Research Article

Neurotoxic Effects of \textit{trans}-Glutaconic Acid in Rats

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\textit{trans}-Glutaconic acid (tGA) is an unsaturated C5-dicarboxylic acid which may be found accumulated in glutaric aciduria type I, whose pathophysiology is still uncertain. In the present work it was investigated the \textit{in vitro} effect of increasing tGA concentrations on neurochemical and oxidative stress parameters in rat cerebral cortex. We observed that Na$^+$, K$^+$-ATPase activity was reduced by tGA, but not creatine kinase, respiratory chain complex IV, and ATP synthase activities. On the other hand, tGA significantly increased lipid peroxidation (thiobarbituric acid-reactive species levels and spontaneous chemiluminescence), as well as protein oxidative damage (oxidation of sulfhydryl groups). tGA also significantly decreased nonenzymatic antioxidant defenses (TRAP and reduced glutathione levels). Our data suggest that tGA may be neurotoxic in rat brain.

1. Introduction

Glutaryl-CoA dehydrogenase deficiency (OMIM: number 231670), also known as glutaric aciduria type I, is an autosomal recessive metabolic disorder due to a blockade in the catabolic pathway of the amino acids lysine, hydroxylysine, and tryptophan. This disease was first described by Goodman et al. [1] and is biochemically characterized by tissue accumulation of predominantly glutaric acid and, to a lesser degree, of 3-hydroxyglutaric and \textit{trans}-glutaconic (tGA) acids [2, 3]. Clinically, affected patients present macrocephaly, progressive dystonia, and dyskinesia, symptoms that are apparent within the first year of life [4, 5]. Degeneration of caudate and putamen following encephalopathic crises and frontotemporal atrophy at birth is also commonly seen in these patients [6, 7]. Children presenting residual glutaryl-CoA dehydrogenase activity of up to 30% (low excretors) present low or undetectable excretion of glutaric acid, but the neurological signs may be found similar in these individuals, regardless of the amount of glutaric acid excreted [8–11].

The primary cause of neurological alterations in glutaric acidemia type I is still not defined. It has been demonstrated that brain tissue exposure to the main accumulating metabolites, glutaric, and 3-hydroxyglutaric acids results in excitotoxicity [12–15], oxidative stress induction [16–18], and/or disruption of energy homeostasis [12, 19–24]. Furthermore, it has been recently showed that lysine administration to a knockout mice model of glutaric aciduria type I (Gcdh\textsuperscript{−/−}) results in oxidative damage and energy impairment in the brain of these animals, as well as in compromised neurodevelopmental and cognitive behavior [25–28]. On the other hand, very little is known regarding the direct toxicity of tGA. In this scenario, and it has been demonstrated that tGA is able to inhibits glutamate decarboxylase activity in rat and rabbit brains, human mitochondrial NAD(P)(+) -dependent malic enzyme, and dopamine beta-monooxygenase activities in purified preparations, as well as inducing mitochondrial permeability transition in rat liver mitochondrial preparations, provoking apoptosis in immature oligodendrocytes,
and reducing cell viability of primary cultures of mice cerebral neocortical neurons [29–34].

Therefore, the aim of the present work was to investigate the in vitro effects of tGA on various biochemical parameters in cerebral cortex of young rats. The investigated parameters were the values of Na$^+$, K$^+$-ATPase, creatine kinase (CK), complex IV and ATP synthase activities, carbonyl and sulfhydryl content, thiobarbituric acid-reactive substances (TBA-RS) levels, spontaneous chemiluminescence, reduced glutathione (GSH) concentrations, and total nonenzymatic antioxidant capacity of the tissue (TRAP) in cerebral cortex of young rats.

2. Material and Methods

2.1. Reagents. All chemicals were purchased from Sigma (St. Louis, MO, USA). tGA was dissolved in the day of the experiments in the incubation medium used for each technique, and the solution had its pH adjusted to 7.4. The final concentrations of tGA in the medium ranged from 0.01 to 1.0 mM.

Parallel experiments were always carried out with negative controls (blanks) in the presence or absence of tGA and also with or without cortical supernatants in order to detect artifacts caused by this organic acid in the assays. Therefore, any interference of tGA on the reactions used to measure the biochemical parameters would be identified.

2.2. Animals. Thirty-day-old male Wistar rats obtained from the Central Animal House of the Departamento de Bioquímica, Federal University of Rio Grande do Sul (UFRGS), were used. Rats were kept with dams until weaning at 21 days of age. The animals had free access to water and food and were housed in a 12:12 light/dark cycle in an air-conditioned constant temperature (22 ± 1°C) colony room. The “Principles of Laboratory Animal Care” (NIH publication number 80-23, revised 1996) were followed in all experiments and the Ethics Committee for Animal Research of UFRGS, Porto Alegre, Brazil, approved the experimental protocol. All efforts were made to minimize the number of animals used and their suffering.

2.3. Tissue Preparation and Incubation for Oxidative Stress Parameters. On the day of the experiments the animals were killed by decapitation without anesthesia, and the brain was rapidly excised on a Petri dish placed on ice. The olfactory bulbs, brain stem, medulla, cerebellum, hippocampus, corpus callosum, and striatum were discarded, and the cerebral cortex was homogenized away from the subcortical structures, weighed and homogenized in 10 volumes (1:10, w/v) of 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl. Homogenates were centrifuged at 750 $\times$ g for 10 min at 4°C to discard nuclei and cell debris [35]. The pellet was discarded, and the supernatant, a suspension of mixed and preserved organelles, including mitochondria, was separated and incubated in 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl at 37°C for one hour with tGA at concentrations of 0.01, 0.1, or 1 mM. Controls did not contain this metabolite in the incubation medium. Immediately after incubation, aliquots were taken to measure the values of carbonyl and sulfhydryl content, TBA-RS levels, spontaneous chemiluminescence, GSH concentrations, and TRAP.

2.4. Preparation of Synaptic Plasma Membrane from Rat Cerebral Cortex. Cerebral cortex was homogenized in 10 volumes of 0.32 mM sucrose solution containing 5.0 mM HEPES and 1.0 mM EDTA. The homogenate was preincubated at 37°C for 1 h in the absence or presence of 0.01, 0.1, or 1 mM tGA. Membranes were prepared afterwards according to the method of Jones and Matus [36] using a discontinuous sucrose density gradient consisting of successive layers of 0.3, 0.8, and 1.0 mM. After centrifugation at 69,000 $\times$ g for 2 h, the fraction at the 0.8–1.0 mM sucrose interface was taken as the membrane enzyme preparation.

2.5. Tissue Preparations for Determination of Respiratory Chain Complex IV and Creatine Kinase Activities. For the determination of the activities of the respiratory chain complex IV, cerebral cortex was homogenized (1:20, w/v) in SETH buffer (250 mM sucrose, 2.0 mM EDTA, 10 mM Trizma base, and 50 U/mL heparin), pH 7.4. The homogenates were centrifuged at 800 $\times$ g for 10 min, and the supernatants were kept at –70°C until being used for enzyme activity determination. For total creatine kinase activity determination, the cerebral cortex was homogenized (1:10 w/v) in isosmotic saline solution [37].

2.6. Mitochondrial Preparations. Mitochondrial fractions prepared according to Cassina and Radi [38] were used for the determination of ATP synthase activity, TBA-RS levels, and sulfhydryl content.

2.7. Determination of Na$^+$, K$^+$-ATPase Activity. Na$^+$, K$^+$-ATPase activity was evaluated according to Tsakiris and Delconstantinos [39]. Released inorganic phosphate (Pi) was measured by the method of Chan et al. [40]. Enzyme-specific activity was expressed as nmol Pi released-min$^{-1}$-mg protein$^{-1}$.

2.8. Determination of Bioenergetics Parameters. The activity of respiratory chain complex IV was determined according to Rustin et al. [41] slightly modified, as described in details in a previous report [42]. ATP synthase activity was measured in mitochondrial preparations from cerebral cortex, according to Rustin et al. [41]. Complex IV and ATP synthase activities were calculated as nmol-min$^{-1}$-mg protein$^{-2}$.

CK activity was measured in total homogenates according to Hughes [43] with slight modifications, as described by Schuck et al. [37]. Results are expressed as $\mu$mol of creatine min$^{-1}$-mg protein$^{-1}$.

2.9. Determination of Protein Carbonyl Formation Content. Protein carbonyl content, a marker of oxidized proteins, was measured spectrophotometrically according to Reznick and Packer [44]. The results were calculated as nmol of carbonyls...
protein-bound sulfhydryl content is inversely correlated to 2.10. Sulfhydryl (Thiol) Group Oxidation. This assay was performed according to Aksenov and Markesbery [45]. The protein-bound sulfhydryl content is inversely correlated to oxidative damage to proteins. Results were reported as nmol TNB-mg of protein⁻¹.

2.11. Determination of TBA-RS Levels. TBA-RS was determined according to the method of Esterbauer and Cheeseman [46]. A calibration curve was performed using 1,1,3,3-tetramethoxypropane, and each curve point was subjected to the same treatment as supernatants. Some experiments were performed in the absence or presence of reduced glutathione (GSH; 100 μM), melatonin (100 μM), the nitric oxide synthase inhibitor Nω-nitro-L-arginine methyl ester (L-NAME; 500 μM), trolox (5 μM), or a combination of catalase (Cat; 10 mU·mL⁻¹) plus superoxide dismutase (SOD; 10 mU·mL⁻¹). TBA-RS values were calculated as nmol of TBA-RS-mg protein⁻¹.

2.12. Spontaneous Chemiluminescence. Samples were assayed for spontaneous chemiluminescence in a dark room by the method of Gonzalez-Flecha et al. [47]. Results were calculated as cpm-mg protein⁻¹.

2.13. Determination of TRAP. TRAP, representing the total nonenzymatic antioxidant capacity of the tissue, was determined by measuring the chemiluminescence intensity of luminol induced by 2,2′-azo-bis-(2-aminopropane) (ABAP) according to the method of Lissi et al. [48]. TRAP values were expressed as nmol of trolox-mg of protein⁻¹.

2.14. GSH Levels Quantification. GSH levels were measured according to Browne and Armstrong [49]. Some experiments were performed in the presence or absence of melatonin (100 μM), L-NAME (500 μM), trolox (5 μM), or Cat (10 mU·mL⁻¹) plus SOD (10 mU·mL⁻¹). Calibration curve was prepared with standard GSH (0.01-1 mM), and the concentrations were calculated as nmol-mg protein⁻¹.

The oxidation of a commercial solution of GSH (200 μM) was also tested by exposing this solution to 1 mM tGA for one hour in a medium devoid of brain supernatants. After tGA exposure, 7.4 mM ω-phthalaldehyde was added to the vials, and the mixture was incubated at room temperature during 15 minutes.

2.15. Protein Determination. Protein was measured by the method of Lowry et al. [50] using bovine serum albumin as standard.

2.16. Statistical Analysis. Results are presented as mean ± standard deviation. Assays were performed in duplicate, and the mean was used for statistical analysis. Data was analysed using one-way analysis of variance (ANOVA) followed by the post hoc Duncan multiple range test when F was significant.

Differences between groups were rated significant at P < 0.05. All analyses were carried out in an IBM-compatible PC computer using the Statistical Package for the Social Sciences (SPSS) software.

3. Results

tGA inhibits Na⁺, K⁺-ATPase activity in synaptic plasma membranes from rat cerebral cortex.

Initially, we tested the influence of tGA on Na⁺, K⁺-ATPase activity in synaptic plasma membranes prepared from cerebral cortex. Figure 1 shows that the incubation of tGA with purified synaptic membrane preparations caused a significant inhibition of Na⁺, K⁺-ATPase activity.

3.1. tGA Does Not Interfere on Important Parameters of Brain Bioenergetics. We also investigated the effect of tGA on important parameters of brain bioenergetics. It is observed in Table 1 that the presence of up to 1.0 mM tGA in the incubation medium does not disturb creatine kinase, respiratory chain complex IV, and ATP synthase activities in rat cerebral cortex.

3.2. Protein Oxidative Damage Is Induced by tGA. We then evaluated the effect of tGA on carbonyl formation and sulfhydryl oxidation in cortical homogenates in order to evaluate protein oxidative damage. We found that tGA provoked a significant decrease of sulfhydryl content at 0.1 and 1.0 mM in cortical homogenates (Figure 2(a)), although it did not affect protein carbonyl content. Furthermore, sulfhydryl content was not altered by tGA in brain mitochondrial preparations (Table 2).
3.3. tGA Induces Lipid Peroxidation. The influence of tGA on the lipid peroxidation parameters TBA-RS levels and spontaneous chemiluminescence was investigated. Figure 2(b) shows that tGA at 1 mM significantly increased TBA-RS levels in cortical homogenates. Similar results were obtained for spontaneous chemiluminescence.

We also tested the effect of the antioxidants GSH (100 μM), melatonin (100 μM), L-NAME (200 μM), trolox (5 μM), or a combination of Cat plus SOD (10 μU mL⁻¹ each) on tGA-elicited increase of TBA-RS levels (Figure 3). These antioxidants were coincubated with 1.0 mM of the organic acid and brain cortical homogenates for 60 min, after which the levels of TBA-RS were measured. We observed that GSH and melatonin fully prevented tGA-induced increased lipid peroxidation, whereas the combination of Cat plus SOD partially prevented such effects. Trolox and L-NAME did not prevent tGA effect.

It was also observed that tGA did not affect TBA-RS levels in brain mitochondrial preparations (Table 2).

3.4. Nonenzymatic Antioxidant Defenses Are Decreased by tGA. We assessed the in vitro effect of tGA on cortical GSH levels and TRAP. We found that this organic acid significantly decreased GSH levels and TRAP at the highest tested concentration (1.0 mM) (Figure 4). Furthermore, tGA-induced effect was totally prevented by the free-radical scavengers melatonin and trolox and by the combination of Cat plus SOD, but not by L-NAME (Figure 3).

We then investigated whether tGA-induced GSH levels decrease was secondary to a direct oxidative attack. We

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**Table 1:** In vitro effect of trans-glutaconic acid (tGA) on creatine kinase (CK), respiratory chain complex IV, and ATP synthase activities in cerebral cortex of 30-day-old rats.

| Group | CK (μmol·min⁻¹·mg of protein⁻¹) | Complex IV (nmol·min⁻¹·mg of protein⁻¹) | ATP synthase (nmol·min⁻¹·mg of protein⁻¹) |
|-------|---------------------------------|----------------------------------------|----------------------------------------|
| Control | 11.28 ± 0.20                     | 114.72 ± 7.42                          | 476.00 ± 31.23                        |
| 0.1 mM tGA | 10.66 ± 0.78                     | 116.46 ± 2.57                          | —                                      |
| 1 mM tGA | 11.04 ± 1.18                     | 107.14 ± 3.29                          | 508.75 ± 15.47                        |

The experiments were performed in triplicate, and data represent mean ± standard error of the mean (n = 4-5). It was not observed any difference among groups (ANOVA and Student’s t-test). CK: μmol·min⁻¹·mg of protein⁻¹; complex IV and ATP synthase: nmol·min⁻¹·mg of protein⁻¹.

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**Table 2:** In vitro effect of trans-glutaconic acid (tGA) on thiobarbituric acid-reactive species (TBA-RS) levels and sulfhydryl (SH) content in mitochondrial preparations from cerebral cortex of 30-day-old rats and glutathione (GSH) content in a commercial GSH preparation.

| Group | TBA-RS (nmol·mg of protein⁻¹) | SH (nmol·mg of protein⁻¹) | GSH (fluorescent units) |
|-------|-------------------------------|---------------------------|-------------------------|
| Control | 6.44 ± 0.59                   | 55.98 ± 2.30              | 2941 ± 144.3            |
| 1 mM tGA | 6.16 ± 0.40                   | 56.22 ± 3.59              | 2640 ± 70.35            |

The experiments were performed in triplicate, and data represent mean ± standard error of the mean (n = 4-5). It was not observed any difference between groups (Student’s t-test for paired samples). TBA-RS and SH: nmol·mg of protein⁻¹; GSH: fluorescent units.
ATPase activity, a crucial enzyme for maintaining ion homeostasis compared to 1.0 mM GA. Therefore exposed a commercial solution of GSH (200 μM) to 1.0 mM tGA for 60 min in the absence of cortical supernatants. It was observed that this organic acid per se did not oxidize highly purified GSH (Table 2).

4. Discussion

tGA, or (E)-pentene-1,5-dioic acid, is an unsaturated C5-dicarboxylic acid that is found in patients affected by glutaric aciduria type I. Its role on this organic aciduria pathogenesis is under debate, since there are patients affected by glutaric aciduria type I who do not present tGA accumulation [3]. On the other hand, several tGA toxic effects have already been reported [29–34].

Herein we present data demonstrating that Na⁺, K⁺-ATPase activity, a crucial enzyme for maintaining ion homeostasis and membrane potential in cells [51], was inhibited by tGA at 0.1 and 1.0 mM concentrations. Inhibition of Na⁺, K⁺-ATPase activity has been described in various diseases, including cerebral ischemia [52, 53], neurodegenerative [54], and metabolic disorders [55, 56]. Furthermore, studies have indicated that Na⁺, K⁺-ATPase inhibition may result in cellular death by activating apoptotic cascades and neuronal damage probably due to amplification of potassium homeostasis impairment [57].

Considering that this enzyme is present at high concentrations in brain cellular membranes and consumes about 40–50% of the ATP generated [58], our next step was to evaluate the effects of tGA on some important bioenergetics parameters in order to identify whether ATP deficit could contribute to Na⁺, K⁺-ATPase activity inhibition caused by this organic acid. It was observed that tGA does not affect the activities of respiratory chain complex IV, creatine kinase, and ATP synthase. However, at this point, it should be mentioned that effects of tGA on glycolysis Krebs cycle enzyme activities, and the other respiratory chain complexes activities cannot be ruled out.

It was further investigated the in vitro effect of tGA on oxidative stress parameters, since Na⁺, K⁺-ATPase activity is highly dependent of critical sulphhydril groups present in its catalytic site, rendering this enzyme activity highly susceptible to oxidative damage [59, 60]. We showed that tGA in vitro induced oxidative damage to proteins, as observed by sulphhydril content decrease, and to lipids, since it increased TBA-RS levels and spontaneous chemiluminescence. Interestingly, carbonyl content was not altered by the presence of tGA in the incubation medium, suggesting that particularly sulphhydril groups are prone to protein oxidative damage by this organic acid. On the other hand, light emitted in the spontaneous chemiluminescence assay mainly arises from oxidized lipids due to an increase in reactive oxygen or nitrogen species production, and TBA-RS levels reflects the amount of malondialdehyde formation, an end product of

![Figure 3: In vitro effect of the antioxidants GSH (100 μM), melatonin (100 μM), L-NAME (500 μM), trolox (5 μM), and catalase (Cat) plus superoxide dismutase (SOD; 10 μM mL⁻¹ each) on trans-gluataconic (tGA) induced alterations on thiobarbituric-acid reactive substances (TBA-RS) and glutathione (GSH) levels in cerebral cortex of 30-day-old rats. Cortical homogenates were preincubated for 15 min with the antioxidants before the addition of the 1 mM tGA. The experiments were performed in triplicate, plotted as mean ± S.E.M. (n = 4–5), and are expressed as nmol-mg protein⁻¹. *P < 0.05 and **P < 0.01 compared to control group; †P < 0.05 compared to tGA group (Duncan multiple range test).]

![Figure 4: In vitro effect of trans-gluataconic acid (tGA) on the nonenzymatic antioxidant markers total-radical trapping antioxidant potential (TRAP) and glutathione (GSH) levels in rat cerebral cortex of 30-day-old rats. The experiments were performed in triplicate, plotted as mean ± S.E.M. (n = 5–6), and are expressed as nmol-mg protein⁻¹. *P < 0.05 and ***P < 0.001 compared to control group (Duncan multiple range test).]
membrane fatty acid peroxidation [61]. In this scenario, it is tempting to speculate that tGA oxidized lipids from cell and organelle membranes, which could lead to an impairment of membrane fluidity. Consequently, integral proteins such as Na⁺, K⁺-ATPase could also be affected, having their functioning impaired.

Furthermore, it was observed that GSH and melatonin fully prevented tGA-induced increased lipid peroxidation, since these compounds prevented TBA-RS increase elicited by this organic acid, whereas the combination of Cat plus SOD only partially prevented such effects. On the other hand, trolox (a hydrophilic vitamin E analogue) and L-NAME did not prevent tGA effect. It is speculated that lipid oxidation induced by tGA occurs due to an increase of reactive oxygen species.

We then assessed nonenzymatic antioxidant defenses by measuring GSH levels and TRAP and observed that both parameters were decreased by tGA in vitro. Since these parameters are suitable to evaluate the capacity of a tissue to prevent and respond to oxidative damage [35, 61, 62], it is likely that tGA impairs rat cortical antioxidant defenses. Moreover, tGA-induced effect on GSH levels was totally prevented by the free-radical scavengers melatonin and trolox and by the combination of Cat plus SOD, but not by L-NAME, suggesting that mainly peroxyl, alkoxyl, and hydroxyl radicals were involved in the reduction of GSH levels provoked by tGA. Considering that tGA was not able to oxidize a commercial GSH solution in the absence of brain supernatants in the incubation medium, it is unlikely that this organic acid is per se a direct oxidant agent, corroborating the idea that it probably provoked lipid and protein oxidative damage by increasing free radical generation.

Since the alterations elicited by tGA on TBA-RS and sulphhydril content in whole homogenates, which contain the whole cell machinery, were not observed when this organic acid was supplemented to mitochondrial preparations, it is feasible that oxidative stress induced by tGA was probably mediated by cytosolic mechanisms. For instance, some putative mechanisms could involve xanthine oxidase, cytosolic NADPH oxidase, lysosomes, peroxisomes, and others [63].

Oxidative stress is an imbalance between the total tissue antioxidant (enzymatic and nonenzymatic) defenses and reactive species generation in cell leading to a deleterious cell condition [61]. In this study, we showed evidences that tGA induces oxidative stress in cerebral cortex in vitro, since it increased oxidative damage and decreased antioxidant defenses. It should be emphasized that the brain is highly susceptible to damage induced by increased reactive species generation, since it has low cerebral antioxidant defenses as compared to other tissues [64]. Furthermore, it may be also speculated that the inhibition of Na⁺, K⁺-ATPase activity elicited by tGA reported in the present study could be secondary to increased oxidation of critical sulphhydril groups at the catalytic site of the enzyme.

Taken together, our results provide evidences that tGA is toxic to brain cells in vitro, by causing alterations in cell ion balance, and probably neurotransmission, as well as oxidative stress in rat cerebral cortex. We cannot at this point establish whether our data have a pathophysiological significance for glutaric aciduria type I. tGA accumulates in urine for excretion [65, 66], and excretion of this acid may become prominent, exceeding that of 3-hydroxyglutaric acid, during episodes of ketosis [4, 67]. Some studies indicated that the diagnostic relevance of tGA is limited [68], since tGA excretion in urine may be inconsistently found. However, it should be highlighted that even glutaric acid, known to be the major accumulating in glutaric aciduria type I, may be excreted at discrete or even normal concentrations (low excretor patients), as above mentioned.

5. Conclusion
Considering that the main signs and symptoms of affected patients are neurological [3], and in case the present findings are confirmed in vivo in animal experiments and also in tissues of patients accumulating tGA, it may be speculated that tGA toxicity could collaborate to the brain damage characteristic of glutaric aciduria type I affected patients.

Conflict of Interests
The authors declare that there are no possible conflict of interests.

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