Contribution of Reactive Oxygen Species to 3-Hydroxyglutarate Neurotoxicity in Primary Neuronal Cultures from Chick Embryo Telencephalons

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ABSTRACT

Glutaryl-CoA dehydrogenase deficiency is an autosomal recessively inherited neurometabolic disorder with a distinct neuropathology characterized by acute encephalopathic crises during a vulnerable period of brain development. 3-Hydroxyglutarate (3-OH-GA), which accumulates in affected patients, has been identified as an endogenous neurotoxin mediating excitotoxicity via *N*-methyl-D-aspartate receptors. As increased generation of reactive oxygen species (ROS) and nitric oxide (NO) plays an important role in excitotoxic neuronal damage, we investigated whether ROS and NO contribute to 3-OH-GA neurotoxicity. 3-OH-GA increased mitochondrial ROS generation in primary neuronal cultures from chick embryo telencephalons, which could be prevented by MK-801, confirming the central role of *N*-methyl-D-aspartate receptor stimulation in 3-OH-GA toxicity. ROS increase was reduced by α -tocopherol and—less effectively—by melatonin. α -Tocopherol revealed a wider time frame for neuroprotection than melatonin. Creatine also reduced neuronal damage and ROS formation but only if it was administered ≥ 6 h before 3-OH-GA. NO production revealed only a slight increase after 3-OH-GA incubation. NO synthase inhibitor *N*^ω-nitro-L-arginine prevented NO increase but did not protect neurons against 3-OH-GA. The NO donor *S*-nitroso-*N*-acetylpenicillamine revealed no effect on 3-OH-GA toxicity at low concentrations (0.5–5 μ M), whereas it potentiated neuronal damage at high concentrations (50–500 μ M), suggesting that

weak endogenous NO production elicited by 3-OH-GA did not affect neuronal viability. We conclude from our results that ROS generation contributes to 3-OH-GA neurotoxicity *in vitro* and that radical scavenging and stabilization of brain energy metabolism by creatine are hopeful new strategies in glutaryl-CoA dehydrogenase deficiency. (*Pediatr Res* 50: 76–82, 2001)

Abbreviations

3-OH-GA, 3-hydroxyglutarate
DHR, dihydrorhodamine-123
GCDH, glutaryl-CoA dehydrogenase (EC 1.3.99.7)
GDD, glutaryl-CoA dehydrogenase deficiency
HBS, magnesium- and zinc-free HEPES buffered saline
L-NAME, *N*^ω-nitro-L-arginine
MK-801, (5R,10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine
NMDA, *N*-methyl-D-aspartate
NO, nitric oxide
nNOS, neuronal nitric oxide synthase; synonym, nitric oxide synthase type I (EC 1.14.13.39)
NO⁻, nitroxyl anion
NO⁺, nitrosonium ion
ROS, reactive oxygen species
SNAP, *S*-nitroso-*N*-acetylpenicillamine

GDD (synonym, glutaric aciduria type I) is a neurometabolic disease caused by autosomal recessively inherited deficiency of GCDH (EC 1.3.99.7), which catalyzes the dehydrogenation and decarboxylation of glutaryl-CoA to crotonyl-CoA in the catabolic pathways of the amino acids L-tryptophan, L-lysine, and L-hydroxylysine (1). Deficient GCDH activity results in an

accumulation of 3-OH-GA, glutarate, and glutaconate in brain tissue and body fluids, mostly reaching micromolar concentrations, but during acute encephalopathic crisis they can rise to considerably higher levels (1–2 mM) (2, 3). GDD is clinically characterized by acute encephalopathic crises precipitated mostly between age 6 and 18 mo by intercurrent illnesses (1). If not treated adequately, irreversible destruction of vulnerable brain regions, *i.e.* striatum and cortex, will result, leading to a dystonic dyskinetic movement disorder (1–3).

The pathomechanism of the acute neuronal damage in GDD has been subject to intense debates. The first pathophysiologic

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concepts hypothesized a reduced production of γ -aminobutyric acid (4), and a role for quinolinic acid (5), which could not be confirmed. Evidence from recent *in vitro* studies points to an excitotoxic sequence in GDD, with 3-OH-GA acting as the main neurotoxin *via* NMDA receptors (6, 7). NMDA receptors play an important role in the development of the CNS (8, 9), and even transient NMDA receptor blockade enhances apoptosis in immature rat brains (10), thus limiting the application of recently available NMDA receptor antagonists to emergency treatment during acute encephalopathic crises.

The brain is at a particular risk from damage by ROS as it has an extremely high rate of oxygen consumption, a high content of oxidizable polyunsaturated fatty acids, and a weak defense system against ROS (11, 12). Increased ROS generation was previously demonstrated in excitotoxicity (11–14), pointing to an impaired brain energy metabolism secondary to acquired mitochondrial dysfunction (11, 15). A previous *in vitro* study demonstrated decreased phosphocreatine concentrations after 3-OH-GA exposure in rat cortical cultures, suggesting an involvement of decreased energy buffering capacity in 3-OH-GA neurotoxicity (6). Increased NO production after activation of Ca^{2+} -dependent nNOS also contributes to NMDA receptor-mediated excitotoxicity, but its exact role is still controversially discussed [reviewed by Dawson *et al.* (16)]. Whereas neurodegeneration by NO is mostly dependent on the generation of peroxynitrite (17), neuroprotection by activated nNOS has been addressed to redox-related species of NO, NO^- and NO^+ (18, 19).

The aim of the present study was to delineate the role of ROS and NO in 3-OH-GA neurotoxicity, and to determine whether the prevention of ROS and NO production might open new neuroprotective options.

METHODS

Cell culture. Primary neuronal cultures from 7-d-old chick embryo telencephalons were prepared as previously described (20). Briefly, cerebral hemispheres were dissociated, and a cell suspension was seeded with a density of 4×10^4 cells/cm² either onto culture flasks (Nunc, Hamburg, Germany) or onto Petri dishes (Nunc) with glass coverslips, previously coated with poly-L-lysine (Sigma Chemical Co., Deisenhofen, Germany). Cell cultures were maintained in Dulbecco's modified Eagle medium supplemented with 20% fetal bovine serum (LifeTechnologies, Eggenstein, Germany) at 37°C and 5% CO₂ in a humidified atmosphere. Cultured neurons were used for the experiments at d 6 *in vitro*. We previously demonstrated that cultured chick embryo neurons expressed NMDA receptors at this time point and were susceptible to 3-OH-GA (7). Animal care was approved by and followed the guidelines of the Institutional Review Board.

Treatment protocol. Cultured neurons were exposed to 1 mM 3-OH-GA (kind gift of Prof. W. Buckel, Department of Microbiology, University of Marburg, Germany) at d 6 *in vitro* for 1 h. 3-OH-GA and all other applied substances were dissolved in HBS, containing 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂ · 2 H₂O, 10 mM D-glucose, 10 mM HEPES, and 10 μ M glycine in deionized water (all adjusted at pH 7.4 with 1

mM NaOH) to retain full NMDA receptor function. Positive controls were performed by exposure to 0.3 mM NMDA for 1 h, negative controls by exposure to HBS alone for 1 h. α -Tocopherol, melatonin, creatine monohydrate, and NMDA were all purchased from Sigma Chemical Co., L-NAME from RBI (Natick, MA, U.S.A.), MK-801 from Tocris (Bristol, U.K.), and SNAP from Molecular Probes (Eugene, OR, U.S.A.). α -Tocopherol (0.1–10 μ M) or melatonin (5–500 μ M) were applied each for 1 h, either 1 h before 3-OH-GA exposure (preincubation), together with 3-OH-GA (coincubation), or immediately after 3-OH-GA exposure (postincubation). In this concentration range α -tocopherol does not interfere with signal transduction pathways by mechanisms that are independent of its antioxidant properties (21). Creatine monohydrate (0.01–1 mM) was administered 2–72 h before, during, and after 3-OH-GA exposure. L-NAME (100 μ M) was applied 1 h before and during 3-OH-GA exposure. Cultured neurons were treated with MK-801 (10 μ M) 1 h before and during exposure to 3-OH-GA. Furthermore, neurons were exposed to SNAP (0.5–500 μ M) during and after 3-OH-GA exposure.

Assessment of neuronal viability. Cell viability was determined by trypan blue (0.4% in PBS) exclusion method 1–72 h after 3-OH-GA exposure as previously described (7). The uptake of trypan blue indicates membrane leakage, an end point of neuronal degeneration. The number of stained (non-viable) and unstained (viable) neurons were counted under a microscope. Only darkly stained neurons were considered damaged. A total number of 500–600 neurons were counted in eight randomly chosen subfields per group ($n = 8$).

Apoptotic cell damage was investigated by nuclear staining with DNA fluorochrome Hoechst 33258 (Sigma Chemical Co.) in sister cultures as previously described (7). Briefly, neurons were fixed with methanol 1–72 h after exposure to 3-OH-GA, and then were incubated with 10 μ g/mL Hoechst 33258 for 15 min. Nuclear morphology was evaluated by fluorescence microscopy (Axiovert 100; Zeiss, Oberkochen, Germany). The percentage of apoptotic neurons revealing chromatin condensation or nuclear fragmentation was determined in eight randomly chosen subfields evaluating 500–600 single neurons. All cell counts were performed without knowledge of the respective treatments.

Measurement of mitochondrial ROS. Mitochondrial ROS production was determined as previously described (22). In brief, ROS production was monitored 1–24 h after the beginning of exposure to 3-OH-GA or NMDA using the lipophilic nonfluorescent dye DHR (Molecular Probes), which accumulates in mitochondria and is oxidized by ROS to the positively charged fluorescent dye rhodamine-123 (23–25). This treatment protocol was chosen to delineate the time-course of ROS production after exposure. In further experiments the mechanism of elicited ROS generation was investigated by treatment with either MK-801, α -tocopherol, melatonin, or creatine monohydrate. To record fluorescence, cultured neurons were loaded with 5 μ M DHR for 30 min in the dark and then gently washed three times with PBS. Digital video imaging of rhodamine-123 fluorescence was conducted using an Axiovert 100 inverted microscope (Zeiss), equipped with a 40 $\times$ fluorescence objective (Zeiss), a charge-coupled device camera

(C 2400–87), and Argus-50/CA software (all from Hamamatsu, Herrsching, Germany). To avoid photobleaching, cells were only shortly exposed (approximately 5 s) to excitation light. Fluorescence intensity of rhodamine-123 (excitation, 490 nm; emission, 510 nm) was measured in single neurons ($n = 100$) and was expressed in an arbitrary scale as fluorescence units. The unspecific background was subtracted from the images.

NO measurement. NO production was determined by measurement of nitrite, a stable oxidation breakdown product of NO, using the Griess reagent (26). The principle of this assay is a quantitative conversion of sulfanilic acid to a diazonium salt by reaction with nitrite in acid solution. The diazonium salt is then coupled to *N*-(1-naphthyl)ethylenediamine, thereby forming an azo dye that can be spectrophotometrically determined from its absorbance at 548 nm. A mixture of 0.1 mL of Griess reagent (Molecular Probes), 0.3 mL of culture media of sister cultures ($n = 6$), and 2.6 mL of deionized water was incubated for 30 min at room temperature, and nitrite concentrations were calculated using a standard curve. Nitrite levels were expressed as x -fold of control.

Data analysis. All data were expressed as mean $\pm$ SD except for measurements of ROS (mean $\pm$ SEM). All experiments were performed in triplicate. Figures show representative experimental data for one of three independent experiments. A $p < 0.05$ was considered significant. One-way ANOVA followed by Scheffé's test (for three or more groups) or t test (for two groups) was used to determine the statistical significance of any difference.

RESULTS

3-OH-GA increases ROS production mediated by NMDA receptors. Exposure to 1 mM 3-OH-GA for 1 h increased mitochondrial ROS generation as determined by rhodamine-123 fluorescence (Fig. 1A). Mitochondrial ROS increased to maximum immediately after the 1-h exposure (time point 1 h) and subsequently decreased to control level within the subsequent 3 h (time point 4 h) (Fig. 1A). Exposure to 0.3 mM NMDA for 1 h as a positive control revealed a stronger and more prolonged ROS increase than with 3-OH-GA (Fig. 1A). Consistently, NMDA decreased cell viability ($29 \pm 7\%$) much more strongly than 3-OH-GA ($60 \pm 5\%$; control: $91 \pm 2\%$). 3-OH-GA- or NMDA-induced ROS increase was totally prevented by MK-801, suggesting an NMDA receptor-mediated mechanism (Fig. 1B). 3-OH-GA-induced neuronal damage appeared at 8–12 h after treatment (trypan blue exclusion), reaching a maximum at 24–72 h after treatment (Fig. 1C). Trypan blue-stained neurons mostly revealed necrotic features. In contrast, Hoechst 33258 staining revealed only a mild increase in apoptotic neurons between 12 and 48 h after treatment.

α -Tocopherol protects neurons against 3-OH-GA neurotoxicity. Furthermore, we investigated the effects of the radical scavengers melatonin and α -tocopherol on ROS generation and cell viability in our toxicity model. Coincubation with melatonin increased cell viability at a concentration of 500 μ M, whereas 5–50 μ M melatonin as well as preincubation and postincubation did not reveal significant neuroprotection (Fig.

Figure 1. 3-OH-GA and NMDA increased ROS production via NMDA receptor stimulation. Primary neuronal cultures from chick embryo telencephalons were exposed to 1 mM 3-OH-GA or 0.3 mM NMDA for 1 h. ROS were determined by rhodamine-123 fluorescence ($n = 100$); cell viability ($n = 8$) was evaluated 24 h after treatment by trypan blue exclusion or Hoechst 33258 staining. Figures show representative experimental data from one of three independent experiments. *A*, maximal ROS increase appeared immediately after 3-OH-GA exposure (1 h), which declined to control level within the next 3 h (4 h). NMDA evoked a stronger and more prolonged ROS increase than 3-OH-GA. * $p < 0.05$ vs control (one-way ANOVA followed by Scheffé's test); # $p < 0.05$ 3-OH-GA vs NMDA (t test). Data are expressed as mean $\pm$ SEM. *B*, 3-OH-GA (1 mM)- and NMDA (0.3 mM)-induced ROS increase was prevented by MK-801 (10 μ M), suggesting NMDA receptor-mediated ROS generation. Data show ROS generation at 1 h. * $p < 0.001$ vs NMDA or 3-OH-GA alone (t test). Data are expressed as mean $\pm$ SEM. *C*, time scale of neuronal damage after exposure to 1 mM 3-OH-GA for 1 h. Trypan blue-stained neurons mostly revealed necrotic features, whereas apoptotic neurons increased only weakly (Hoechst 33258), thus necrosis was the predominant mode of cell death. Control levels of trypan blue (9–11%) and apoptotic Hoechst-stained neurons (4–6%) were similar at each time (not shown). * $p < 0.05$ vs control (one-way ANOVA followed by Scheffé's test).

Figure 2. α -Tocopherol rather than melatonin protected cultured chick embryo neurons against 3-OH-GA neurotoxicity by decreasing evoked mitochondrial ROS. Neurons were exposed to 1 mM 3-OH-GA for 1 h, and were pre-, co-, or postincubated with either α -tocopherol or melatonin. Trypan blue exclusion ($n = 8$) was evaluated 24 h after exposure, whereas rhodamine-123 fluorescence ($n = 100$) was determined immediately after exposure to 3-OH-GA (1 h). Experiments were performed in sister cultures. Figures show representative data from one of three experiments. *A*, coincubation with 500 μ M melatonin increased cell viability after exposure to 3-OH-GA. $**p < 0.01$ vs 3-OH-GA (one-way ANOVA followed by Scheffé's test), $###p < 0.01$, $####p < 0.001$ vs coincubation with 500 μ M melatonin (t test). Data are mean $\pm$ SD. *B*, α -tocopherol prevented neuronal damage induced by 3-OH-GA in a concentration-dependent manner and comprised a wider therapeutic window than melatonin. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ vs 3-OH-GA (one-way ANOVA followed by Scheffé's test), $###p < 0.01$, vs coincubation with 10 μ M α -tocopherol (t test). Data are mean $\pm$ SD. *C*, coincubation with 10 μ M α -tocopherol (α -T) decreased 3-OH-GA-evoked ROS formation more strongly than 500 μ M melatonin (M). Rhodamine-123 fluorescence was determined at 1 h. $**p < 0.01$, $***p < 0.001$ vs 3-OH-GA, $###p < 0.01$ melatonin vs α -tocopherol. Data were expressed as mean $\pm$ SEM.

2A). On the other hand, α -tocopherol revealed neuroprotection at concentrations of ≥ 1 μ M (Fig. 2B). Although coincubation was determined to be most effective, pretreatment and post-treatment with α -tocopherol also reduced neuronal damage (Fig. 2B). Consistently, 3-OH-GA-elicited ROS increase was reduced more effectively by α -tocopherol than melatonin (Figs. 2C and 3).

Pretreatment with creatine reduces 3-OH-GA neurotoxicity. Creatine application reduced neuronal damage after exposure to 1 mM 3-OH-GA. The neuroprotective effect of creatine was only observed at concentrations of ≥ 0.1 mM and only if creatine was administered ≥ 6 h before 3-OH-GA (Fig. 4A). In parallel to this, creatine decreased ROS generation only if it was applied ≥ 6 h before exposure to 3-OH-GA (Figs. 3 and 4B).

No evidence for a contribution of endogenous NO to 3-OH-GA neurotoxicity. NO production was determined by measurement of nitrite (26). Nitrite concentrations increased only slightly after exposure to 0.01–1 mM 3-OH-GA (Fig. 5A). Application of 100 μ M L-NAME decreased nitrite levels of neuronal cultures being treated with 3-OH-GA or vehicle below control level (Fig. 5B). However, the decrease in nitrite

Figure 3. Representative photomicrographs of ROS production after exposure to 3-OH-GA (1 h) in the presence or absence of α -tocopherol, melatonin, or creatine. ROS production was determined by rhodamine-123 fluorescence (images B, D, F, H, J, L, N) in cultured chick embryo neurons. Corresponding transmission light microscopy images show the location of fluorescent cells (images A, C, E, G, I, K, M). Transmission light and fluorescent photomicrographs were collected by a charge-coupled device camera and digitalized to 256×256 pixels. Scale bar, 20 μ m. *A*, *B*, HBS-treated control revealed only a weak rhodamine-123 fluorescence, indicating low spontaneous ROS production in cultured neurons. *C*, *D*, rhodamine-123 fluorescence remarkably increased after exposure of cultured neurons to 1 mM 3-OH-GA for 1 h, indicating increased ROS production elicited by 3-OH-GA. *E*, *F*, coincubation with α -tocopherol (10 μ M) prevented 3-OH-GA-induced ROS increase. *G*, *H*, coincubation with melatonin (500 μ M) only partially reduced 3-OH-GA-induced ROS increase. Preincubation with 1 mM creatine for 24 h (*I*, *J*) had no effect on 3-OH-GA-induced ROS increase, whereas an extension of the preincubation period to 24 h (*K*, *L*) or 72 h (*M*, *N*) revealed a remarkable reduction of 3-OH-GA-induced ROS production by 1 mM creatine.

Figure 4. Pretreatment with creatine reduced neuronal damage and ROS increase after 3-OH-GA exposure. Neurons were exposed to 1 mM 3-OH-GA for 1 h. Trypan blue exclusion (24 h after treatment) and rhodamine-123 fluorescence (1 h) were determined in sister cultures. Figures show representative experimental data from one of three experiments. *A*, creatine pretreatment increased cell viability ($n = 8$) in a concentration- and time-dependent way after 3-OH-GA exposure. $*p < 0.05$, $***p < 0.001$ vs 3-OH-GA (one-way ANOVA followed by Scheffé's test). Data are mean $\pm$ SD. *B*, creatine pretreatment reduced mitochondrial ROS generation ($n = 100$) evoked by 3-OH-GA. $**p < 0.01$ vs 3-OH-GA (one-way ANOVA followed by Scheffé's test). Data are mean $\pm$ SEM.

concentrations by L-NAME did not protect 3-OH-GA-treated cultures but even slightly increased neuronal damage (Fig. 5C). However, a protective role of NO could not be confirmed by cotreatment with the NO donor SNAP. At low concentrations (0.5–5 μ M), SNAP did not affect 3-OH-GA neurotoxicity, whereas it had a potentiating effect at high concentrations (50–500 μ M) (Table 1).

DISCUSSION

Recently, we have demonstrated that primary neuronal cultures from chick embryo telencephalons (20) were susceptible to 3-OH-GA (7). 3-OH-GA neurotoxicity has also been con-

Figure 5. NO did not contribute to 3-OH-GA neurotoxicity. Neurons were exposed to 3-OH-GA for 1 h in the presence or absence of L-NAME. Nitrite concentrations ($n = 6$) and trypan blue exclusion ($n = 8$) were determined in sister cultures 24 h after exposure. Figures show representative experimental data from one of three experiments. Data are mean $\pm$ SD. *A*, NO increased only slightly after 3-OH-GA exposure. NO production was determined by spectrophotometric analysis of nitrite concentrations in culture media. Nitrite levels were expressed as x-fold of control. $*p < 0.05$ vs control (one-way ANOVA followed by Scheffé's test). *B*, L-NAME (100 μ M) decreased basal and 3-OH-GA-evoked NO production. $*p < 0.05$ comparison between groups as indicated (*t* test). *C*, L-NAME (100 μ M) did not protect against 3-OH-GA. $*p < 0.05$ with vs without L-NAME (*t* test).

firmed in other culture systems (6–7). Nevertheless, to date nothing is known about the downstream events of 3-OH-GA neurotoxicity.

We found that increased mitochondrial ROS production is involved in the excitotoxic cascades in 3-OH-GA neurotoxicity. ROS maximally increased immediately after 3-OH-GA exposure and was prevented by MK-801, suggesting an NMDA receptor-mediated mechanism. Neuronal cell death appeared as early as 8–12 h after 3-OH-GA exposure, reaching a maximum after 24–72 h, revealing that ROS increase preceded neuronal damage. Damaged neurons predominantly revealed necrotic features, thus confirming previous observations (6, 7).

Furthermore, we found evidence that both therapeutic approaches, *i.e.* radical scavenging with α -tocopherol and melatonin, and stabilization of energy metabolism with creatine reduced or even prevented increased ROS generation and subsequent neuronal damage after 3-OH-GA exposure. The neuroprotective profile of the applied substances differed greatly concerning the extent of neuroprotection and the therapeutic window. α -Tocopherol revealed a stronger neuroprotective effect than melatonin, protecting neurons at 500-fold lower concentration than melatonin. Furthermore, melatonin reduced neuronal damage and mitochondrial ROS formation only if it was coincubated with 3-OH-GA, whereas pretreatment and posttreatment with α -tocopherol was also partially protective, thus revealing a wider therapeutic window for α -tocopherol than for melatonin. Our results are in line with a previous report that demonstrated that melatonin protected primary cultures of cerebellar granule neurons from kainate-induced but not from NMDA-induced excitotoxicity (27), suggesting that ROS induction *via* non-NMDA receptors was more susceptible to an inhibition with melatonin than that *via* NMDA receptors.

Alternatively, the difference between α -tocopherol and melatonin might point to the generation of certain ROS after 3-OH-GA exposure. The oxidant-sensitive dye DHR is known to be oxidized by hydrogen peroxide and hydroxyl radicals (23, 24). Hydrogen peroxide, which can be formed by dismutase of the superoxide anion (28), is generally considered to be poorly reactive, and its toxicity is usually assumed to be a consequence of its conversion to hydroxyl radicals by the Fenton and Haber-Weiss reactions (29). However, the weak neuroprotective effect of the potent hydroxyl radical scavenger melatonin

(30) questions a remarkable contribution of hydroxyl radicals in our model. Growing evidence points to a sensitivity of DHR to oxidation by superoxide anions (31–33), which play an important role in NMDA receptor-mediated neurotoxicity (13, 14). Increased superoxide anion generation by 3-OH-GA is indirectly supported by a potentiating effect of high concentrations of NO donor SNAP on 3-OH-GA toxicity, which is suggestive of peroxynitrite formation (17). Peroxynitrite, which can also be detected by DHR (34), is formed by superoxide anions and NO and is highly toxic (17). However, to date we can only suggest that superoxide anions and peroxynitrite are involved in 3-OH-GA toxicity. Neuroprotection by α -tocopherol, which is the major endogenous lipophilic antioxidant (35) and is considered as the premier peroxy radical scavenger (36), points to an involvement of lipid peroxidation in 3-OH-GA neurotoxicity (37). Further studies are required to elucidate which specific ROS are involved in this cascade.

Mitochondrial dysfunction is supposed to be a key event in excitotoxicity, resulting in disturbed energy metabolism and increased mitochondrial ROS production (11). A recent *in vitro* study found evidence for decreased phosphocreatine concentrations with 3-OH-GA exposure in rat cortical cultures, suggesting an involvement of impaired energy buffering capacity (6). Creatine administration was shown to increase intracellular creatine and phosphocreatine concentrations as well as the phosphocreatine to nucleoside triphosphates ratio (38, 39) and to protect neurons against different neurotoxins, such as NMDA (40), malonate (40), and 3-nitropropionic acid (41), as well as against hypoxia (38) and anoxia (42). Our results revealed neuroprotection of creatine by decreasing mitochondrial ROS generation. Interestingly, creatine application revealed a certain limitation as it protected neurons only if administered ≥ 6 h before 3-OH-GA exposure. This is in line with previous reports (39, 42) and might be explained by the time required for creatine transport and phosphocreatine formation by creatine kinase (43). Creatine administration prevented phosphocreatine depletion after 3-OH-GA exposure (44), and stimulated mitochondrial respiration (45). Therefore, we suggest that creatine administration decreased 3-OH-GA-evoked ROS production *via* stabilization of brain energy metabolism by increasing energy buffering capacity. ROS might secondarily decrease phosphocreatine *via* inhibition of creatine kinase (46). As creatine kinase inhibits the mitochondrial permeability transition, which has been linked to excitotoxic cell death, a decreased creatine kinase activity would further amplify neuronal damage (47). However, incomplete protection by creatine might alternatively point to an involvement of ROS-independent mechanisms in 3-OH-GA toxicity.

In contrast to ROS, we did not find evidence for a contribution of NO to 3-OH-GA neurotoxicity. The NOS inhibitor L-NAME, which prevented NO increase, did not protect neurons against 3-OH-GA neurotoxicity but even slightly enhanced it, suggesting a protective role of NO. However, this protective effect was not confirmed by treatment with the NO donor SNAP, which did not affect 3-OH-GA toxicity at low concentrations but potentiated it at high concentrations. Thus, protection by weak nNOS activation is most likely independent from endogenous NO in 3-OH-GA toxicity, and alternative

Table 1. Effect of NO donor SNAP on 3-OH-GA neurotoxicity

Concentration of SNAP (μ M)	Cell viability (%)	
	SNAP	3-OH-GA + SNAP
0	89.3 $\pm$ 4.6	59.0 $\pm$ 7.9*
0.5	90.1 $\pm$ 4.6	61.1 $\pm$ 6.1*
5	88.8 $\pm$ 6.2	56.2 $\pm$ 5.3*
50	85.4 $\pm$ 4.9	48.5 $\pm$ 7.2*†
500	79.1 $\pm$ 6.3*	36.3 $\pm$ 8.1*†

Neuronal cultures were exposed to 1 mM 3-OH-GA for 1 h in the presence or absence of 0.5–500 μ M SNAP (24 h). Cell viability was determined 24 h after exposure to 3-OH-GA by trypan blue exclusion ($n = 8$). * $p < 0.05$ vs control; † $p < 0.05$ vs 3-OH-GA alone (one-way ANOVA followed by Scheffé's test).

mechanisms should be considered. It was previously shown that activated NOS protected neurons against excitotoxicity under certain experimental conditions (18, 19), which was ascribed to an attenuation of NMDA receptor activity by redox-related species of NO, NO⁻ and NO⁺ (18, 19), or peroxynitrite at low concentration (48). An inhibitory effect of NO⁻, NO⁺, or peroxynitrite on NMDA receptor channel function cannot be excluded from our results. Furthermore, reduction of NO levels by L-NAME might indirectly enhance 3-OH-GA toxicity *via* increasing superoxide anions owing to reduced formation of peroxynitrite. However, this mechanism seems unlikely, as peroxynitrite is more toxic than superoxide anions (17, 28). Further experiments are required to investigate in detail the involvement of NO-related species in 3-OH-GA toxicity.

Taken together, we demonstrated that increased mitochondrial ROS production contributed to 3-OH-GA neurotoxicity, which could be reduced or even prevented by radical scavenging and energy buffering, thus providing new neuroprotective approaches to GDD. Recently, we reported a GDD patient with severe neurologic deterioration despite early detection and therapy (49), suggesting that some high-risk patients will suffer irreversible neurodestruction under the current therapeutic regimens. This points to the necessity to further improve the therapeutic strategies after a better understanding of GDD pathophysiology is obtained.

REFERENCES

- Hoffmann GF, Athanassopoulos S, Burlina AB, Duran M, deKlerck JBC, Lehnert W, Leonard JV, Monavari AA, Müller E, Muntau AC, Naughten ER, Plecko-Starting B, Superti-Furga A, Zschocke J, Christensen E 1996 Clinical course, early diagnosis, treatment, and prevention of disease in glutaryl-CoA dehydrogenase deficiency. *Neuropediatrics* 27:115–123
- Goodman SI, Norenberg MD, Shikes RH, Breslich DJ, Moe PG 1977 Glutaric aciduria: biochemical and morphological considerations. *J Pediatr* 90:746–750
- Land JM, Goulder P, Johnson A, Hockaday J 1992 Glutaric aciduria type I: an atypical presentation together with some observations upon treatment and the possible cause of cerebral damage. *Neuropediatrics* 23:322–326
- Stokke O, Goodman SI, Moe PG 1976 Inhibition of brain glutamate decarboxylase by glutarate, glutaconate, and beta-hydroxyglutarate: explanation of the symptoms in glutaric aciduria? *Clin Chim Acta* 66:411–415
- Heyes MP 1987 Hypothesis: a role for quinolinic acid in the neuropathology of glutaric aciduria type I. *Can J Neurol Sci* 14:441–443
- Ullrich K, Flott-Rahmel B, Schluff P, Musshoff U, Das A, Lücke T, Steinfeld R, Christensen E, Jakobs C, Ludolph A, Neu A, Röper R 1999 Glutaric aciduria type I: pathomechanism of neurodegeneration. *J Inher Metab Dis* 22:392–403
- Kölker S, Ahlemeyer B, Krieglstein J, Hoffmann GF 2000 Maturation-dependent neurotoxicity of 3-hydroxyglutaric and glutaric acids *in vitro*: a new pathophysiologic approach to glutaryl-CoA dehydrogenase deficiency. *Pediatr Res* 47:495–503
- Herkert M, Röttger S, Becker CM 1998 The NMDA receptor subunit NR2B of neonatal rat brain: complex formation and enrichment in axonal growth cones. *Eur J Neurosci* 10:1553–1562
- Komuro H, Rakic P 1993 Modulation of neuronal migration by NMDA receptors. *Science* 260:95–97
- Ikonomidou C, Bosch F, Miksa M, Bittigau P, Vöckler J, Dikranian K, Tenkova TI, Stefovskaya V, Turski L, Olney JW 1999 Blockade of NMDA receptors and apoptotic neurodegeneration in the developing brain. *Science* 283:70–74
- Dugan LL, Sensi SL, Canzoniero LM, Handran SD, Rothman SM, Lin TS, Goldberg MP, Choi DW 1995 Mitochondrial production of reactive oxygen species in cortical neurons following exposure to *N*-methyl-D-aspartate. *J Neurosci* 15:6377–6388
- Mattson MP, Barger SW, Begley JG, Mark RJ 1995 Calcium, free radicals, and excitotoxic neuronal death in primary cell culture. *Methods Cell Biol* 46:187–216
- Patel M, Day BJ, Crapo JD, Fridovich I, McNamara JO 1996 Requirement for superoxide in excitotoxic cell death. *Neuron* 16:345–355
- Lafon-Cazal M, Pietri S, Culcasi M, Bockaert J 1993 NMDA-dependent superoxide production and neurotoxicity. *Nature* 364:535–537
- Schinder AF, Olson EC, Spitzer NC, Montal M 1996 Mitochondrial dysfunction is a primary event in glutamate neurotoxicity. *J Neurosci* 16:6125–6133
- Dawson TM, Gonzalez-Zulueta M, Kusel J, Dawson VL 1998 Nitric oxide: diverse actions in the central and peripheral nervous system. *Neuroscientist* 4:96–112
- Beckman JS, Koppenol WH 1996 Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and the ugly. *Am J Physiol* 271:C1427–C1437
- Lipton SA, Choi YB, Pan ZH, Lei SZ, Chen HSV, Sucher NJ, Loscalzo J, Singel DJ, Stamler JS 1993 A redox-based mechanism for the neuroprotective and neurodestructive effect of nitrite oxide and related nitroso-compounds. *Nature* 364:626–632
- Kim WK, Choi YB, Rayudu PV, Das P, Asaad W, Arnel DR, Stamler JS, Lipton SA 1999 Attenuation of NMDA receptor activity and neurotoxicity by nitroxyl anion, NO⁻. *Neuron* 24:461–469
- Pettmann B, Louis JC, Sensenbrenner MCL 1979 Morphological and biochemical maturation of neurones cultured in the absence of glial cells. *Nature* 281:378–380
- Boscoboinik DO, Chatelain E, Bartoli GM, Stauble B, Azzi A 1994 Inhibition of protein kinase C activity and vascular smooth muscle cell growth by *D*- α -tocopherol. *Biochim Biophys Acta* 1224:418–426
- Ahlemeyer B, Krieglstein J 1998 Retinoic acid reduces staurosporine-induced apoptotic damage in chick embryonic neurons by suppressing reactive oxygen species production. *Neurosci Lett* 246:93–96
- Gow AJ, Branco F, Christofidou-Solomidou M, Black-Schultz L, Albelda SM, Muzykantor VR 1999 Immunotargeting of glucose oxidase: intracellular production of H₂O₂ and endothelial oxidative stress. *Am J Physiol* 277:L271–L281
- Royal JA, Ischiropoulos H 1993 Evaluation of 2,7-dichlorofluorescein and dihydrodihydroamine-123 as fluorescent probes for intracellular H₂O₂ in cultured endothelial cells. *Arch Biochem Biophys* 302:348–355
- Henderson LM, Chappell JB 1993 Dihydrodihydroamine-123: a fluorescent probe for superoxide generation? *Eur J Biochem* 217:973–980
- Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR 1982 Analysis of nitrate, nitrite and ¹⁵N-nitrate in biological fluids. *Anal Biochem* 126:131–138
- Giusti P, Gusella M, Liparetti M, Milani D, Zhu W, Vicini S, Manev H 1995 Melatonin protects primary cultures from cerebellar granule neurons from kainate but not from NMDA excitotoxicity. *Exp Neurol* 131:39–46
- Fridovich I 1997 Superoxide anion radical (O₂⁻), superoxide dismutases, and related matters. *J Biol Chem* 272:18515–18517
- Olanow CW 1993 A radical hypothesis for neurodegeneration. *Trends Neurosci* 16:439–444
- Tan DX, Chen LD, Poeggeler B, Manchester LC, Reiter RJ 1993 Melatonin: a potent, endogenous hydroxyl radical scavenger. *Endocr J* 1:57–60
- Rothe G, Emmendorffer A, Oser A, Roesler J, Valet G 1991 Flow cytometric measurement of the respiratory burst activity of phagocytes using dihydrodihydroamine-123. *J Immunol Methods* 138:133–135
- Bueb JL, Gallois A, Schneider JC, Parini JP, Tschirhart E 1995 A double-labelling fluorescent assay for concomitant measurements of [Ca²⁺]_i and O₂⁻ production in human macrophages. *Biochim Biophys Acta* 1244:79–84
- Ostrovikov S, Franck P, Capiaumont J, Douset B, Belleville F 1998 Effects of H₂O₂ on the growth, secretion, and metabolism of hybridoma cells in culture. *In Vitro Cell Dev Biol Anim* 34:259–264
- Gilad E, Cuzzocrea S, Zingarelli B, Salzman AL, Szabo C 1997 Melatonin is a scavenger of peroxynitrite. *Life Sci* 60:PL169–PL174
- Sun AY, Chen YM 1998 Oxidative stress and neurodegenerative disorders. *J Biomed Sci* 5: 401–414.
- Liebler DC 1993 Peroxyl radical trapping reactions of α -tocopherol in biomimetic systems. In: Packer L, Fuchs J (eds) *Vitamin E in health and disease*. Marcel Dekker, New York, pp 85–95
- Halliwell B, Gutteridge JM 1985 Oxygen radicals and the nervous tissue. *Trends Neurosci* 6:22–26
- Holtzman D, Togliatti A, Khait I, Jensen F 1998 Creatine increases survival and suppresses seizures in the hypoxic immature rat. *Pediatr Res* 44:410–414
- Dechent P, Pouwels PJ, Wilken B, Hanefeld F, Frahm J 1999 Increase of total creatine in human brain after oral supplementation of creatine-mono-hydrate. *Am J Physiol* 277:R698–R704
- Malcon C, Kaddurah-Daouk R, Beal MF 2000 Neuroprotective effects of creatine administration against NMDA and malonate toxicity. *Brain Res* 860:195–198
- Matthews RT, Yang L, Jenkins BG, Ferrante RJ, Rosen BR, Kaddurah-Daouk R, Beal MF 1998 Neuroprotective effects of creatine and cyclocreatine in animal models of Huntington's disease. *J Neurosci* 18:156–163
- Wilken B, Ramirez JM, Probst I, Richter DW, Hanefeld F 1998 Creatine protects the central respiratory system under anoxic conditions. *Pediatr Res* 43:8–14
- Happe HK, Murrin LC 1995 *In situ* hybridization analysis of CHOT1, a creatine transporter, in the rat central nervous system. *J Comp Neurol* 35:94–103
- Das AM, Lücke T, Ullrich K 2000 Glutaric aciduria I: creatine supplementation restores creatine phosphate in mixed cortex cells incubated with 3-hydroxy-glutarate. *J Inher Metab Dis* 23(suppl 1):100(abstr)
- O'Gorman E, Beutner G, Wallimann T, Brdiczka D 1996 Differential effects of creatine depletion on the regulation of enzyme activities and on creatine-stimulated mitochondrial respiration in skeletal muscle, heart, and brain. *Biochim Biophys Acta* 1276:161–170
- Cagnoli CM, Atabay C, Kharlamova E, Manev H 1995 Melatonin protects neurons from singlet oxygen-induced apoptosis. *J Pineal Res* 18:222–226
- O'Gorman E, Beutner G, Dolder M, Koretsky AP, Brdiczka D, Wallimann T 1997 The role of creatine kinase inhibition of mitochondrial permeability transition. *FEBS Lett* 414:253–257
- Kim WK, Rayudu PV, Mullins ME, Stamler JS, Lipton SA 1996 Down regulation of NMDA receptor activity in cortical neurons by peroxynitrite. In: Moncada S, Stamler JS, Gross S, Higgs EA (eds) *The biology of nitric oxide*. Portland Press, London, p 26
- Kölker S, Ramaekers V, Zschocke J, Hoffmann GF 2001 Acute encephalopathy despite early therapy in a patient with homozygosity for E365K in the glutaryl-CoA dehydrogenase gene. *J Pediatr* 138:277–279