Published in final edited form as:

Neurosurgery. 2006 December ; 59(6): 1313–1324. doi:10.1227/01.NEU.0000249218.65332.BF.

Metabolic Remodeling of Malignant Gliomas for Enhanced Sensitization during Radiotherapy: An In Vitro Study

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Abstract

OBJECTIVE—To investigate a novel method to enhance radiosensitivity of gliomas via modification of metabolite flux immediately before radiotherapy. Malignant gliomas are highly glycolytic and produce copious amounts of lactic acid, which is effluxed to the tumor microenvironment via lactate transporters. We hypothesized that inhibition of lactic acid efflux would alter glioma metabolite profiles, including those that are radioprotective. ¹H magnetic resonance spectroscopy (MRS) was used to quantify key metabolites, including those most effective for induction of low-dose radiation-induced cell death.

METHODS—We inhibited lactate transport in U87-MG gliomas with α -cyano-4-hydroxy-cinnamic acid (ACCA). Flow cytometry was used to assess induction of cell death in treated cells. Cells were analyzed by MRS after ACCA treatment. Control and treated cells were subjected to low-dose irradiation, and the surviving fractions of cells were determined by clonogenic assays.

RESULTS—MRS revealed changes to intracellular lactate on treatment with ACCA. Significant decreases in the metabolites taurine, glutamate, glutathione, alanine, and glycine were observed, along with inversion of the choline/phosphocholine profile. On exposure to low-dose radiation, ACCA-pretreated U-87MG cells underwent rapid morphological changes, which were followed by apoptotic cell death.

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DISCLOSURE

None of the authors received any financial support in conjunction with the generation of this study. None of the authors has a financial conflict of interest.

CONCLUSION—Inhibition of lactate efflux in malignant gliomas results in alterations of glycolytic metabolism, including decreased levels of the antioxidants taurine and glutathione and enhanced radiosensitivity of ACCA-treated cells. Thus, in situ application of lactate transport inhibitors such as ACCA as a novel adjunctive therapeutic strategy against glial tumors may greatly enhance the level of radiation-induced cell killing during a combined radio- and chemotherapeutic regimen.

Keywords

α -cyano-4-hydroxycinnamic acid; Glioma therapy; Lactate; Magnetic resonance spectroscopy; Tumor metabolism

Glioblastoma multiforme (GBM) is the most common of all primary brain tumors. It is also the most aggressive, with patient survival being dismal with conventional therapies. Despite numerous clinical approaches, little progress in prolonging patient survival has been achieved in the past 20 years. Similar to most highly malignant tumors, these aggressive gliomas are highly glycolytic, producing large amounts of lactic acid as a metabolic by-product. This aberrant metabolic phenotype is known as aerobic glycolysis because it occurs even in the presence of oxygen (2, 27, 30, 39). In contrast, normal cells produce lactate mainly under anaerobic conditions (anaerobic glycolysis).

Thus, such malignant tumors require an effective mechanism for the rapid disposal of accumulating lactic acid to the tumor microenvironment. Tumors, as well as normal tissue, use a family of transmembrane transporters (monocarboxylate transporter isoforms 1 to 9) (14, 15) for this purpose, in which isoforms with different substrate affinities are expressed in a tissue-specific manner throughout the organism.

Previous studies from our laboratory (26) indicated differential expression of these isoforms in normal brain versus malignant glioma, in which the Type 3 isoform was predominantly expressed in normal tissue, whereas Type 1 and 2 isoforms were highly expressed in GBMs. Our in vitro studies using small interfering ribonucleic acids indicated that selective blocking of lactate efflux (by targeting glioma-expressed mono-carboxylate transporter isoforms) induced critical metabolite changes in the tumors, resulting in cell death (26). Intracellular pH changes were observed (a fourfold increase in H^+ concentrations, resulting in a pH change from 7.4 to 6.8) as well as indications of altered cofactor profiles (oxidized nicotinamide adenine dinucleotide [NAD^+]/reduced nicotinamide adenine dinucleotide [$NADH$] and oxidized nicotinamide adenine dinucleotide phosphate [$NADP^+$]/reduced nicotinamide adenine dinucleotide phosphate [$NADPH$]). Thus, tumor-specific blocking of these transporters presented an attractive anticancer therapeutic target that may debilitate such malignant tumors, either via disruption of energy flux through the tumor cells or via metabolite remodeling within the tumors.

α -cyano-4-hydroxycinnamic acid (ACCA; CHC; mw 189.2) is a known competitive inhibitor of mammalian lactate transporters (13, 36) that also inhibits pyruvate entry into mitochondria (13). We hypothesized that application of ACCA to malignant gliomas might disrupt energy-generating metabolic cascades (both aerobic glycolysis and oxidative phosphorylation), promoting both induction of apoptosis and disruption of pyruvate-derived antioxidant biosynthesis via mitochondria-derived $NADH$. Disruption of glycolysis would also affect the pentose phosphate pathway-derived $NADPH$ /glutathione-generating mechanisms and $NADH$ synthesis at the glyceraldehyde-3-P-dehydrogenase step of the glycolytic pathway, thus depleting the overall capacity of the tumor to defend against free radical-induced cell damage.

We reasoned that a combined experimental strategy of inhibiting lactate efflux with concomitant treatment with radiation would result in the targeted gliomas being highly radiosensitized towards free radical-induced damage and also energetically debilitated because of the disruption of key metabolic pathways. Thus, a combined protocol of chemotherapy and radiotherapy should provide a more effective GBM-targeting strategy.

Radiotherapy has been one of the more effective modalities for therapy of malignant glioma, and dosages of 60 Gy are routinely used (10, 21). However, these can result in toxicity to the surrounding normal brain tissue, including leukoencephalopathy and necrosis (40). Therefore, strategies to enhance radiosensitivity of targeted glioma would permit lower radiation doses to be used, thereby vastly minimizing the frequently observed normal tissue damage while maintaining the efficacy of radiotherapy treatment. Low-dose radiotherapy using doses between 0.5 and 1.0 Gy has been suggested as a potential strategy to develop improved radiotherapeutic regimens while minimizing excessive radiation-induced injury (3, 17, 22, 25).

In the present investigation, we first established that ACCA acts only as a cell-surface inhibitor of lactate transporters in the model U-87MG glioma cells and that it does not internalize. Thus, we reasoned that a combined strategy of first using ACCA as an inhibitor to disrupt glioma metabolism would reduce cellular radioprotectant generation and that subjecting the targeted cells to low-dose irradiation would then provide an avenue for an improved chemoradiotherapy regimen against malignant glioma while significantly minimizing damage to normal tissues. Using in vitro studies, we first used magic angle spinning (MAS) ^1H magnetic resonance spectroscopy (MRS) to demonstrate that radioprotective metabolites are significantly reduced when lactate efflux is blocked in glioma cells and that the treated cells are highly susceptible to low-dose radiation-induced cellular damage, resulting in cell death.

MATERIALS AND METHODS

Cell Line and Reagents

Human malignant glioma cell line U-87 MG was from the American Type Culture Collection (Manassas, VA) and was maintained in Dulbecco modified Eagle's medium/F-12 (DMEM/F12) media supplemented with 10% fetal bovine serum (FBS) and antibiotics (penicillin-streptomycin). All cell culture reagents and serum were purchased from the Invitrogen Corporation (Carlsbad, CA).

ACCA

ACCA is a competitive inhibitor of lactate/pyruvate transporters. 10-mol/L solutions of ACCA were freshly prepared in DMEM/F12 medium in the absence of serum. The pH levels of the solutions were adjusted to 7.4 with NaOH and then sterilized by passing through 0.22- μm syringe filter units (Millex-GV, Milheim, France).

Determining the 30% lethal dose for U-87MG Cells Treated with ACCA

Preliminary studies were conducted to determine the 30% lethal dose (LD_{30}) of U-87 MG cells on exposure to ACCA. U-87 MG cells were seeded in 24-well cell culture plates (Corning Costar #3524; Corning Incorporated Life Sciences, Acton, MA) at a density of 5×10^4 cells per well in DMEM/F12 media supplemented with 10% FBS and were incubated for 24 hours at 37°C. Approximately 24 hours after seeding, the spent media were removed from the plates and mixed with the 10.0-mol/L ACCA stock solution to prepare final concentrations ranging from 0.5 mmol/L to 5.0 mol/L (0.5, 1, 5, 10, 25, and 50 mmol/L, and 1.0 and 5.0 mol/L) and were immediately added back to the plates. Examination of cell

morphology and trypan blue dye exclusion analysis 24 hours after ACCA addition revealed a lack of cell viability at ACCA concentrations of 25 mmol/L and greater. The experiment was then repeated with a more narrow range of ACCA to more accurately determine LD₅₀. In brief, cells were reseeded in 24-well plates as before, and the cells were exposed to ACCA concentrations of 5, 10, 20, and 25 mmol/L. Control cells contained growth media only, in the absence of ACCA. Cell viability was assessed as before by morphological analysis, flow cytometry, and trypan blue dye exclusion assays.

Morphological Analysis

The cells were analyzed by light microscopy before ACCA treatment and 6, 18, and 24 hours after ACCA treatment. Digital histomicrographs (Nikon, Garden City, NY) were taken before and after treating the cells for 24 hours with ACCA at 5-, 10-, 20-, and 25-mmol/L concentrations.

Cell Viability Analysis

Cell viability was measured with trypan blue assay and enumerated with a hemocytometer.

Assay for Cellular Uptake of ACCA by U-87MG Cells

Replica plated U-87 MG cells (80% confluent) were exposed for 0 minutes, 1 hour, or 18 hours to 10-mmol/L ACCA in DMEM/F12 media supplemented with 10% FBS. In brief, for the 0-minute exposure, cells were exposed to the ACCA and then the plates were immediately washed twice in cold Dulbecco's phosphate-buffered saline (D-PBS). The plate was then stored at -20°C until use. For the 1- and 18-hour exposures, the cells were maintained for the duration in media containing 10-mmol/L ACCA and were then washed as before and stored. To test for internalization of ACCA, U87-MG cells were semipermeabilized for 20 minutes with an ice-cold solution of 40-μmol/L digitonin in D-PBS containing 10-mmol/L ACCA. Then, they were rinsed as before and stored at -20°C. The plates were thawed and scraped into microcentrifuge tubes in 1.0 ml of D-PBS, and cell extracts were prepared by repeated freeze-thaw lysis. The lysates were then centrifuged at 13,000 × *g* for 5 minutes, and the clarified supernatant was assayed for the presence or absence of ACCA by ultraviolet-visible spectrometry (Lambda 25; PerkinElmer, Wellesley, MA). A wavelength scan of 0.05-mmol/L ACCA in D-PBS was used to correlate the spectra.

Flow Cytometry

Apoptosis or necrosis in U-87 MG cells was measured by labeling the cells with fluorescein isothiocyanate (FITC)-labeled Annexin V (BD Biosciences, San Diego, CA) and propidium iodide (PI) (BD Biosciences) according to the manufacturer's instructions. In summary, culture supernatant was recovered from each well, and the treated cells were washed once in phosphate-buffered saline (PBS) and trypsin-ethylenediamine tetra-acetic digested acid. One milliliter of fresh media containing 10% FBS was then added to neutralize the trypsin. The cells were recovered and pooled with the previously recovered culture supernatant from respective wells. Fifty-microliter aliquots of the cell suspension were removed for cell viability assay. The remaining cell suspensions were centrifuged at 150 × *g* for 5 minutes (1000 rpm; Sorvall RT6000D), the supernatant was discarded, and the cell pellet was resuspended and centrifuged twice using PBS. The cells were then washed twice in binding buffer (10-mmol/L *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, 140-mmol/L NaCl, 2.5-mmol/L CaCl₂, pH 7.4) and then resuspended in 100 μl of fresh binding buffer. One μg/ml of Annexin V FITC and 1 μg/ml of PI were added to the cell suspensions, and the cells were analyzed by flow cytometry (Facsan; BD Biosciences). Excitation was set at 530 nm (FL1) for FITC and 620 nm (FL2) for PI. At least 20,000 events were recorded, and

ungated events were accumulated for data. Cell populations that displayed high FITC and low PI intensities (lower right quadrant of the cytogram) were defined as apoptotic, whereas those that displayed low FITC and high PI intensities (upper left quadrant of the cytogram) and those that were in the upper right quadrant of the cytogram (high FITC and high PI staining) were defined as necrotic.

MRS

MRS measurements of ACCA-treated U-87 MG cells were carried out in a Bruker high-resolution MAS ^1H MRS (HR-MAS ^1H MRS). In brief, U-87 MG cells maintained in 10% FBS-supplemented DMEM/F12 media were exposed to 10-mmol/L ACCA for 6, 18, or 24 hours. The cells were recovered at each time point by brief trypsinization, washed once in media containing serum, and then washed in PBS. The cells were then pelleted at $100 \times g$ in 1.5-ml microcentrifuge tubes (800 rpm, 5 min; 5415D microcentrifuge; Eppendorf North America, Westbury, NY). Aliquots of the cell pellets were mixed with D_2O containing 0.7% TMS at a 1:1 (v/v) ratio and placed in zirconium tubes (2.9-mm diameter, 10- μL sample capacity). The tubes were then placed in a Bruker 11.7-T Avance 500 MR spectrometer (Bruker BioSpin Corp., Billerica, MA) with the sample being maintained at 4°C and spun at 4.2 kHz at 54.7 degrees relative to the longitudinal magnetic field (B_0). Internal chemical shift reference was set to 0.00 parts per million with TMS, and the D_2O signal was used to adjust for temporal variations in the magnetic field (e.g., locking). After a presaturation pulse for water suppression, cell spectra were acquired with a Carr-Purcell-Meiboom-Gill rotor-synchronized pulse sequence (repetition time, 3500 ms; τ , 0.15 ms; bandwidth, 8 kHz; 16,000 complex points; 256 averages; total acquisition time, 15 min 38 s) (7). Spectral analyses were completed on the resultant neuro-chemical profiles.

HR-MAS ^1H MRS Data Analyses

Each spectrum was analyzed using LCModel software (SW Provencher, Göttingen, Germany) using a linear combination of 27 neurochemical model spectra (basis set) to fit the tissue metabolite spectrum and calculate absolute concentration values for each metabolite. The basis set was custom made using calibrated phantoms of individual neurochemicals in buffer (31, 32). Because assessment of the number of cells contained in each sample was not performed, the magnetic resonance visible metabolites are reported in terms of ratios rather than absolute concentration. Because we found a good correlation between N-acetyl-aspartyl-glutamine (NAAG) and sample size, the metabolite-to-NAAG ratio was used for most of the analyses, whereas the metabolite-to-total lipid ratio was used for profiling intracellular lactate levels. After MRS analysis, the metabolite peak profiles were analyzed by scanning densitometry (Un-Scan-It, version 5.1; Silk Scientific, Orem, UT) via a manual digitizing mode to calculate the relative concentrations.

Calculation of the Plating Efficiency and Survival Fraction of U-87 MG Cells

U-87 MG cells were plated in 35-mm tissue culture plates (six plates per experiment; Becton Dickinson Labware, Franklin Lakes, NJ) at a cell density of 140 cells per plate. The plates were then incubated for 24 hours at 37°C to allow for cell adherence and growth. Each plate was checked at this point and then every second day for the next 14 to 21 days. Subsequently, the plates were washed with cold PBS, and the surviving colonies were stained in situ with 2% crystal violet in 95% ethanol for enumeration. Cell colonies consisting of more than 50 cells were scored as representing surviving cells using an automated cell counter (ColCount; Oxford Optronix Ltd., Oxford, England). Surviving fractions of the U-87 MG cells were calculated based on the mean plating efficiency of the six replica plates. Experiments were then repeated to verify reproducibility of the data.

Plating efficiency was calculated based on the formula:

$$PE = \frac{\text{colonies counted}}{\text{numbers of cells seeded}} \times 100$$

Normalized survival fractions were calculated from the formula:

$$SF = \frac{(\text{colonies counted})/\text{number of cells seeded}}{\text{mean PE at 0 Gy}}$$

Irradiation Experiments

Based on the expected surviving fraction, U-87 MG cells were seeded into 35-mm tissue culture plates at cell densities of 200, 400, and 800 cells per plate and incubated at 37°C to allow for cell adherence. The plates were then irradiated at a dose rate of 0.53 Gy/minute using a Theratron ⁶⁰Co 780 γ irradiator (29.56 mmHg) (Atomic Energy Corporation, Ottawa, Canada). Three of the seeded plates served as controls and did not receive any radiation. The other plates were divided into sets of six and were irradiated at the following single dosages: 0.5, 1.0, 1.5, 2.0, and 3.0 Gy. After irradiation, the cells were reincubated at 37°C; thereafter, each plate was checked every second day for 14 to 21 days. Subsequently, the depleted media was discarded and the dishes prepared for counting as described previously. Surviving fractions were calculated based on sham (nonirradiated) series of seeded plates.

Combined Chemotherapy and Radiotherapy Treatments

Cells were prepared using methods similar to those described above for the irradiated series. Approximately 6 hours before irradiation, but after adherence of the seeded cells, ACCA was added to each plate to achieve a final concentration of 10 mmol/L. Three of the seeded plates served as controls and received no irradiation, but did receive ACCA. The other plates were divided into sets of six and were irradiated at single dosages of 0.5, 1.0, 1.5, 2.0, or 3.0 Gy. As described previously, the plates were reincubated at 37°C, and then each plate was checked every second day for the next 14 to 21 days. The plates were then processed for colony counting as described before. Surviving fractions were calculated as described for our sham (nonirradiated) series.

Data Analysis for Survival Assays

Clonogenic survival data were fitted to the linear quadratic model (24). Surviving fractions measured at the tested doses were fitted with the linear quadratic equation using nonlinear least-square regression, using the software programs SPSS 12.1 (SPSS, Inc., Chicago, IL) and SigmaPlot 8.0 (Systat Software, Inc., San Jose, CA) to produce the best-fit parameters. All parameters were fitted simultaneously, and estimates of uncertainty were expressed as likelihood confidence intervals.

RESULTS

ACCA Imparts Significant Viability Changes in U-87MG Glioma

The primary aim of this study was to test for enhanced radiosensitization of glioma when lactate efflux was inhibited. Thus, as a first step, LD₃₀ values were determined for U-87 MG cells treated with ACCA, the lactate transporter inhibitor under study. A lower lethal dose (LD₃₀) was selected (rather than a 50% lethal dose) to compensate for additional lethality that would accumulate during downstream irradiation treatments. The endpoints of the study were based on the morphological assessment, cell viability, and flow cytometry of treated

cells. Twenty-four hours after exposure to ACCA, cell viability was assessed via flow cytometry and trypan blue dye exclusion assays.

Ugated fluorescence-activated cell sorter analysis showed progressive apoptotic and necrotic cell death as ACCA exposure levels were increased. Untreated but confluent U-87MG cell populations (control cells) harbored an apoptotic/necrotic cell fraction of 17.5% (upper right, upper left, and lower right quadrants, Fig. 1, 0 mmol/L). This did not alter significantly at 5-mmol/L ACCA (Fig. 1, 5 mmol/L). However, as the ACCA concentration was increased to 10-mmol/L and higher concentrations, the apoptotic and necrotic cell fractions in combination showed an exponential increase (31% at 10 mmol/L; 87.5% at 20 mmol/L; 94.7% at 25 mmol/L; Fig. 1). Based on the flow cytometry data, ACCA at a concentration of 10 mmol/L (corresponding to an approximate LD₃₀) was used for HR-MAS ¹H MRS analysis and for combined ACCA/irradiation experiments.

Cells treated with 5-mmol/L ACCA did not display any morphological differences from untreated (control) cells (Fig. 2A). However, at 10-mmol/L ACCA, the cells displayed morphological changes, including shortened, spindle-shaped cell processes, membrane blebbing, and shrunken cell bodies (Fig. 2B). At ACCA concentrations of 20 mmol/L (Fig. 2C) and greater (data not shown), U-87 MG cells detached from the dish surface, with significant loss of cellular morphology (Fig. 2C).

Cell viability of ACCA-treated cell populations were further evaluated and enumerated by trypan blue exclusion assays to compare with flow cytometry data; the viabilities at high-ACCA treatments were 11 ± 3% (20 mmol/L) and 3 ± 3% (25 mmol/L), thus confirming the cytotoxicity of ACCA at 20-mmol/L and higher concentrations. However, at lower ACCA concentrations (0- to 10-mmol/L ACCA), the trypan blue assays indicated cell viabilities that were higher than those observed with flow cytometry: 96.5 ± 0.5% (0 mmol/L); 82.5 ± 1.5% (5 mmol/L); and 72.0 ± 0.2% (10 mmol/L) (values indicate percent viability ± standard deviation).

In U-87MG Glioma, the Inhibitory Effect of ACCA Is Limited to Lactate Transport Only

ACCA is an inhibitor of lactate transport at the cell surface (13, 36) and of pyruvate transport in isolated mitochondria (13). Because it has not been clearly established whether or not ACCA can be readily internalized by mammalian cells, specifically by malignant tumors (36), we tested the ability of ACCA to enter the glioma cells under study. Wavelength scans of U87-MG glioma cell extracts clearly indicated that, at physiological pH levels, 10-mmol/L ACCA is not internalized either by lactate transporters or via membrane diffusion, even after 18 hours of exposure to the inhibitor (Fig. 3).

MRS Experiments

For a successful combined chemo- and radiotherapy approach, rapid or significant drops in radioprotective metabolites (9, 12, 20, 35, 37) and in key metabolites involved in cellular energy generation should be observed in ACCA-treated U-87MG glioma cells. Thus, we examined the ACCA-treated cells at multiple time points for changes in antioxidants/radioprotectants (i.e., changes in taurine and glutathione). To obtain an indirect measure of ACCA's effect on cellular metabolism and mitochondrial function, intracellular glutamate, alanine, and glycine levels were also evaluated, which should be affected by inhibition of lactate efflux from glioma or by inhibition of pyruvate entry into mitochondria. Thus, overall energy metabolism should be adversely affected by inhibition of lactate efflux from these highly glycolytic glioma cells.

ACCA Inhibits Lactate Efflux in U-87 MG Glioma

MRS analysis of proliferating glioma cells indicated a large flux in intracellular lactate levels (doublet of peaks centered at 1.35 parts per million (Fig. 4). On exposure to 10-mmol/L ACCA (6 h after ACCA treatment), an initial (maximal) drop in intracellular lactate was observed as glycolytic metabolism was rapidly down-regulated in response to inhibition of lactate efflux. However, intracellular lactate then continued to accumulate during the next 12 hours as glioma cells maintained a suboptimal level of glycolysis. Between 12 and 24 hours after ACCA treatment, intracellular lactate levels dropped again, most likely because of a disruption of glycolysis and redirection of metabolites to alternate pathways in response to continued inhibition of lactate efflux by ACCA. These effects were more pronounced when the total lipid ratio was used to normalize the peak profiles, whereas the use of NAAG for normalization resulted in a lower magnitude of change.

ACCA Imparts Significant Changes in Cellular Antioxidant Levels

Levels of two antioxidant chemicals were significantly reduced when analyzed by MRS of ACCA-treated U-87MG cells, namely, taurine and reduced glutathione. Both taurine and reduced glutathione concentrations showed an approximate 40% drop at 6 hours after ACCA treatment when normalized against NAAG (Fig. 5A, Table 1). Twenty-four hours after ACCA treatment, both antioxidants showed a marked reduction by approximately 90%, diminishing to almost undetectable levels in the spectra (Fig. 5A, Table 1).

ACCA Treatment Affects Mitochondrial Bioenergetic Functions

Cellular glycine (Fig. 5A) levels also showed a reduction (40%) that paralleled those of taurine and glutathione at 6 hours after ACCA treatment (normalized to NAAG), whereas glutamate (Fig. 5B) and alanine (Fig. 5C) levels indicated little or no change. However, all three metabolites showed significant reductions 24 hours after ACCA treatment, with glycine showing the most significant change (90%), followed by lesser reductions for glutamate (70%) and alanine (70%). It is interesting to note the parallel changes in glycine, taurine, and reduced glutathione; glycine is produced as a metabolic by-product during biosynthesis of taurine from serine via cysteine (Fig. 6, Pathway E), and glycine serves as a precursor for glutathione biosynthesis via glutamate (Fig. 6, Pathway G). Thus, MRS profiles indicate depletion of glycine, resulting in debilitation of the taurine biosynthetic pathway and suppression of the biosynthesis of glutathione.

ACCA Affects Choline/Phosphocholine Ratio in U-87MG Glioma

Changes to choline and phosphocholine concentrations are known biomarker alterations that are observed during chemotherapeutic treatment of tumors (11). Treatment of U-87MG glioma with ACCA also induced changes in choline and phosphocholine levels in a time-dependent fashion; of the choline-phosphocholine pair, the former constituted the major metabolite before ACCA treatment (2:1, choline:phosphocholine) (Fig. 5D), whereas both were observed at equimolar concentrations 6 hours after ACCA treatment (1:1). At 24 hours after treatment, the choline/phosphocholine ratio was significantly inverted (1:4). Overall, a ninefold change in the choline/phosphocholine ratio was observed after prolonged exposure of cells to ACCA (Fig. 5D, Table 1). It should be noted that the total choline + phosphocholine (Cho + pCho) values and the (Cho + pCho)/NAAG ratios were not significantly changed after ACCA treatment (data not shown).

Clonogenic survival of U-87MG cells subjected to low-dose irradiation is adversely affected in the presence of ACCA. The clonogenic survival of U-87 MG glioma cells subjected to low-dose radiation alone (Fig. 7) could be described by the linear quadratic model throughout the entire dosage range (0–3.0 Gy). In contrast, combined treatment of U-87 MG

glioma cells with ACCA (10 mmol/L) and low-dose radiation displayed an exponential relationship throughout most of the dosage range (0 to 2.0 Gy) (Fig. 7). Comparison of the two curves indicates that the maximum effect of combined ACCA and low-dose irradiation on clonogenic survival occurred between dosages of 0 Gy and 1.0 Gy. Further changes in clonogenic survival were not evident at higher radiation doses.

DISCUSSION

A primary metabolic phenotype frequently observed among highly malignant tumors is their enhanced glycolytic metabolism, even in the presence of ample tissue oxygen (39). Termed “aerobic glycolysis,” wherein glucose is rapidly and quantitatively catabolized to lactic acid, this aberrant phenomenon is the primary principle behind clinical (18) fluoro-deoxyglucose-positron emission tomographic imaging of malignant tumors (8).

Among the few treatment modalities available for these high-grade tumors, radiotherapy remains a primary treatment of choice. Delivered to the tumor mass either via external irradiation (e.g., gamma knife radiosurgery) or via the use of radio isotope “seeds” placed in the postsurgical resection cavity, the treatment is often quite effective. However, the radiation sensitivity of surrounding normal tissue restricts the therapeutic dosage that can be applied (29). Therefore, additional strategies, including more frequent radio fractionation schedules, and combination therapies, which target key signaling pathways that are up-regulated in tumors, are currently being examined. These aim to affect radio resistance or radiation response of the targeted tumors while sparing the surrounding normal tissue (33). Thus, novel chemotherapy regimens that enhance radiosensitivity of targeted glioma cells to impart greater radiation-induced cell death while protecting the surrounding normal tissue from radiation-induced cell damage can be useful in developing additional clinical strategies to target these highly malignant, often radio-resistant tumors.

Building on our previous research (26), we investigated whether or not inhibition or disruption of glycolytic metabolism (by inhibition of the tumor’s capacity to efflux lactic acid) would alter the metabolite profile of targeted glioma and make them more susceptible to low-dose radiation-induced deoxyribonucleic acid (DNA) damage by preventing recovery from such damage. Our rationale was based on the premise that a disrupted metabolic scheme in glioma would debilitate the tumor’s bioenergetic functions, depriving the cells of nicotinamide cofactors (NADH and NADPH) necessary for regeneration of key radioprotective metabolites (e.g. taurine and glutathione) and metabolites that scavenge reactive oxygen species (e.g., pyruvate).

In addition, it has been proposed that rapidly proliferating cells likely resort to aerobic glycolysis to reduce oxidative stress during phases of the cell cycle when maximal metabolic rates occur (5). Furthermore, we hypothesized that disruption of the glucose-to-lactate (glycolytic) metabolic pathway would result in enhanced reactive oxygen species generation because the tumors would have to enhance their mitochondrial respiration for energy generation to survive. Overall, key metabolite profiles that impart radioprotective properties to the tumor should be adversely impacted, resulting in increased radiosensitivity. Thus, a strategy of first exposing glioma to inhibitors of glycolysis and then to low-dose irradiation should drastically affect the clonogenic survival of the tumor cells.

We first inhibited tumor glycolysis with ACCA, which has been reported to harbor low binding affinity ($K_i = 0.5$ mmol/L) for lactate transporters in highly glycolytic tumors in vitro (36). The inhibitor had no impact on U-87 MG glioma cell function or survival at concentrations up to 5 mmol/L, corresponding to previous reports for other mammalian cell systems, including glial cells in vitro (38). However, at 10-mmol/L and higher

concentrations, ACCA inhibited U-87 MG glioma cell metabolism and survival, as indicated by flow cytometric, morphological, and MRS analysis (Figs. 1, 2, and 5). When K_m data (5 mmol/L) for lactate (36) and intratumoral lactate concentrations (5–11 mmol/L) (34, 38) of highly glycolytic tumors are considered, our data indicate that a 10-fold degree of inhibition of lactate efflux rates is necessary to significantly affect the survival of U-87MG glioma cells in vitro.

Evidence has not been clear on whether or not ACCA can be internalized by highly glycolytic tumor cells (36), in which case the observed metabolite changes and cell-death may also occur because of direct inhibition of pyruvate entry into mitochondria (Fig. 6). Our ACCA permeability studies (Fig. 3) clearly indicated that the inhibitory effects of ACCA were extracellular in these highly glycolytic tumors. Thus, the observed metabolite profiles are the result of the inhibition of lactate efflux and not caused by disruption of mitochondrial pyruvate entry.

MRS profiling indicated an overall reduction in all metabolites at 24 hours after ACCA treatment, indicating general disruption of both mitochondrial and glycolytic metabolism as a result of long-term inhibition of lactate efflux (Figs. 4 and 5). Most likely because of disruption of nicotinamide cofactor ($NAD^+/NADH$ and $NADP^+/NADPH$) cycling, this would result in reduced biosynthesis of cysteine and glutamate, both of which are necessary for the synthesis of taurine and glutathione, the key radioprotective metabolites in mammalian tissues (Fig. 6) (16, 19).

MRS profiles of ACCA-treated glioma indicated progressive reductions in both taurine and glutathione levels after ACCA treatment (Fig. 5A, Table 1). Examination of the metabolic pathways (Fig. 6) showed that inhibition of lactate efflux directly affected the recycling of NADH to NAD^+ (Step B, Fig. 6). Resultant increases in NADH inhibited the 3-P-glycerate to serine conversion (Step E, Fig. 6), with concomitant reductions in cysteine and glycine biosynthesis. Thus, biosynthesis of taurine (via cysteine) and glutathione (via glycine and glutamate, Pathway G, Fig. 6) were inhibited as a result. These effects were clearly observed at 6 hours after ACCA addition for glycine, glutathione, and taurine (Fig. 5A, Table 1).

Minimal changes in glutamate or alanine concentrations 6 hours after ACCA addition (Fig. 5, B and C, Table 1) can also be explained via the metabolic schema, where enhanced redirection of pyruvate into mitochondrial respiration (Step C, Fig. 6) attributable to inhibition of lactate efflux (Step B, Fig. 5) can maintain the cellular glutamate levels (Path G, Fig. 6) and direct synthesis of alanine via pyruvate and glutamate (Step F, Fig. 6).

Increased mitochondrial respiration would also enhance synthesis of reactive oxygen species during respiration (1), further exacerbating free radical-induced DNA damage on the cells. Overall, the combination of disruption of tumor glycolysis and low-dose irradiation resulted in enhanced loss of survival in the U-87 MG glioma cells.

A disruption of nicotinamide cofactor ($NAD^+/NADH$ and $NADP^+/NADPH$) profiles would also directly affect key signaling pathways that are involved in DNA damage repair. For example, DNA-dependent protein kinase (23) and proteolytic poly(adenosine 5'-diphosphate-ribose) polymerase (6) need to be activated for both recognition and repair of radiation-induced DNA damage. Thus, a disruption of nicotinamide cofactor profiles, which regulate activation of proteolytic poly(adenosine 5'-diphosphate-ribose) polymerase, would be unavailable to the ACCA-treated glioma, essentially trapping the tumor cells in a lethal cycle of metabolite- and radiation-induced growth arrest.

An initial redirection of metabolite flux towards the pentose-phosphate pathway (6 hr after ACCA treatment) should have resulted in enhanced production of the reduced nicotinamide

NADPH, the primary cofactor involved in reducing glutathione (oxidized glutathione to glutathione conversion) (4). Along with enhanced synthesis of glutamate, the precursor amino acid for glutathione biosynthesis (Fig. 6), this should have enhanced the reduced glutathione levels 6 hours after the ACCA treatment stage. In contrast, a 40% reduction in glutathione was observed during MRS analysis. Although the molecular basis is not clear at present, this observation further supports the usefulness of metabolic remodeling before low-dose radiation-based glioma therapy.

Furthermore, it has been reported that treating GBMs with sublethal irradiation alone can promote migration and invasiveness of glioma cells (40), which implies that radiotherapy of glioma may be counterproductive as a single treatment modality. Thus, a combined strategy of metabolic pathway-targeting chemotherapy and low-dose radiotherapy as described here will most likely enhance the efficacy of a chemo- and radiotherapeutic regimen.

Overall, this study provides a metabolic scheme for enhancing the radiosensitivity of highly glycolytic tumors via disruption of an aberrant metabolic pathway commonly observed in malignant tumors. Small-molecule chemical inhibitors such as ACCA, with a low affinity for lactate transporters, will be preferable for targeting glioma in vivo in a combined chemo- and radiotherapeutic strategy because such inhibitors would have minimal physiological effects on transient neuronal–astrocyte lactate-shuttling pathways during synaptic activation (18). Thus, an extracellular, low-affinity inhibitor of lactate transport would be effective and would also harbor fewer side effects in an orthotopic experimental treatment strategy.

A crucial question will be whether or not this approach with ACCA is applicable in vivo. In this regard, ongoing studies in an orthotopic nude rat brain model have indicated that ACCA, when applied in situ (via osmotic pumps), is nontoxic at the concentrations (up to 20 mmol/L) used in the current study. Preliminary experiments are underway in an orthotopic nude rat glioma model (using U-87 MG glioma) to establish the efficacy of the studies described above in enhancing survival.

Because of the diffuse nature of malignant glioma, conventional radiotherapy at 60 Gy does not affect the residual tumor beyond the surgical margin (28). Numerous clinical trials of combined conventional chemotherapy during and after radiation therapy have been performed, but the outcomes have not been optimal. Thus, a combined protocol of chemotherapeutics specifically targeting glioma metabolism and then lower-dose radiation, as suggested here, may provide better outcomes in future clinical settings.

Acknowledgments

Research support was provided by grants from the American Cancer Society IRG-85-003–14 (SPM), LEARN Foundation, MI (SPM), and the Fund for Medical Research and Education (FMRE), Wayne State University School of Medicine. We thank Michael Joiner, Ph.D., for his invaluable advice in planning the radiobiological studies and for his critical review of the manuscript. We also thank Murali Guthikonda, M.D., Setti Rengachary, M.D., Prahlad Parajuli, Ph.D., and Pingyang Yu, M.D., for their invaluable support and assistance during this project. Ken Thompson, B.S., and Tom Owoc, B.F.A., are acknowledged for help with digital file conversions in preparing figures for this manuscript.

References

1. Ahmad IM, Aykin-Burns N, Sim JE, Walsh SA, Higashikubo R, Buettner GR, Venkataraman S, Mackey MA, Flanagan SW, Oberley LW, Spitz DR. Mitochondrial O₂*- and H₂O₂ mediate glucose deprivation-induced stress in human cancer cells. *J Biol Chem*. 2005; 280:4254–4263. [PubMed: 15561720]
2. Baggetto LG. Deviant energetic metabolism of glycolytic cancer cells. *Biochimie*. 1992; 74:959–974. [PubMed: 1477140]

3. Beauchesne PD, Bertrand S, Branche R, Linke SP, Revel R, Dore JF, Pedoux RM. Human alignant glioma cell lines are sensitive to low radiation doses. *Int J Cancer*. 2003; 105:33–40. [PubMed: 12672027]
4. Berg, JM.; Tymoczko, JL.; Stryer, L. *Biochemistry*. New York: W.H. Freeman; 2002.
5. Brand K. Aerobic glycolysis by proliferating cells: Protection against oxidative stress at the expense of energy yield. *J Bioenerg Biomembr*. 1997; 29:355–364. [PubMed: 9387096]
6. Chalmers A, Johnston P, Woodcock M, Joiner M, Marples B. PARP-1, PARP-2, and the cellular response to low doses of ionizing radiation. *Int J Radiat Oncol Biol Phys*. 2004; 58:410–419. [PubMed: 14751510]
7. Cheng LL, Ma MJ, Becerra L, Ptak T, Tracey I, Lackner A, Gonzalez RG. Quantitative neuropathology by high resolution magic angle spinning proton magnetic resonance spectroscopy. *Proc Natl Acad Sci U S A*. 1997; 94:6408–6413. [PubMed: 9177231]
8. Delbeke D, Martin WH. Metabolic imaging with FDG: A primer. *Cancer J*. 2004; 10:201–213. [PubMed: 15383201]
9. Estrela JM, Ortega A, Obrador E. Glutathione in cancer biology and therapy. *Crit Rev Clin Lab Sci*. 2006; 43:143–181. [PubMed: 16517421]
10. Fiveash JB, Spencer SA. Role of radiation therapy and radiosurgery in glioblastoma multiforme. *Cancer J*. 2003; 9:222–229. [PubMed: 12952307]
11. Gillies RJ, Morse DL. In vivo magnetic resonance spectroscopy in cancer. *Annu Rev Biomed Eng*. 2005; 7:287–326. [PubMed: 16004573]
12. Gupta RC, Win T, Bittner S. Taurine analogues; a new class of therapeutics: Retrospect and prospects. *Curr Med Chem*. 2005; 12:2021–2039. [PubMed: 16101502]
13. Halestrap AP, Denton RM. Specific inhibition of pyruvate transport in rat liver mitochondria and human erythrocytes by alpha-cyano-4-hydroxy-cinnamate. *Biochem J*. 1974; 138:313–316. [PubMed: 4822737]
14. Halestrap AP, Meredith D. The SLC16 gene family-from monocarboxylate transporters (MCTs) to aromatic amino acid transporters and beyond. *Pflugers Arch*. 2004; 447:619–628. [PubMed: 12739169]
15. Halestrap AP, Price NT. The proton-linked monocarboxylate transporter (MCT) family: Structure, function and regulation. *Biochem J*. 1999; 343:281–299. [PubMed: 10510291]
16. Inoue M, Sato EF, Nishikawa M, Park AM, Kira Y, Imada I, Utsumi K. Mitochondrial generation of reactive oxygen species and its role in aerobic life. *Curr Med Chem*. 2003; 10:2495–2505. [PubMed: 14529465]
17. Joiner MC, Marples B, Lambin P, Short SC, Turesson I. Low-dose hypersensitivity: Current status and possible mechanisms. *Int J Radiat Oncol Biol Phys*. 2001; 49:379–389. [PubMed: 11173131]
18. Kasischke KA, Vishwasrao HD, Fisher PJ, Zipfel WR, Webb WW. Neural activity triggers neuronal oxidative metabolism followed by astrocytic glycolysis. *Science*. 2004; 305:99–103. [PubMed: 15232110]
19. Kirsch M, De Groot H. NAD(P)H, a directly operating antioxidant? *FASEB J*. 2001; 15:1569–1574. [PubMed: 11427489]
20. Lafleur MV, Woldhuis J, Loman H. Effects of sulphhydryl compounds on the radiation damage in biologically active DNA. *Int J Radiat Biol Relat Stud Phys Chem Med*. 1980; 37:493–498. [PubMed: 6447117]
21. Laperriere N, Zuraw L, Cairncross G. Cancer Care Ontario Practice Guidelines Initiative Neuro-Oncology Disease Site Group: Radiotherapy for newly diagnosed malignant glioma in adults: A systematic review. *Radiother Oncol*. 2002; 64:259–273. [PubMed: 12242114]
22. Marples B. Is low-dose hyper-radiosensitivity a measure of G2-phase cell radiosensitivity? *Cancer Metastasis Rev*. 2004; 23:197–207. [PubMed: 15197323]
23. Marples B, Cann NE, Mitchell CR, Johnston PJ, Joiner MC. Evidence for the involvement of DNA-dependent protein kinase in the phenomena of low dose hyper-radiosensitivity and increased radioresistance. *Int J Radiat Biol*. 2002; 78:1139–1147. [PubMed: 12556341]
24. Marples B, Joiner MC. The response of Chinese hamster V79 cells to low radiation doses: Evidence of enhanced sensitivity of the whole cell population. *Radiat Res*. 1993; 133:41–51. [PubMed: 8434112]

25. Marples B, Wouters BG, Collis SJ, Chalmers AJ, Joiner MC. Low-dose hyper-radiosensitivity: A consequence of ineffective cell cycle arrest of radiation-damaged G2-phase cells. *Radiat Res.* 2004; 161:247–255. [PubMed: 14982490]
26. Mathupala SP, Parajuli P, Sloan AE. Silencing of monocarboxylate transporters via small interfering ribonucleic acid inhibits glycolysis and induces cell death in malignant glioma: An in vitro study. *Neurosurgery.* 2004; 55:1410–1419. [PubMed: 15574223]
27. Mathupala SP, Rempel A, Pedersen PL. Aberrant glycolytic metabolism of cancer cells: A remarkable coordination of genetic, transcriptional, post-translational, and mutational events that lead to a critical role for type II hexokinase. *J Bioenerg Biomembr.* 1997; 29:339–343. [PubMed: 9387094]
28. Matsutani M. Chemoradiotherapy for brain tumors: Current status and perspectives. *Int J Clin Oncol.* 2004; 9:471–474. [PubMed: 15616877]
29. Mornex F, Nayel H, Taillandier L. Radiation therapy for malignant astrocytomas in adults. *Radiother Oncol.* 1993; 27:181–192. [PubMed: 8210454]
30. Pedersen PL. Tumor mitochondria and the bioenergetics of cancer cells. *Prog Exp Tumor Res.* 1978; 22:190–274. [PubMed: 149996]
31. Provencher, SW. [Accessed 12/12/05] LCMModel Manual. <http://sprovencher.com/pages/lcmmanual.shtml>
32. Provencher SW. Estimation of metabolite concentrations from localized in vivo proton NMR spectra. *Magn Reson Med.* 1993; 30:672–679. [PubMed: 8139448]
33. Robson T, Worthington J, McKeown SR, Hirst DG. Radiogenic therapy: Novel approaches for enhancing tumor radiosensitivity. *Technol Cancer Res Treat.* 2005; 4:343–361. [PubMed: 16029055]
34. Roslin M, Henriksson R, Bergstrom P, Ungerstedt U, Bergenheim AT. Baseline levels of glucose metabolites, glutamate and glycerol in malignant glioma assessed by stereotactic microdialysis. *J Neurooncol.* 2003; 61:151–160. [PubMed: 12622454]
35. Shimizu T, Iwanaga M, Yasunaga A, Urata Y, Goto S, Shibata S, Kondo T. Protective role of glutathione synthesis on radiation-induced DNA damage in rabbit brain. *Cell Mol Neurobiol.* 1998; 18:299–310. [PubMed: 9590560]
36. Spencer TL, Lehninger AL. L-lactate transport in Ehrlich ascites-tumour cells. *Biochem J.* 1976; 154:405–414. [PubMed: 7237]
37. Venkatachalam SR, Chattopadhyay S. Natural radioprotective agents: An overview. *Curr Org Chem.* 2005; 9:389–404.
38. Volk C, Kempfski B, Kempfski OS. Inhibition of lactate export by quercetin acidifies rat glial cells in vitro. *Neurosci Lett.* 1997; 223:121–124. [PubMed: 9089688]
39. Warburg, O.; Dickens, F. Kaiser Wilhelm-Institut für Biologie. *The Metabolism of Tumours: Investigations from the Kaiser-Wilhelm Institute for Biology, Berlin-Dahlem.* London; Constable: 1930.
40. Wild-Bode C, Weller M, Rimner A, Dichgans J, Wick W. Sublethal irradiation promotes migration and invasiveness of glioma cells: Implications for radiotherapy of human glioblastoma. *Cancer Res.* 2001; 61:2744–2750. [PubMed: 11289157]

FIGURE 1.

Cytograms showing that ACCA imparted significant cell death in U87-MG gliomas at concentrations of 10 mmol/L and above. Flow cytometric analyses of U87-MG gliomas exposed to 0- to 25-mmol/L ACCA at 5-mmol/L increments. Percent cell number for each quadrant is indicated at the corners of each cytogram. Cell populations with high FITC and low PI (*lower right quadrant*) are defined as apoptotic. Those with low FITC and high PI (*upper left quadrant*) and high FITC and high PI (*upper right quadrant*) are defined as necrotic.

FIGURE 2.

Cellular morphology of U87-MG gliomas exposed to ACCA (cellular morphology 72 h after exposure to ACCA). *A*, at 0- and 5-mmol/L ACCA, the cells did not show significant morphological changes. *B*, at 10-mmol/L ACCA, cells were still attached to culture vessels, but displayed a rounded morphology. *C*, at 20-mmol/L ACCA, all cells had detached from the culture vessel (original magnification, $\times 100$).

FIGURE 3.

Graph showing that ACCA is not internalized by U87-MG glioma cells. Spectrometric analyses of whole-cell lysates of U87-MG gliomas exposed to 10-mmol/L ACCA for 0 minutes to 18 hours in the absence of digitonin, and 20 minutes in the presence of digitonin. The spectra are overlaid with a solution spectrum for ACCA (0.05 mmol/L) for peak identification.

FIGURE 4.

Graph showing that ACCA inhibits lactate efflux and glycolytic metabolism. MRS analysis of U87-MG glioma intracellular lactate profile (doublet of peaks centered at 1.35 parts per million) for control cells (*green*), 6 hours after exposure to 10-mmol/L ACCA (*black*), 18 hours after exposure to ACCA (*blue*), and 24 hours after exposure to ACCA (*red*).

FIGURE 5.

Key metabolites of U-87 MG gliomas are altered after ACCA treatment. MRS analyses of taurine, glutathione, and glycine (*A*); glutamate (*B*); alanine (*C*); and choline and phosphocholine (*D*). Stacked plots are shown with the chemical shift on the x-axis, and successive spectra are outlined along an oblique y-axis. Notations for *arrows* are indicated above the spectra profiles. Samples were analyzed in quadruplicate for the 0-hour time point (control), and 6- and 24-hour samples were analyzed in triplicate. The x-axis indicates the chemical shift in parts per million.

FIGURE 6.

Illustration demonstrating key metabolites and the metabolic pathways that are altered after exposure of U-87 MG gliomas to ACCA. The key metabolites examined by MRS are outlined (*green background*). The primary glycolytic metabolic pathway (*A* $\rightarrow$ *B*) is inhibited on exposure of cells to ACCA (*red background*), which blocks lactate efflux. The metabolism can then shift to the pentose-phosphate pathway (*D*) and towards mitochondrial respiration (*C*). Concomitant biosynthetic pathways for amino acids can be up-regulated (*E–G*) at least during the initial period of exposure to ACCA and before cells undergo metabolic crisis because of long-term exposure to ACCA. Key reducing cofactors (NADPH and NADH) are indicated (*yellow background*).

FIGURE 7.

Graph showing clonogenic survival of irradiated U-87 MG glioma alone and in combination with previous exposure to ACCA. Clonogenic survival was measured as described in the Methods section for cells exposed to low-dose radiation alone (●) or treated with ACCA before low-dose radiation (○). The data were normalized to calculate the survival fraction.

TABLE 1Metabolite levels after α -cyano-4-hydroxycinnamic acid treatment^a

	0 hours	6 hours	24 hours
Taurine	5.79 $\pm$ 0.55	3.54 $\pm$ 0.84 ($\downarrow$ 40%)	0.74 $\pm$ 0.10 ($\downarrow$ 90%)
Glutathione	16.46 $\pm$ 4.28	10.04 $\pm$ 1.76 ($\downarrow$ 40%)	4.64 $\pm$ 2.08 ($\downarrow$ 70%)
Glutamate	6.23 $\pm$ 1.24	5.97 $\pm$ 1.47 ($\leftrightarrow$)	1.75 $\pm$ 0.82 ($\downarrow$ 70%)
Alanine	3.46 $\pm$ 0.76	3.01 $\pm$ 1.04 ($\leftrightarrow$)	1.20 $\pm$ 0.25 ($\downarrow$ 70%)
Glycine	5.10 $\pm$ 0.64	3.19 $\pm$ 0.21 ($\downarrow$ 40%)	0.49 $\pm$ 0.12 ($\downarrow$ 90%)
Choline/P-choline	1.87 $\pm$ 0.35 (2:1)	0.79 $\pm$ 0.09 (1:1)	0.20 $\pm$ 0.05 (1:4)

^aTaurine, glutathione, glutamate, alanine, and glycine concentrations are given in relative units $\pm$ standard deviation, normalized to N-acetyl aspartate glutamate (NAAG) levels. The relative units for N-acetyl aspartate glutamate were 1.0, 1.65, and 1.27 at 0, 6, and 24 hours, respectively. The approximate changes in metabolite concentrations are indicated next to the relative values. The percent changes are relative to the values at 0 hours. $\downarrow$, drop in value; $\leftrightarrow$, no change. Choline:phosphocholine ratios are given in parentheses next to their values.