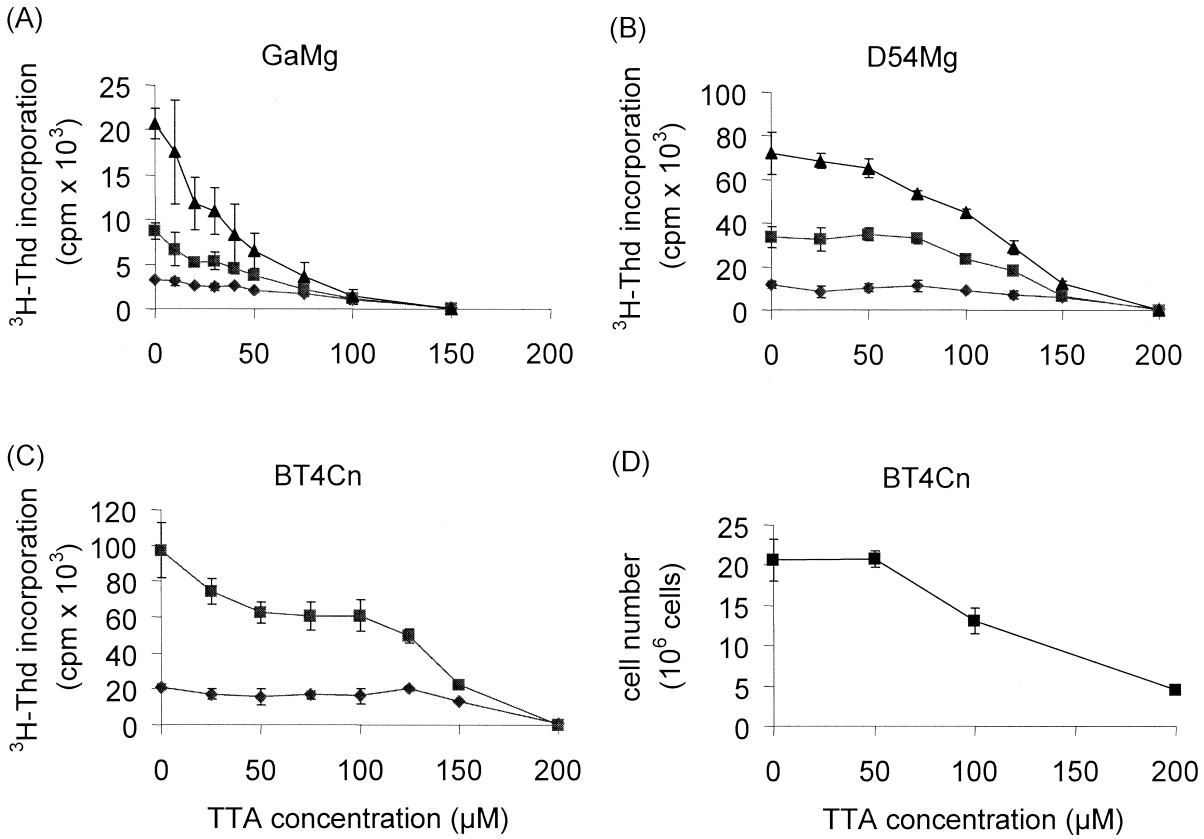

Fig. 1. Effects of TTA on [^{3}H]thymidine incorporation in three glioma cell lines: GaMg (A), D54Mg (B), and BT4Cn (C). [^{3}H]Thymidine incorporation was measured after 2 (—◆—), 4 (—■—), and 6 (—▲—) days of TTA treatment (A–C). BT4Cn cell number (D) was measured after 6 days. Each point, with error bars, represents the mean ± SD of triplicates.

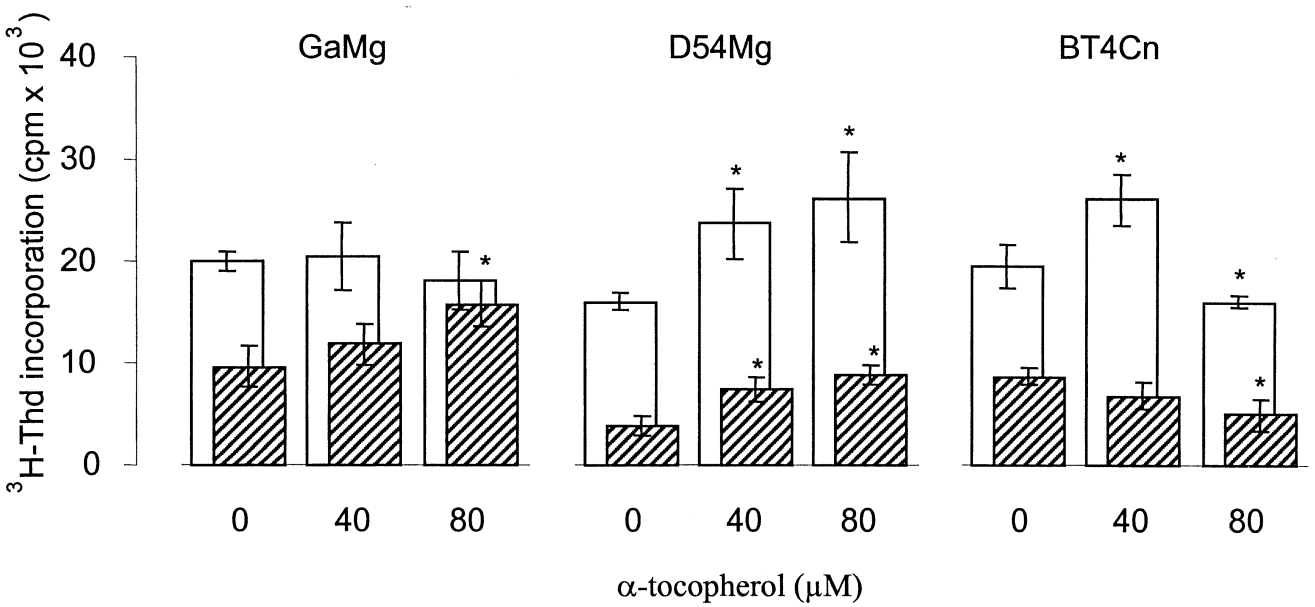

Fig. 2. Effect of α-tocopherol on TTA-mediated reduction in [^{3}H]thymidine incorporation in glioma cells. GaMg, D54Mg, and BT4Cn cells were grown for 4 days without (open bars) or with (striped bars) 40, 125, and 125 μM TTA, respectively, supplemented with α-tocopherol. The TTA doses were based on the concentrations known to give 50% reduction in [^{3}H]thymidine incorporation. Each bar represents the mean ± SD (N = 4). *Significantly different compared to 0 μM α-tocopherol $P < 0.05$.

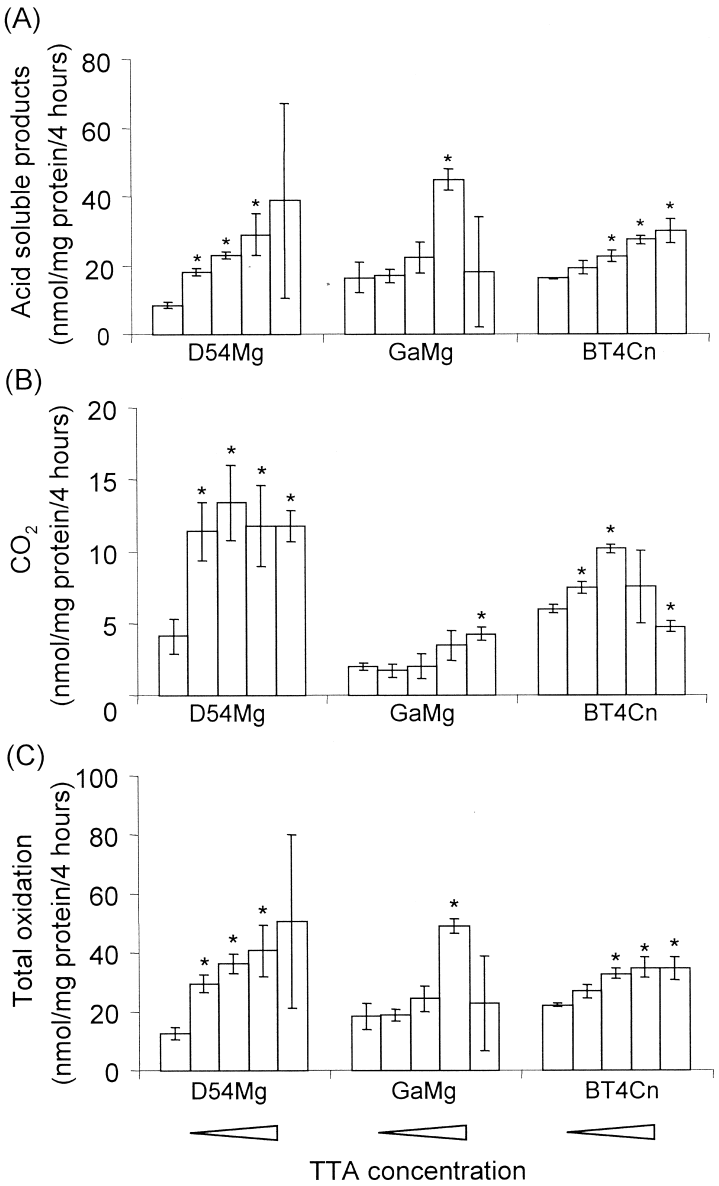

Fig. 3. Oxidation of [1-^{14}C]palmitic acid in glioma cell cultures. D54Mg, GaMg, and BT4Cn cells were grown for 6 days in growth medium supplemented with different concentrations of TTA (0, 50, 100, 150, and 200 μM). The cultures were then incubated for 4 hr with 100 μM [1-^{14}C]-labeled palmitic acid. Each bar represents the mean ± SD of triplicates. [1-^{14}C]-Labeled acid-soluble products (A) and CO_2 (B) were assayed in the same cultures. Total [1-^{14}C]palmitic acid oxidation (C) was calculated as the sum of acid-soluble products and CO_2. *Significantly different from control $P < 0.05$.

Increased CO_2 production was evident in GaMg cells treated with 150 and 200 μM TTA. CO_2 production also increased in the rat BT4Cn line although, at concentrations exceeding 100 μM, this effect was clearly reversed (Fig. 3B). Generally, the stimulating effect of TTA on the oxidation of [1-^{14}C]palmitic acid was more evident in the human cell lines than in the rat cell line.

3.3. Enzyme activities

To further localize the TTA-mediated effects on fatty acid oxidation, we measured the activities of key enzymes in both mitochondrial (CPT I and CPT II) and peroxisomal (FAO) β-oxidation. Due to the reduction in protein amounts from TTA-treated cell cultures, it was not possible to measure enzyme activities in GaMg and D54Mg cells at TTA doses higher than 50 and 150 μM, respectively.

CPT I activity was not affected by TTA in the absence of malonyl-CoA, but in the presence of 20 μM malonyl-CoA this activity increased 3.7-fold in homogenates of TTA-treated (100 μM) D54Mg cells compared to control cells (Table 1). Although TTA stimulated CPT I activity in the absence of malonyl-CoA in BT4Cn cells, this stimulation was more evident in the presence of malonyl-CoA (Table 1). Thus, in both D54Mg and BT4Cn cells, TTA treatment decreased the malonyl-CoA sensitivity of CPT I. This is in contrast to what we previously observed in rat liver [44].

Table 1
Effect of TTA on the activity of CPT I in two glioma cell lines

		−Malonyl-CoA	+Malonyl-CoA
D54Mg	control	1.16 ± 0.06	0.09 ± 0.01
	TTA	1.28 ± 0.13	0.33 ± 0.06*
BT4Cn	control	0.21 ± 0.03	0.04 ± 0.01
	TTA	0.38 ± 0.08*	0.16 ± 0.03*

Cells were grown for 6 days in growth medium supplemented with 100 μM TTA. After homogenization, a postnuclear fraction was prepared for enzyme activity measurements. The CPT I activity was measured with and without addition of 20 μM malonyl-CoA. The specific enzyme activities (recorded as nmol/min/mg protein) are presented as means ± SD (n = 3–6).

* Significantly different from control $P < 0.05$.

In D54Mg cells, TTA increased CPT II activity dose-dependently up to 150 μM (Fig. 4B). No change in CPT II activity was present in GaMg cells grown in 50 μM TTA (Fig. 4A). CPT II activity in BT4Cn cells was increased by the TTA treatment (2.5-fold at 100 μM TTA), although the induction was reversed in doses exceeding 100 μM TTA (Fig. 4C).

Peroxisomal FAO activity in GaMg and D54Mg cells was not significantly affected by TTA treatment (Fig. 4, A and B). In contrast, FAO activity in BT4Cn cells increased in a dose-dependent manner up to 4-fold induction in 100 μM TTA, without giving an additional increase at higher TTA concentrations (Fig. 4C).

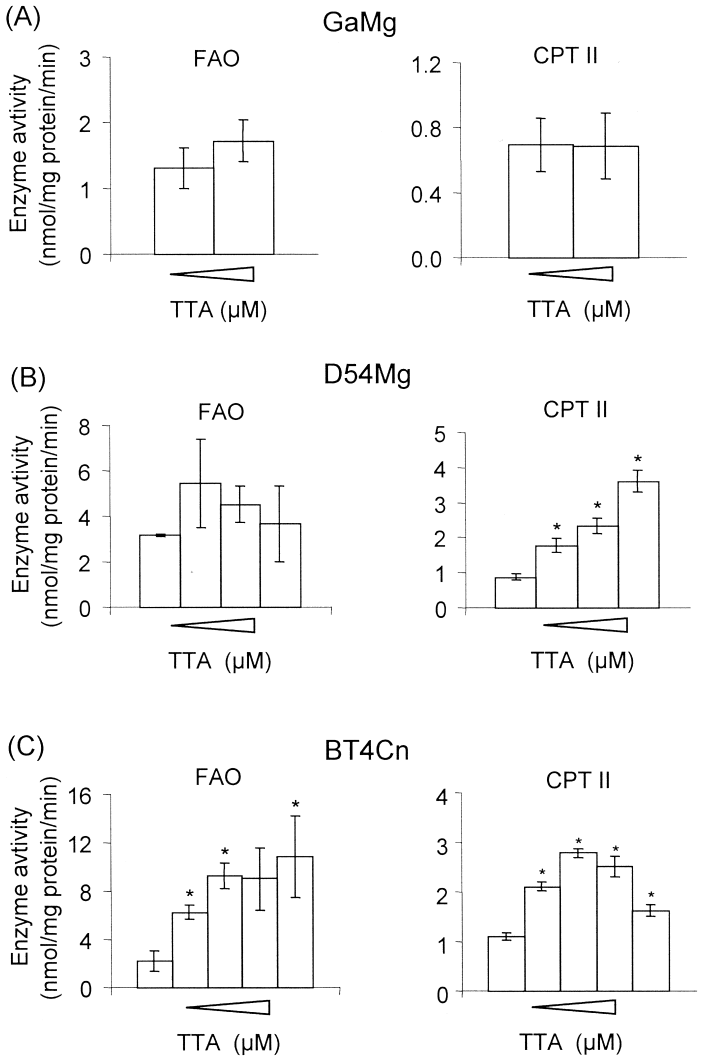

Fig. 4. Effects of TTA on the activities of FAO and CPT II. GaMg (A), D54Mg (B), and BT4Cn (C) cells were grown for 6 days in growth medium supplemented with different concentrations of TTA (A: 0 and 50 μM; B: 0, 50, 100, and 150 μM; C: 0, 50, 100, 150, and 200 μM). The cultures were then harvested and suspended in dH_2O before they were maintained at −80° until the enzyme activities were assayed. Each bar represents the mean ± SD of triplicates. *Significantly different from control $P < 0.05$.

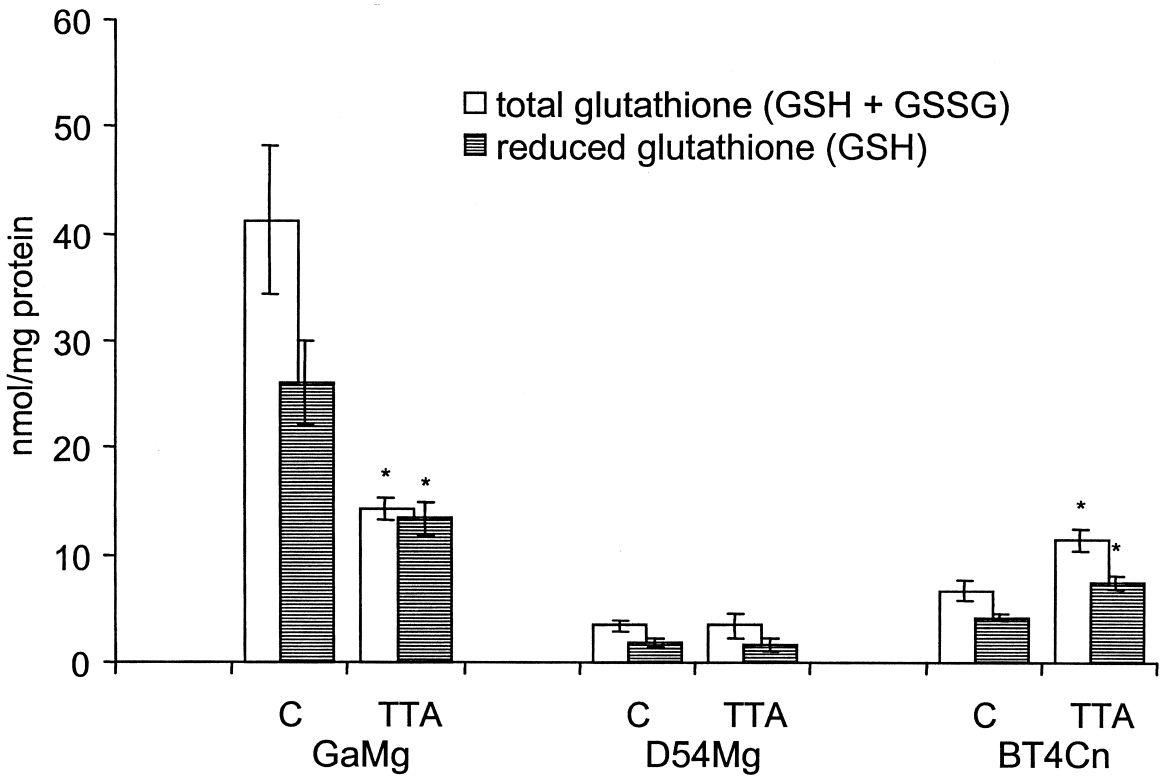

Fig. 5. Changes in cellular content of glutathione. Cells were grown for 6 days in the absence (C) or presence of 100 μM TTA. Following harvesting, the cells were extracted with an equal volume of cold 5% sulfosalicylic acid containing 50 μM dithioerythritol, and then frozen. Determination of glutathione was performed as described in "Materials and Methods". The bars give mean values ± SD of triplicates. *Significantly different from control $P < 0.05$.

3.4. Glutathione and associated enzymes

The cellular glutathione content decreased in TTA-treated GaMg cells while remaining unaffected in D54Mg cells (Fig. 5). In the rat glioma line BT4Cn, the cellular glutathione content was positively affected by TTA (Fig. 5). Glutathione peroxidase activity was not affected by TTA in the two human cell lines, but was 4.8-fold higher in untreated D54Mg cells compared to untreated GaMg cells (Table 2). Glutathione reductase activity was not affected in D54Mg cells, but decreased in GaMg cells after TTA treatment (Table 2). TTA did not affect the activity of glutathione *S*-transferase in D54Mg cells (Table 2). The rat glioma line BT4Cn differed from the two human lines as the activities of glutathione peroxidase, glutathione reductase, and glutathione *S*-transferase were increased by TTA (Table 2).

3.5. Oxidative damage in glioma cells in vitro and in vivo

Fig. 6 shows that TTA, at a dose giving an approximate 50% reduction in [^{3}H]thymidine incorporation (Fig. 1), induced a slightly increased production of MDA in GaMg and D54Mg cells, but not in BT4Cn cells, after 2 days.

Administration of TTA increased the survival time of rats with intracranially implanted BT4Cn gliomas.[1] In con-

[1]Berge K, Tronstad KJ, Flindt EN, Rasmussen TH, Bjerkvig R, Madsen L, Kristiansen K, Berge RK, manuscript in preparation.

Table 2
Effects of TTA activities of glutathione-associated enzymes in three glioma cell lines

	GaMg		D54Mg		BT4Cn	
	Control	TTA	control	TTA	control	TTA
GPx	4.9 ± 2.5	7.6 ± 6.2	23.4 ± 5.9	25.1 ± 3.3	41.9 ± 7.5	62.1 ± 5.0*
GR	40.2 ± 4.0	19.7 ± 3.3*	55.9 ± 9.0	62.0 ± 0.7	16.7 ± 2.2	31.4 ± 0.1*
GST	ND	ND	10.5 ± 4.0	8.9 ± 0.1	18.9 ± 8.8	40.2 ± 2.4*

Cells were grown for 6 days in growth medium supplemented with 100 μM TTA. The cells were homogenized by suspending in dH_2O followed by freezing at −80°. The specific enzyme activities are given as nmol/min/mg protein (mean ± SD of triplicates).
ND = not determined, GPx = glutathione peroxidase, GR = glutathione reductase, GST = glutathione *S*-transferase.
* Significantly different from control $P < 0.05$.

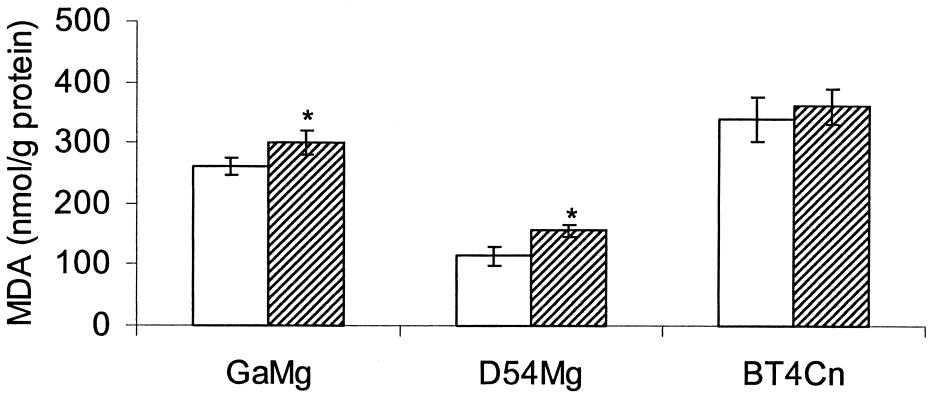

Fig. 6. Effect of TTA on production of MDA in glioma cells. GaMg, D54Mg, and BT4Cn cells were grown for 2 days without (control, open bars) or with (striped bars) 25, 150, and 150 μM TTA, respectively. Each bar represents the mean ± SD (N = 3). *Significantly different from control $P < 0.05$.

trol rats, the α-tocopherol content in glioma tissue was the same as in normal brain tissue, but there was a tendency toward a decrease in glioma tissue from TTA-treated rats (Fig. 7A), although this was not statistically significant. Fig. 7B shows that MDA production was relatively high in the glioma tissue compared to normal brain tissue, and a moderate increase was seen in the TTA-treated rats compared to the control rats given palmitic acid. The discrepancy between the *in vivo* and *in vitro* measurements may be due to the duration of the treatment, since the cell cultures were treated for 2 days and the rats with intracranial tumor were treated for approximately two weeks.

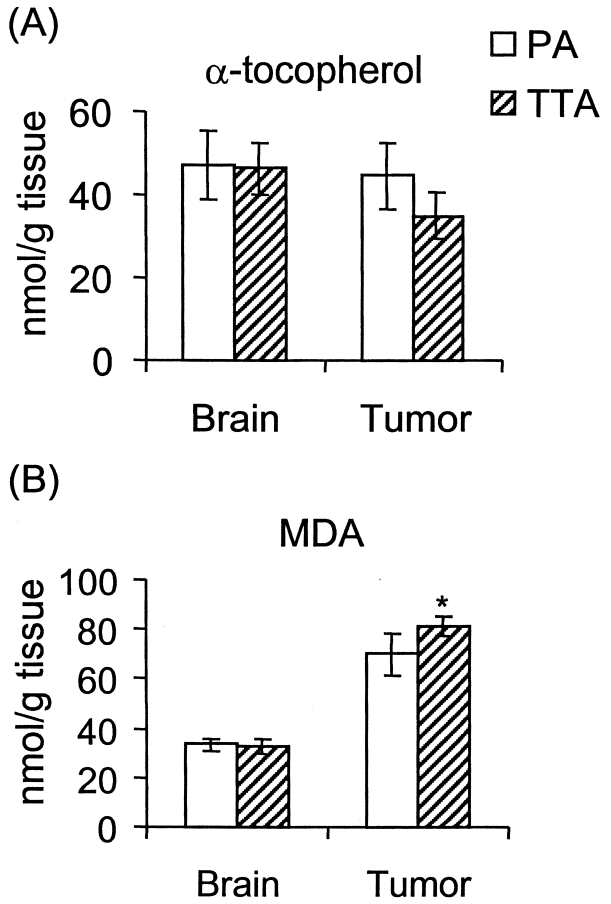

Fig. 7. Effects of TTA on α-tocopherol (A) and lipid peroxidation (B) in normal brain and intracranially implanted BT4Cn glioma tissue. The experiments were performed as described in "Materials and Methods". Control rats were given palmitic acid (PA). The bars represent the means ± SD (N = 3–6). *Significantly different from control $P < 0.05$.

3.6. Cytochrome c in cytosol

Cytochrome *c* was measured in the cytosolic fraction of BT4Cn cells after 0, 3, 6, 12, and 18 hr of TTA treatment (Fig. 8). Up to 12 hr, the amount of cytosolic cytochrome *c* was approximately 2% of the amount present in the total homogenate. After 12 hr, the cytosolic cytochrome *c* concentration exceeded 10% of the amount present in the total homogenate.

4. Discussion

This study demonstrates that TTA reduces the growth of glioma cells, as can be seen from the reduced [^{3}H]thymidine incorporation and the reduction in cell number (Fig. 1). However, the cell lines differed in their degree of sensitivity. GaMg cells clearly appeared most sensitive, while D54Mg and BT4Cn cells tolerated higher doses (Fig. 1). Different mechanisms seemed to be involved in the TTA-mediated growth reduction in the different cell lines.

All three glioma cell lines were able to oxidize palmitic acid as a source of energy (Fig. 3). A TTA-mediated increase in palmitic acid oxidation was evident in all three cell lines, although in GaMg cells this effect was not seen with TTA concentrations lower than 150 μM (Fig. 3). The dose-dependent increase in palmitic acid oxidation in the D54Mg cells can be explained by increased mitochondrial oxidation, since the activity of CPT II increased (Fig. 4B) and the malonyl-CoA sensitivity of CPT I decreased (Table 1). The mitochondrial capacity to oxidize palmitic acid to CO_2 was

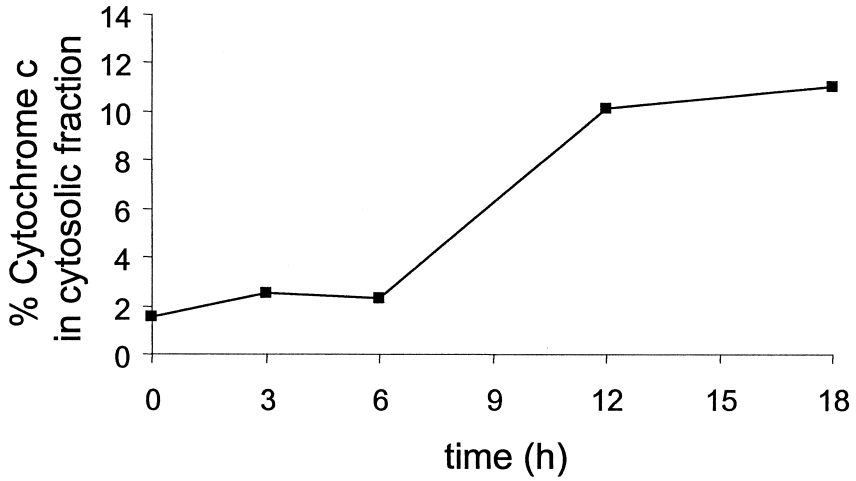

Fig. 8. Effect of TTA on amount of cytochrome *c* in cytosol. BT4Cn cells were treated with 200 μM TTA. Cytochrome *c* was determined in homogenates and cytosolic fractions after different time intervals. The amount of cytosolic cytochrome *c* is displayed as a percent of the amount of cytochrome *c* present in the total homogenate. Each point represents the mean of duplicates.

maximized at low doses of TTA (Fig. 3B), although the oxidation of palmitic acid to acid-soluble products was further stimulated at higher concentrations (Fig. 3A). The dose–response of CO_2 production in BT4Cn (Fig. 3B) resembled the dose–response of mitochondrial CPT II activity (Fig. 4C). This is in accordance with the fact that mitochondria are the main cellular producers of CO_2, and indicates that increased mitochondrial oxidation can account for the TTA-mediated changes in CO_2 production in BT4Cn cells. However, it seems likely that the small increase in the production of acid-soluble products (Fig. 3A) can be attributed to increased peroxisomal β-oxidation, since the FAO activity responded rather vigorously compared to the CPT II activity (Fig. 4C). Although the activities of FAO and CPT II could not be obtained in GaMg cells at TTA doses exceeding 50 μM, the increased CO_2 production indicates that elevated mitochondrial oxidation contributes to the observed stimulation of palmitic acid oxidation in this cell line as well (Fig. 3). These results indicate that the TTA-mediated increase in fatty acid oxidation in the human glioma line D54Mg can be explained by increased mitochondrial oxidation, while peroxisomal activity is more important in the rat glioma line BT4Cn. This is in line with the observation that peroxisomal activation is more evident in rodent cells than in human cells after treatment with peroxisome proliferators such as TTA.

Increased oxidative stress leading to lipid peroxidation is thought to be the mechanism behind growth reduction mediated by several PUFAs (reviewed in [11]). In our studies, α-tocopherol was supplemented to the growth medium to investigate whether improved antioxidant status could counteract TTA-mediated growth reduction. α-Tocopherol specifically counteracted the inhibiting effects of TTA on [^{3}H]thymidine incorporation in GaMg cells (Fig. 2). In contrast, α-tocopherol seemed to improve the general growth conditions for D54Mg cells without interacting with the mechanisms behind the effect of TTA on [^{3}H]thymidine incorporation. α-Tocopherol did not enhance [^{3}H]thymidine incorporation in TTA-treated BT4Cn cells.

Glutathione peroxidase activity, which is reported to protect against PUFA-induced cell death [3], appeared to be very low in the GaMg cells (Table 2). It was previously suggested that low glutathione peroxidase activity contributes to the sensitivity of cancer cells to PUFAs [45], and from our data this suggestion may also be valid for TTA. TTA seemed to weaken the glutathione machinery in GaMg cells, whereas the glutathione content was unchanged in D54Mg cells (Fig. 5 and Table 2). Interestingly, both human cell lines responded to TTA by increasing MDA production (Fig. 6). In contrast, the glutathione system in the BT4Cn line seemed to be stimulated upon TTA treatment (Fig. 5 and Table 2). However, the activity of the H_2O_2-producing enzyme FAO was significantly increased in TTA-treated BT4Cn cells (Fig. 4C). Although TTA did not increase MDA production in BT4Cn cells in culture, the tendency toward a decrease in α-tocopherol may account for the increase in MDA observed in BT4Cn tumor tissue from TTA-treated rats (Fig. 7).

Cytosolic long-chain acyl-CoA esters inhibit mitochondrial adenine nucleotide translocators, resulting in an intramitochondrial ADP deficiency [46]. *In vitro*, such a deficiency is a potent stimulator of mitochondrial ROS production [47]. It has been suggested that this situation results in accumulation of electrons along the electron transfer chain in the inner mitochondrial membrane, which subsequently leads to increased superoxide production [48]. It has also been proposed that the level of long-chain fatty acyl-CoA may play a role in palmitic acid-induced apoptosis, and that CPT I, which is situated in the mitochondrial outer membrane, removes long-chain fatty acyl-CoA from the cytoplasm and thus protects against this type of apoptosis [49,50]. Paumen *et al*. [49] used etomoxir to inhibit CPT

I, which enhanced palmitic acid-induced cell death. The antiapoptotic Bcl-2 protein was found to directly interact with CPT I in a yeast two-hybrid system [50]. As we have shown in this work, TTA stimulates fatty acid oxidation (Figs. 3 and 4) and could thus change the concentration of cellular acyl-CoAs. On the other hand, TTA itself is hardly oxidized and may therefore accumulate in the cell as TTA-CoA [51], which may cause intramitochondrial ADP deficiency, increased ROS production, and apoptosis. Furthermore, TTA caused a release of cytochrome *c* into cytosol in BT4Cn cells (Fig. 8), possibly affecting the accumulation of electrons in the electron transfer chain and the mitochondrial production of ROS. Another consequence may be the induction of apoptosis, since cytosolic cytochrome *c* is known to bind to apoptosis protease activation factor-1 and activate an apoptotic program [52–54]. These considerations are very interesting since several cell lines, when treated with TTA, show characteristic apoptotic features such as blebbing and nuclear condensation.[2]

We conclude that the TTA-mediated increase in fatty acid oxidation is due to increased mitochondrial fatty acid oxidation in the human line D54Mg, while both the mitochondria and the peroxisomes contribute to this response in the rat BT4Cn line. The experiments revealed insufficient data for us to draw a conclusion as to GaMg, although the increased CO_2 production indicates that mitochondria take part in the oxidative stimulation. Increased oxidative stress can be justified as a possible mechanism behind the reduced growth in the most sensitive glioma cell line, GaMg. However, we cannot exclude that redox conditions may also play a role in D54Mg and BT4Cn. The increased amount of cytochrome *c* in the cytosol of TTA-treated BT4Cn cells may be of major relevance to the antiproliferative effect, since cytosolic cytochrome *c* is known to activate apoptotic cascades. This is in line with preliminary results on the induction of apoptosis in our laboratory. Our observations increase our suspicion that mitochondria are targets for TTA action.

It must be decided whether some of the described TTA-mediated effects actually contribute to the antiproliferative properties or if they only parallel the growth effects. Are the changes in fatty acid metabolism linked to the increased formation of ROS or is the increased lipid peroxidation caused by other factors? These aspects are currently investigation in our laboratory.

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[2] Data not shown.

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