

Glutaric Acid and Its Metabolites Cause Apoptosis in Immature Oligodendrocytes: A Novel Mechanism of White Matter Degeneration in Glutaryl-CoA Dehydrogenase Deficiency

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ABSTRACT

Glutaryl-CoA dehydrogenase deficiency is an inherited metabolic disease characterized by elevated concentrations of glutaric acid (GA) and its metabolites glutaconic acid (GC) and 3-hydroxy-glutaric acid (3-OH-GA). Its hallmarks are striatal and cortical degeneration, which have been linked to excitotoxic neuronal cell death. However, magnetic resonance imaging studies have also revealed widespread white matter disease. Correspondingly, we decided to investigate the effects of GA, GC, and 3-OH-GA on the rat immature oligodendroglia cell line, OLN-93. For comparison, we also exposed the neuroblastoma line SH-SY5Y and the microglia line BV-2 to GA, GC, and 3-OH-GA. Cell viability was measured by metabolism of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium. Flow cytometry was used to assess apoptosis *via* annexin-V, anti-active caspase-3 antibody, and propidium iodide staining. GA, GC, and 3-OH-GA reduced OLN-93 oligodendroglia cell viability in a dose-dependent manner. Toxicity of GA, GC, and 3-OH-GA was abrogated by preincubation with the pan-caspase inhibitor z-VAD-fmk. Apoptosis but not necrosis was detected at various stages (early: annexin-V; effector: caspase-3) after 24–48 h of incubation with GA, GC, or 3-OH-GA in OLN-93 but not in

neuroblastoma or microglia cells. OLN-93 lacked expression of N-methyl-D-aspartate receptors, making classical glutamatergic excitotoxicity an unlikely explanation for the selective toxicity of GA, GC, and 3-OH-GA for OLN-93 cells. GA, GC, and 3-OH-GA directly initiate the apoptotic cascade in oligodendroglia cells. This mechanism may contribute to the white matter damage observed in glutaryl-CoA dehydrogenase deficiency. (*Pediatr Res* 57: 771–776, 2005)

Abbreviations

GA, glutaric acid
GC, glutaconic acid
GDD, glutaryl-CoA dehydrogenase deficiency
GYKI, 4-(8-Methyl-9H-1,3-dioxolo[4,5-h][2,3]benzodiazepin-5-yl)-benzenamine hydrochloride
MK801, dizocilpine
MRI, magnetic resonance imaging
MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium
NMDA, N-methyl-D -aspartate
3-OH-GA, 3-hydroxy-glutaric acid

Glutaryl-CoA dehydrogenase deficiency (GDD; also called glutaric aciduria type I; McKusick 231670) is an inherited metabolic disease resulting from deficiency of glutaryl-CoA dehydrogenase (EC 1.3.99.7). This disease is characterized by

accumulation of glutaric acid (GA), 3-hydroxy-glutaric acid (3-OH-GA), and glutaconic acid (GC). Whereas the concentrations of GA, 3-OH-GA, and GC may rise to millimolar concentrations in various body fluids during acute crises, signs of tissue damage are restricted to the CNS.

Hallmarks of GDD in magnetic resonance imaging (MRI) studies of the brain are atrophy of the basal ganglia and the frontotemporal cortex (1). Two mechanisms that mediate neurotoxicity of GA and 3-OH-GA have been established in neonatal rat brain tissue homogenates, primary chick embryo neuronal cultures, and neonatal rat brain slice cultures: excitotoxicity mediated by N-methyl-D-aspartate (NMDA) receptors (2,3) and mitochondrial production of reactive oxygen species

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(4–6). However, detailed MRI studies have also revealed delayed myelination (1) and a variable degree of periventricular white matter disease (7,8) in addition to the neuronal atrophy observed. In adult-onset GDD, leukoencephalopathy is the leading MRI finding (9). In a mouse model of GDD, there is no evidence of neuronal loss, whereas there is diffuse spongiform white matter disease (10). No pathophysiologic explanation for oligodendroglial cell destruction has been reported so far. To improve clinical understanding of this topic, we investigated the impact of GA, 3-OH-GA, and GC on the immature oligodendrocyte cell line OLN-93 and assessed mechanisms of cellular damage that are triggered by GA, 3-OH-GA, and GC.

METHODS

Cells. The permanent oligodendrocyte cell line OLN-93, derived from spontaneously transformed cells in primary rat brain glia cultures (11), was provided by Dr. C. Richter-Landsberg (Oldenburg, Germany). OLN-93 cells bear the morphologic and antigenic properties (A2B5⁻, galactocerebroside⁺, myelin basic protein⁺, Wolfgram protein⁺, proteolipidprotein⁺) of 5- to 10-d-old (postnatal time) cultured rat brain oligodendrocytes. These resemble the intermediate stage between the oligodendrocyte precursor cell and the mature oligodendrocyte. A more immature phenotype, characterized by cells with a bipolar morphology, was induced by incubation with 10 ng/mL recombinant platelet-derived growth factor-AA (PeproTech, Rocky Hill, NJ) and 10 ng/mL basic fibroblast growth factor (PeproTech) (12,13) for 5 d, whereas maturation, featuring multipolar cell character and intense arborization, was induced by 10 ng/mL ciliary neurotrophic factor (PeproTech), 5 μ M forskolin (Sigma Chemical Co.-Aldrich, Deisenhofen, Germany), and 15 nM 3,3',5'-triodo-L-thyronine (Sigma Chemical Co.-Aldrich) (14).

The human neuroblastoma cell line SH-SY5Y was obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). The rat microglia cell line BV-2 was provided by Dr. E. Blasi (University of Modena and Reggio Emilia, Modena, Italy). All cells were kept at 37°C and 5% CO₂. Cell culture medium that consisted of Dulbecco's modified Eagle's medium (Biochrom, Berlin, Germany) with 4.5 g/L D-glucose (OLN-93) or 3 g/L D-glucose (BV-2/SH-SY5Y), respectively, was supplemented with 10% FCS and 1% human serum albumin.

Reagents. Stock solutions (0.1 M) of GA, GC (Sigma Chemical Co.-Aldrich), and 3-OH-GA (gift of Dr. E. Gerstner, Philipps-University, Marburg, Germany) were prepared in culture medium, adjusted to pH 7.4 with 2 N NaOH, filtered through 0.2- μ m microfilters, and diluted just before use. A 1-mM stock solution of the protein kinase inhibitor staurosporine (Sigma Chemical Co.-Aldrich) was prepared in DMSO. The NMDA receptor antagonist dizocilpine (MK801) and the selective AMPA receptor antagonist 4-(8-methyl-9H-1,3-dioxolo[4,5-h][2,3]benzodiazepin-5-yl)-benzenamine hydrochloride (GYKI 52466) were obtained from Tocris (Bristol, UK).

Assessment of glutamate receptor expression by reverse transcription-PCR. Total RNA from OLN-93 cells was isolated by acidic phenol/chloroform extraction and treated with DNase I. Before reverse transcription, possible DNA contamination was examined by running RNA samples on a 2% agarose

gel, subsequently developed with ethidium bromide. RNA (500 ng) was reverse-transcribed with M-MLV (Moloney murine leukemia virus) reverse transcriptase (Promega, Madison, WI) in 25 μ L of reaction mixture. The resulting cDNA (1 μ L) was amplified by PCR using primer pairs, as depicted in Table 1.

mRNA expressions of NMDA receptor subunit 1 (NR-1), 2A (hNR2A), 2B (GRIN2B), and 2D (GRIN2D) and AMPA receptor subunit GluR3 and GluR4 were studied by reverse transcription-PCR. cDNA amplification was performed in 32 cycles, consisting of denaturation (45 s at 94°C), annealing (1 min at 50 or 56°C), and primer extension (1 min at 72°C). Amplified cDNA was subjected to 5% PAGE and subsequent silver staining.

Annexin-V measurement. A measurable feature of apoptosis is the loss of plasma membrane asymmetry. In normal cells, membrane phospholipids are distributed asymmetrically between the inner and outer leaflets of the plasma membrane. Phosphatidylserine, an aminophospholipid, is normally present in the inner leaflet of the plasma membrane. Early in apoptosis, before loss of membrane integrity, phosphatidylserine translocates from the inner to the outer leaflet, exposing it to the external cellular environment at the surface of the plasma membrane. Phosphatidylserine translocation can be assessed by Ca²⁺-dependent high-affinity binding to Annexin-V. Recombinant human Annexin-V-FITC (Bender Medsystems, Vienna, Austria) was used following the recommended protocol to detect early stage of ongoing apoptosis by flow cytometry (FL-1 channel). For discriminating apoptotic from necrotic events, propidium iodide (50 μ g/mL; Sigma Chemical Co.-Aldrich) was used as a second fluorescent marker, with cells undergoing necrosis with subsequent propidium iodide uptake showing a strong signal in the FL-2 channel. Flow cytometry was performed by a fluorescence-activated cell sorter (FACScan; Becton Dickinson, Heidelberg, Germany), and data were analyzed by Win MDI 2.8 software (Windows Multiple Document Interface for Flow Cytometry; <http://facs.scripps.edu/software.html>).

Caspase-3 detection. Caspase-3 has been recognized as a central player in mediating the effector stage of apoptosis by initiating the process of DNA fragmentation. Activation of caspase-3 in living cells was assessed by the caspase-3 Activity Assay (Oncogene, San Diego, CA) following the manufacturer's instruction. The assay uses a FITC-labeled MAb directed against the cleaved (activated) form of caspase-3. The FITC label allows for direct detection of activated caspase-3 in apoptotic cells by flow cytometry. Samples were analyzed by using the FL-1 channel of the flow cytometer.

Cell-cycle DNA staining with propidium iodide. Cells that have undergone DNA fragmentation lose DNA fragments after ethanol-mediated cell membrane permeabilization and are picked up by their reduced overall DNA content, as measured by flow cytometry (sub-G1 fraction) (15). Cells were resuspended in Hanks' balanced salt solution (Biochrom, Berlin, Germany), chilled 70% ethanol was added to a final concentration of 60%, and the cells were stored at -20°C for 1–2 d. Cells were pelleted and resuspended in 0.5 mL of 70% ethanol and washed and finally resuspended in 1 mL of Hanks' balanced salt solution. Then, 0.5 mL of 0.2 M Na₂HPO₄ adjusted to pH 7.8 with 0.1 M citric acid was added for 5 min. Cells were spun down, the supernatant was discarded, and 50 μ L of DNase-free RNase was added (0.1 mg/mL; Sigma Chemical Co.-Aldrich). After 10 min at room temperature, 200 μ L of PBS that contained 50 μ g/mL propidium iodide was added and allowed to react for 10 min in the dark. Cells were analyzed by FACScan flow cytometer (Becton Dickinson), with 20,000 events per sample being collected. Data analysis was performed by WIN MDI 2.8 and Cylchred program (<http://www.facs-lab.toxikologie.uni-mainz.de>).

Colorimetric viability assay. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) assay was used to estimate cell viability as described (16).

Table 1. Primer pairs of NMDA and AMPA receptor subunits used to assess glutamate receptor expression by PCR

Target	Primer location	Sequence (5'-3')	Size (bp)	GenBank acc. no.	
NR-1	Sense	906–926	GAGAAGGAGAACATCACCGAC	575	L05666
	Antisense	1460–1480	TCCTGTGTGCCGAACCTTGCCA		
hNR2A	Sense	818–837	GATCCACTCTTCTGTCATCT	452	U09002
	Antisense	1250–1269	ATTCGCCGGTCTTTGTTTCAGC		
GRIN2B	Sense	328–349	TCAAGGATGCCACGAGAAG	565	U88963
	Antisense	872–892	GTGGCTTCTTCTTGGTACAG		
GRIN2D	Sense	698–718	ATTGAGGTGCTACTGACGGT	411	NM_000836
	Antisense	1088–1108	GAAACCATAATCACGACGACAG		
GluR3	Sense	71–89	ATGGGGCAAAGCGTGCTCC	328	M85036
	Antisense	381–399	AAGGAGGTCAGGGTGTTCAT		
GluR4	Sense	162–179	ATGAGGATTATTGACAGG	280	M85037
	Antisense	424–442	ATGGCAAACACCCCTCTAG		

Metabolism of the yellow dye MTT by live mitochondria results in a color switch toward violet. MTT is cleaved by all living, metabolically active cells but not dead cells. The amount of formazan generated is directly proportional to the cell number over a wide range, if used in a homogeneous cell population. MTT (Sigma Chemical Co.-Aldrich) was dissolved in PBS at 5 mg/mL (stock solution) and sterilized by filtration. Cells were seeded in flat-bottom 96-well microtiter plates (50,000–100,000 cells/well) and grown for 24 h at 37°C. Then, various concentrations of GA, GC, or 3-OH-GA were added for another 24 h. Finally, 10 μ L of MTT (5 mg/mL) was added to the wells (final concentration 0.5 mg/mL) for 3 h, and the reaction was stopped by addition of 100 μ L of isopropanol that contained 0.04 N HCl. Absorbance was read in a microplate reader (BioRad, Munich, Germany) at 570 nm and a reference wavelength of 650 nm. Data were compared by *t* test, with *p* < 0.05 indicating significance.

RESULTS

GA and its metabolites GC and 3-OH-GA dose-dependently reduce OLN-93 cell viability. When OLN-93 were exposed to 10 mM GA, GC, or 3-OH-GA for 24 h, cell viability decreased sharply (Fig. 1). When assessed by MTT metabolism, the toxicity of all three organic acids was found to be dose dependent, with changes being readily observed at concentrations as low as 0.1 mM (Fig. 2). In contrast, no effects of GA, GC, and 3-OH-GA were seen with the neuroblastoma cell line (SH-SY5Y) or the microglia cell line (BV-2; data not shown).

GA, GC, and 3-OH-GA induce early and late steps of apoptosis. Apoptosis involves a series of sequential events that can be measured *in vitro*. Early during apoptosis, phosphatidylserine (PS) is externalized and thus becomes accessible for Annexin-V. GA-, GC-, or 3-OH-GA-exposed OLN-93 cells

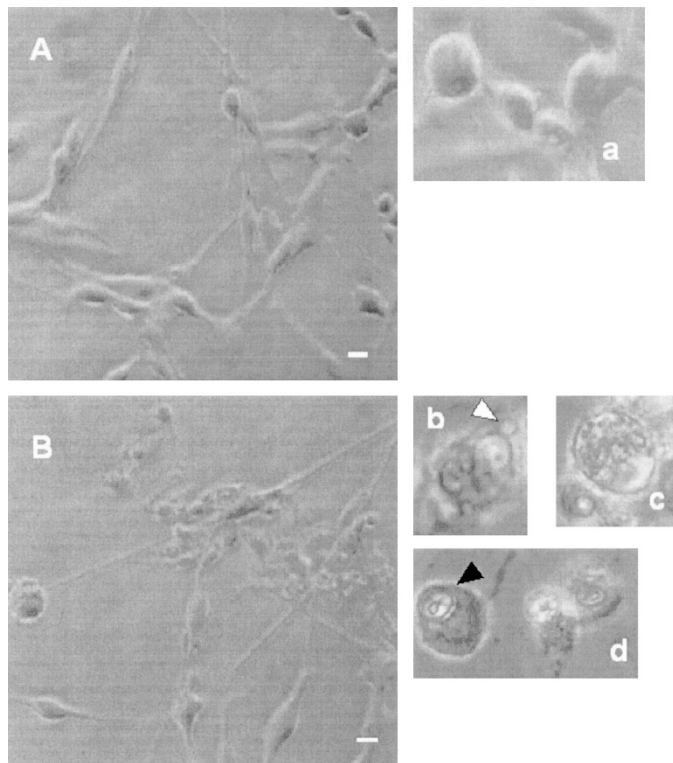


Figure 1. Representative transmission light phase contrast photomicrographs of OLN-93 cell cultures after 24 h of incubation with 10 mM GA. Photomicrographs were taken by Olympus digital camera. Controls (A and a) are intact cells without signs of apoptosis; GA-treated cells (B) show plasma membrane blebbing (b, white arrowhead), chromatin fragmentation (c), and chromatin condensation (d, black arrowhead). White bars = 10 μ m.

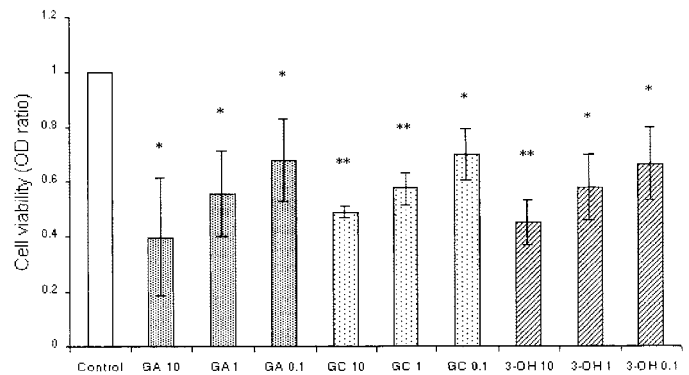


Figure 2. OLN-93 cell viability, as measured by metabolization of MTT (OD ratio), after 24 h of incubation with GA, GC, or 3-OH-GA at concentrations of 0.1, 1, or 10 mM (mean and SD of three independent experiments). **p* < 0.05; ***p* < 0.01.

were found to bind more FITC-labeled Annexin-V than controls (Fig. 3). Activation of the effector caspase-3 was assessed by intracellular binding of a FITC-labeled MAbs specific to the activated (cleaved) form of the enzyme. The percentage of cells that displayed activated caspase-3 was found to increase alongside the concentrations of GA, GC, or 3-OH-GA used (Fig. 4). When the total DNA content of cells was assessed by propidium iodide staining of permeabilized cells, the number of cells with subnormal DNA content secondary to the loss of apoptosis-generated small DNA fragments was significantly increased (Fig. 5).

Toxicity of GA, GC, and 3-OH-GA on OLN-93 cells is abrogated by the pan-caspase inhibitor z-VAD-fmk. For assessing whether inhibition of caspases could block the programmed cell death induced by GA, GC, and 3-OH-GA, the

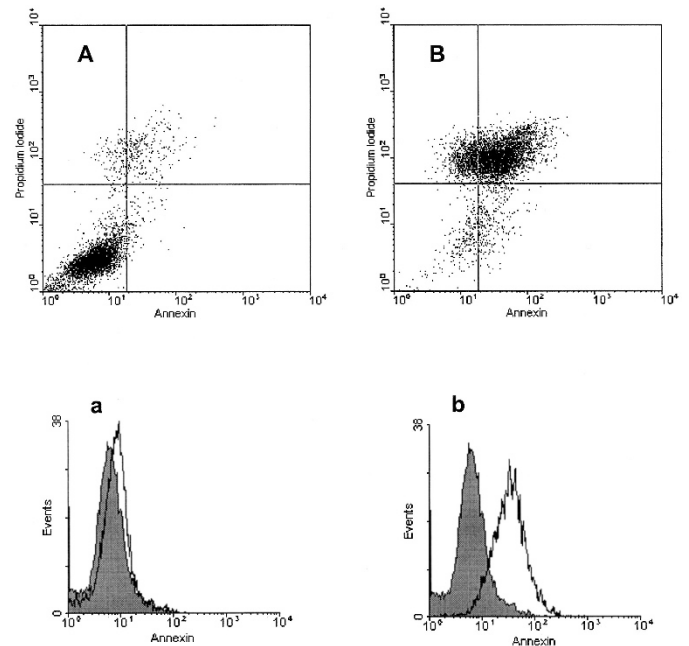


Figure 3. Flow cytometry analysis of annexin-V surface binding (x axis) and propidium iodide uptake (y axis) of OLN-93 exposed to 10 mM GA for 24 h. (A and a) Controls. (B and b) GA-treated cells. (A and B) Dot plots. (a and b) Histograms, showing FITC-annexin-V binding (open curve) vs background control fluorescence (shaded). x axis, fluorescence intensity; y axis, number of cells.

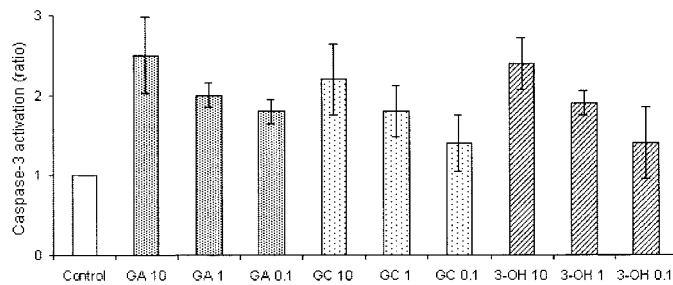


Figure 4. Activation of the effector caspase-3, as assessed by intracellular binding of a FITC-labeled MAb specific for the activated (cleaved) form of the enzyme, using flow cytometry. OLN-93 cells were incubated with GA, GC, or 3-OH-GA at concentrations of 0.1, 1, or 10 mM for 24 h before analysis (mean and SD of three independent experiments). * $p < 0.05$; ** $p < 0.01$.

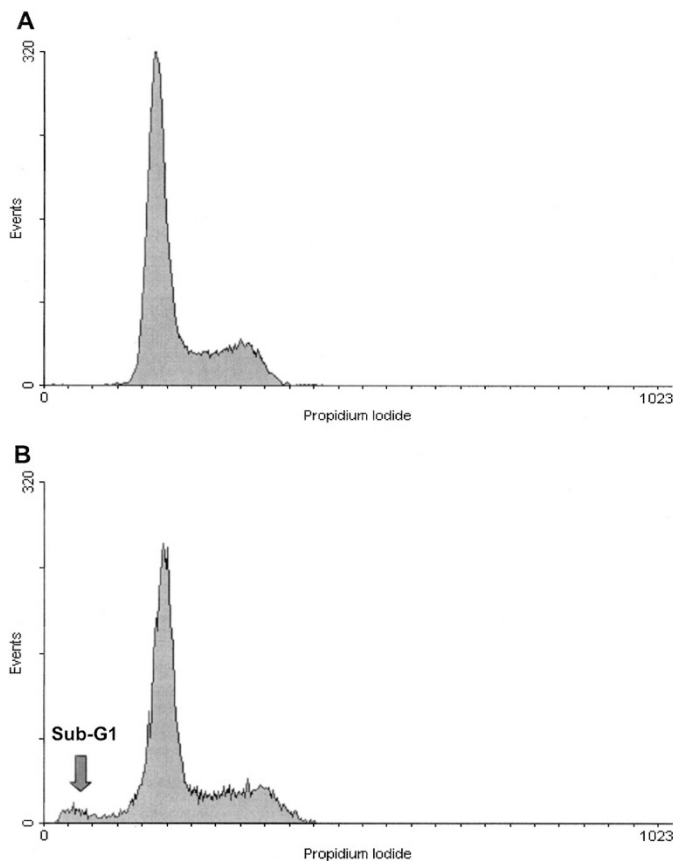


Figure 5. DNA content of control (A) and 10 mM GC-treated OLN-93 cells (B), assessed after permeabilization and subsequent propidium iodide staining, using flow cytometry. The main peak represents cells in G0/G1 phase of the cell cycle. The proportion of cells with subnormal DNA content (sub-G1), secondary to loss of cleaved DNA fragments generated by the apoptotic machinery, increased after 24 h of incubation with 10 mM GC (B), compared with controls (A).

pan-caspase inhibitor z-VAD-fmk (40 μ M final concentration; Promega) was added together with the organic acids tested. This treatment completely abrogated the effects of GA, GC, or 3-OH-GA (Fig. 6).

Excitotoxicity is not a major mechanism of GA-, GC-, and 3-OH-GA-mediated toxicity in OLN-93 cells. Reverse transcription-PCR analysis of OLN-93 cells failed to detect transcripts of the genes coding for the NMDA receptor subunits NR1, NR2A, and NR2B, whereas cells that expressed the

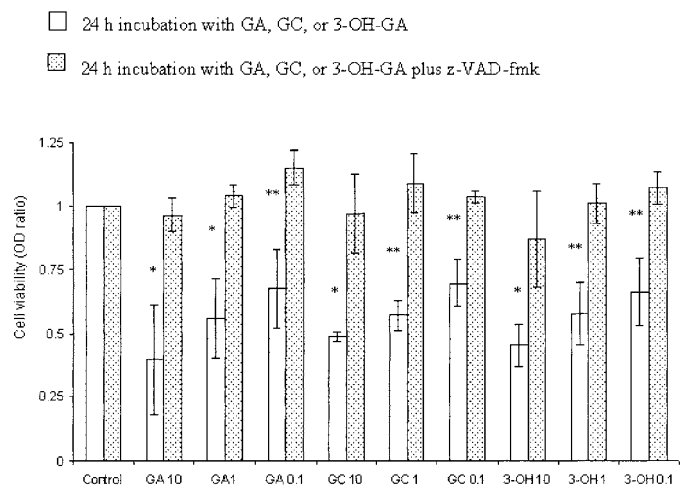


Figure 6. Involvement of caspases in toxicity mediated by GA, GC, or 3-OH-GA (0.1, 1, or 10 mM, 24 h) was assessed using the pan-caspase inhibitor z-VAD-fmk. OLN-93 cell viability was determined *via* metabolization of MTT (OD ratio, mean and SD). z-VAD-fmk (40 μ M final concentration) was added together with the organic acids tested. * $p < 0.05$; ** $p < 0.01$. □ 24 h incubation with GA, GC, or 3-OH-GA, ▨ 24 h incubation with GA, GC, or 3-OH-GA plus Z-VAD-fmk.

NR2D and AMPA receptor subunits GluR3, GluR4, NR1, NR2A, or NR2B transcripts were readily detected in total CNS control RNA. Neither the NMDA receptor antagonist MK801 nor the AMPA receptor antagonist GYKI was able to prevent the reduction of cell viability observed with GA, GC, and 3-OH-GA (data not shown).

Both oligodendrocyte precursor cells and more mature oligodendrocytes are susceptible to GA-, GC-, and 3-OH-GA-mediated toxicity. After OLN-93 were cultured in the presence of platelet-derived growth factor-AA and basic fibroblast growth factor for 5–7 d, an O-2A progenitor-type bipolar morphology emerged, consistent with partial dedifferentiation (14). Conversely, when cultured in the combined presence of ciliary neurotrophic factor, forskolin, and triiodo-thyronine, cells displayed the intensive arborization characteristic of more mature oligodendrocytes. When exposed to GA, 3-OH-GA, or GC, both precursor cells and mature oligodendrocytes showed increased annexin-V binding, which was slightly more pronounced in the precursor cell type (data not shown).

DISCUSSION

These data show that the organic acids GA, GC, and 3-OH-GA specifically induce apoptosis in the immature oligodendroglia cell line OLN-93. GA-, GC-, or 3-OH-GA-mediated oligodendrocyte cell death bears the hallmarks of various steps of apoptosis and can be blocked by the pan-caspase inhibitor z-VAD-fmk.

Until recently, most investigations have focused on the toxicity of GA and 3-OH-GA for neurons, which is thought to involve NMDA receptor-mediated excitotoxicity. As GA poorly penetrates the blood-brain barrier, GDD has been difficult to study in animal models. Targeted deletion of the gene encoding glutaryl-CoA dehydrogenase in murine embryonic stem cells has allowed for the generation of mice with a

biochemical phenotype closely resembling human GDD (10). Surprisingly, these mice show no evidence of neuronal loss but display mild motor deficits. Pathologically, they are characterized by diffuse spongiform white matter disease. White matter abnormalities, including dys- and demyelination, have also been observed in human GDD. In adult-onset GDD, they seem to be the prominent feature on MRI (9). We believe that the hitherto unexplained leukoencephalopathy is related to direct oligodendroglia cell destruction caused by GA and its metabolites.

For neurons, the main mechanism that causes cell damage after GA exposure seems to be necrosis (17). In contrast, the main damage mechanism in oligodendrocytes seems to be apoptosis. GA, GC, and 3-OH-GA dose-dependently induced phosphatidyl-serine externalization, caspase-3 activation, and DNA fragmentation. Moreover, the loss of cell viability caused by GA, GC, and 3-OH-GA could be prevented by the pan-caspase inhibitor z-VAD-fmk, clearly establishing classical (caspase-dependent) apoptosis as the main mechanism responsible for GA-, GC-, and 3-OH-GA-mediated oligodendrocyte cell death.

GA- and 3-OH-GA-triggered neuronal damage has been reported to be mediated by excitotoxicity involving the NMDA receptor (18), although no specific binding to NMDA receptors could be demonstrated (3). Specifically, GA and 3-OH-GA toxicity was found to depend on expression of the NR1 and NR2B subunits of the NMDA receptor. In contrast, OLN-93 oligodendrocytes did not express these NMDA receptor subunits that are essential for NMDA-mediated excitotoxicity (4). Moreover, GA-, 3-OH-GA-, and GC-mediated oligodendrocyte apoptosis could not be prevented by preincubation with the nonselective NMDA receptor antagonist MK801 or the AMPA receptor antagonist GYKI. In various studies, oligodendrocytes have been found to express functional AMPA receptor but little or no NMDA receptor. Overstimulation of AMPA receptors has been shown to induce excitotoxic damage in oligodendroglia *in vitro* and *in vivo* (19,20). However, excitotoxicity does not contribute significantly to apoptosis induced in oligodendrocytes that are exposed to GA, 3-OH-GA, or GC. Bjugstad *et al.* (21) also found that GA/3-OH-GA-mediated cell death is not necessarily mediated by glutamate receptors: neurotoxicity in striatal and cortical cultures of embryonic rat striatum could not be prevented by glutamate antagonists MK801 and NBQX.

Both GA and GC have been shown to directly induce mitochondrial permeability transition (22). Whereas opening the permeability transition pore features prominently among apoptotic pathways (23), induction of apoptosis by various triggers, including branched chain amino acids, may proceed in the absence of mitochondrial permeability transition (24). The role of mitochondria in mediating GA-, 3-OH-GA-, and GC-triggered oligodendrocyte apoptosis remains to be determined.

Oligodendrocytes develop from oligodendrocyte precursor cells in forebrain and cerebellum (25) during late gestational and early postnatal development. Oligodendroglial progenitor cells exist in the subventricular zone and migrate into the intermediate zone and cortical plate, where they differentiate into immature oligodendrocytes, which mature and myelinate

axons (26). Postnatal cerebral ultrasounds of patients with GA-1 often show bilateral subependymal cysts (27). Oligodendrocyte development depends also on interactions with other cells in the CNS, in particular, with neurons that synthesize and release factors that are essential for oligodendrocyte precursor cells, including glutamate (28). Glutamate-mediated activation of AMPA receptors generates large depolarizing currents, inducing immediate expression of genes. Immature oligodendrocytes are more vulnerable and more susceptible to AMPA receptor-mediated damage than mature oligodendrocytes (14,19). Both immature and mature oligodendrocytes seemed to be susceptible to GA-, GC-, or 3-OH-GA-mediated induction of apoptosis, which seemed more pronounced in the precursor cell type. In contrast to OLN-93 cells, SH-SY5Y neuroblastoma or BV-2 microglia cells were resistant to GA, 3-OH-GA, or GC up to concentrations of 10 mM. Whereas neurotoxicity of GA and 3-OH-GA seems to be maturation dependent (17), susceptibility to apoptosis mediated by GA, 3-OH-GA, or GC seems to be more a matter of lineage than maturity. However, the SH-SY5Y and BV-2 cell lines may be poor models for studying neuronal and microglial cells *in vitro*, and experiments involving freshly prepared cells are preferred (2,17,21,29).

In addition to excitotoxicity, GA and its metabolites exert various effects, including inhibition of mitochondrial respiratory chain activity, energy production (30), generation of reactive oxygen species (4,6), depletion of intracellular creatine phosphate stores (3), and inhibition of the Na^+/K^+ -ATPase of the plasma membrane (29). These biochemical disturbances require GA concentrations encountered during metabolic imbalances and may be important for the development of neuronal necrosis associated with metabolic decompensation. Little is known about cerebrospinal fluid and brain tissue concentrations of GA and its metabolites in GDD patients (1), and there is a complete lack of fetal data. Whereas in most experimental settings described in this article, oligodendrocyte apoptosis was observed with concentrations as little as 0.1 mM, neuronal apoptosis was elicited experimentally using higher concentrations, up to 50 mM (2–4,17,21,30). Moreover, although experiments have been performed with GA, 3-OH-GA, or GC, all three substances are present within *in vivo* situations. Morphologic changes of the brain in patients with GDD already develop *in utero* and are readily visualized by ultrasound in the fetus and the asymptomatic newborn (31,32). Therefore, we propose a dual action of GA and its metabolites in patients with GDD: moderately increased concentrations, as encountered *in utero* and in patients who are subjected to rigorous dietary treatment, induce oligodendrocyte apoptosis, whereas peak concentrations during metabolic compensation precipitate neuronal necrosis.

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