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Induction of oxidative stress by the metabolites accumulating in isovaleric acidemia in brain cortex of young rats

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Abstract

The present work investigated the *in vitro* effects of isovaleric acid (IVA) and isovalerylglycine (IVG), which accumulate in isovaleric acidemia (IVAcidemia), on important parameters of oxidative stress in supernatants and mitochondrial preparations from brain of 30-day-old rats. IVG, but not IVA, significantly increased TBA-RS and chemiluminescence values in cortical supernatants. Furthermore, the addition of free radical scavengers fully prevented IVG-induced increase of TBA-RS. IVG also decreased GSH concentrations, whereas IVA did not modify this parameter in brain supernatants. Furthermore, IVG did not alter lipid peroxidation or GSH concentrations in mitochondrial preparations, indicating that the generation of oxidants by IVG was dependent on cytosolic mechanisms. On the other hand, IVA significantly induced carbonyl formation both in supernatants and purified mitochondrial preparations from rat brain, with no effect observed for IVG. Therefore, it is presumed that oxidative damage may be at least in part involved in the pathophysiology of the neuropathology of IVAcidemia.

Keywords: *Isovaleric acid, isovalerylglycine, isovaleric acidemia, oxidative stress, rat cerebral cortex*

Introduction

Isovaleric acidemia (IVAcidemia, MIM 243500) is an autosomal recessive inherited metabolic disorder of leucine metabolism caused by a deficiency of isovaleryl-CoA dehydrogenase (IVD; E.C. 1.3.99.10), a homotetrameric mitochondrial flavoenzyme of the family of acyl-CoA dehydrogenases [1]. IVAcidemia is considered a severe, potentially life-threatening disorder that manifests with acute neonatal encephalopathy with recurrent episodes of vomiting, lethargy and coma in about half of the affected individuals and with poor feeding, tachypnea, dehydration and varying degrees of developmental delay in the other half of the patients [2–5]. IVD deficiency leads to accumulation of the isovaleryl-CoA derivatives isovaleric acid (IVA), 3-hydroxyisovaleric acid (3-OHIVA) and isovalerylglycine (IVG), which are highly excreted in the urine and detected by GC/MS. Furthermore, isovalerylcarnitine accumulated in plasma is detected identified by MS/MS and blood spots [2]. The diagnosis of IVAcidemia is based on clinical symptoms and the presence of high amounts of predominantly IVG, with 3-OHIVA and some other metabolites appearing at lower amounts in urine [5–7]. The incidence of

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IVAcidemia varies from 1/62 500 live births in Germany [8] to 1/250 000 in the USA [4,9–11].

Although clinical and laboratory characteristics have been extensively studied, CT and MRI findings of IVAcidemia have not been described in detail. Cranial CT from a patient with a chronic-intermittent form of isovaleric acidemia revealed symmetrical pallidal lesions, whereas abnormalities in the globi pallidi and corticospinal tracts of the mesencephalon were observed with MRI [12].

Although neurological abnormalities are pronounced in IVAcidemia, the mechanisms of brain damage in this disorder are poorly known. However, it was recently described that exposition of cortical homogenates to IVA provoked a significant inhibition of Na^+, K^+-ATPase activity and that this effect was totally prevented by the antioxidants trolox and creatine [5], suggesting the involvement of peroxyl radicals probably acting on critical groups of the enzyme. In addition, it was demonstrated that 3-OHIVA, which also accumulates in 3-methylglutaconic aciduria, induces lipid peroxidation and carbonyl formation in rat brain preparations [13]. Therefore, in the present study we investigated the *in vitro* influence of IVA and IVG on important parameters of oxidative stress, such as thiobarbituric acid-reactive substances (TBA-RS), chemiluminescence, carbonyl content and glutathione (GSH) levels in cerebral cortex of young rats. We also tested the role of antioxidants on the effects elicited by IVG. It is expected that investigation of the role of these metabolites on the central nervous system function will eventually lead to a better understanding of the pathophysiology of IVAcidemia.

Material and methods

Animals and reagents

Wistar rats of 30 days of life obtained from the Central Animal House of the Department of Biochemistry, ICBS, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS–Brazil, were used. The animals were maintained on a 12:12 h light/dark cycle (lights on 07.00–19.00 h) in an air conditioned constant temperature (22°C ± 1°C) colony room, with free access to water and 20% (w/w) protein commercial chow (SUPRA, Porto Alegre, RS, Brazil). The experimental protocol was approved by the Ethics Committee for animal research of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil and followed the 'Principles of Laboratory Animal Care' (NIH publication 85-23, revised 1985). All efforts were made to minimize the number of animals used and their suffering.

All chemicals were purchased from Sigma (St. Louis, MO), except for IVG which was prepared by Dr Ernesto Brunet. IVA and IVG solutions were prepared on the day of the experiments in the incubation medium used for each technique and pH was adjusted at 7.4. The final concentrations of the metabolites in the medium were 0.01–10 mM. In some experiments, antioxidants were added to the incubation medium at the following final concentrations: 1.5 or 10 μM Trolox (TRO), 200 and 1000 μM melatonin (MEL), 100 and 750 μM GSH, 500 μM N^{ω}-nitro-L-arginine (L-NAME), 1 mM N-acetylcysteine (NAC), combination of SOD plus CAT (2.5 or 15 mU/mL each) and 3 mM creatine (Cr). Chemiluminescence assayed using a Wallac 1409 scintillation counter. TBA-RS, carbonyls and the antioxidant enzyme activities were measured with a double-bean Hitachi U-2001 spectrophotometer with temperature control. GSH levels were measured in a Hitachi F-2000 fluorescence spectrophotometer.

Cerebral cortex supernatant preparation

On the day of the experiments the rats were sacrificed by decapitation without anaesthesia and the brain was rapidly excised on a Petri dish placed on ice. The olfactory bulbs, pons, medulla, cerebellum and striatum were discarded and the cerebral cortex was dissected, 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 *g* for 10 min at 4°C to discard nuclei and cell debris [14]. The pellet was discarded and the supernatant, a suspension of mixed and preserved organelles, including mitochondria, was separated and incubated at 37°C for 1 h with IVA or IVG. Immediately after incubation, aliquots were taken to measure TBA-RS, chemiluminescence, GSH concentrations and carbonyl content.

Preparation of purified mitochondrial fractions

Mitochondrial fractions were prepared from cerebrum (total brain excluding cerebellum, olfactory bulbs, pons and medulla) of 30-day-old rats. The cerebrum was homogenized in 10 volumes of 5 mM potassium phosphate buffer, pH 7.4, containing 0.3 m sucrose, 5 mm MOPS, 1 mM EGTA and 0.1% bovine serum albumin. The homogenate was centrifuged at 1500 *g* for 10 min at 4°C and the pellet was discarded. The supernatant was then centrifuged for a further 10 min at 4°C at 15 000 *g* in order to isolate the mitochondria present in the pellet, which was finally suspended in the same buffer [15]. Disrupted mitochondrial fractions obtained by freezing/thawing three times were incubated at 37°C for 30 min with IVG (0.01–1.0 mM) or IVA (1.0–5 mM). Immediately after incubation, aliquots were used to measure TBA-RS, chemiluminescence, GSH concentrations and carbonyl content.

Thiobarbituric acid-reactive substances (TBA-RS)

TBA-RS was determined according to the method of Esterbauer and Cheeseman [16]. Briefly, 300 μL of cold 10% trichloroacetic acid were added to 150 μL of IVG or IVA pre-treated cerebral cortex supernatants and centrifuged at 300 *g* for 10 min. Three hundred microlitres of the supernatants were transferred to a pyrex tube and incubated with 300 μL of 0.67% TBA in 7.1% sodium sulphate on a boiling water bath for 25 min. The tubes containing the mixture were allowed to cool on running tap water for 5 min. The resulting pink-stained TBA-RS was determined in a spectrophotometer at 532 nm. A calibration curve was performed using 1,1,3,3-tetramethoxypropane, and each curve point was subjected to the same treatment as supernatants. TBA-RS values were calculated as nmol/mg protein and represented as percentage of control.

Chemiluminescence

Samples were assayed for spontaneous chemiluminescence in a dark room by the method of Gonzalez-Flecha et al. [17]. Incubation flasks contained 3.5 mL of 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl were counted for background chemiluminescence during 5 min. An aliquot of 500 μL of cortical supernatants was immediately added and chemiluminescence was measured for 30 min at room temperature. The background chemiluminescence was subtracted from the final luminescence value and the results were calculated as cpm/mg protein and represented as percentage of control.

Reduced glutathione (GSH) content

GSH concentrations were measured according to Browne and Armstrong [18]. IVA or IVG pre-treated cerebral cortex supernatants were diluted in 20 volumes of (1:20, v/v) 100 mM sodium phosphate buffer pH 8.0, containing 5 mM EDTA. One hundred microlitres of this preparation were incubated with an equal volume of o-phthaldialdehyde (1 mg/mL methanol) at room temperature during 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 nm and 420 nm, respectively. A calibration curve was prepared with standard GSH (0.01–1 mM) and the concentrations were calculated as nmol/mg protein and represented as percentage of control.

We also tested whether IVG could oxidize a commercial solution of GSH (200 μM) by exposing this solution to 1.0 mM IVG for 60 min in a medium devoid of brain supernatants. N-Ethylmaleimide (NEM, 150 μM), a classical oxidant of sulfhydryl groups, was used as a positive control. After IVG exposition, 7.4 mM o-phthaldialdehyde (1 mg/mL) was added to the vials and the mixture was incubated at room temperature for 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 nm and 420 nm, respectively.

Determination of protein carbonyl content

PCF (protein carbonyl formation), a marker of protein oxidative damage, was measured spectrophotometrically according to Reznick and Packer [19]. Two hundred microlitres of the aliquots from the incubation were treated with 400 μL of 10 mM 2,4-dinitrophenylhidrazine (DNPH) dissolved in 2.5 N HCl or with 2.5 N HCl (blank) and left in the dark for 1 h. Samples were then precipitated with 500 μL 20% TCA and centrifuged for 5 min at 10 000 *g*. The pellet was then washed with 1 mL ethanol: ethyl acetate (1:1, v/v) and suspended in 550 μL 6 m guanidine prepared in 2.5 N HCl at 37°C for 5 min. The difference between the DNPH-treated and HCl-treated samples (blank) was used to calculate the carbonyl content determined at 365 nm. The results were calculated as nmol of carbonyls groups/mg of protein and represented as percentage of control, using the extinction coefficient of $22\ 000 \times 10^6$ nmol/mL for aliphatic hydrazones.

Protein determination

Protein was measured by the method of Lowry et al. [20] using bovine serum albumin as standard.

Statistical analysis

Unless otherwise stated, results are presented as mean ± standard deviation. Assays were performed in duplicate or triplicate and the mean or median 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. Linear regression analysis was also used to test dose-dependent effects. Only significant F-values are shown in the text. 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.

Results

IVG induces lipid oxidation in rat cortical supernatants

First we investigated the *in vitro* effect of IVA and IVG on lipid peroxidation parameters in cerebral cortex supernatants and purified mitochondrial preparations of 30-day-old rats. Figure 1 shows that IVG [$F_{(5,26)} = 10.735$; $p < 0.001$], but not IVA, significantly increased TBA-RS levels in a dose-dependent manner [$\beta = 0.800$; $p < 0.001$] at 0.5 mM and higher concentrations in brain supernatants. Similarly, chemiluminescence was significantly increased by IVG

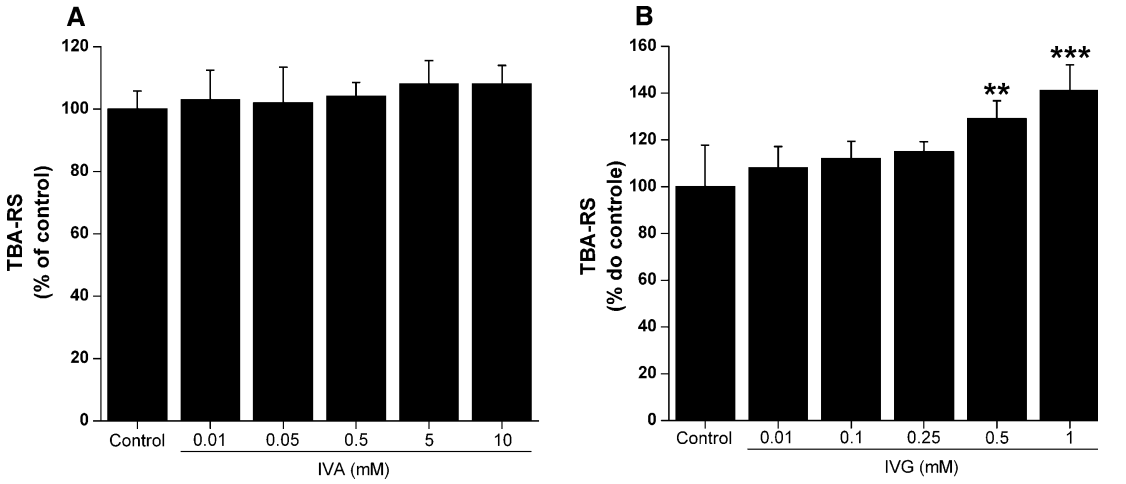

Figure 1. *In vitro* effect of isovaleric acid (IVA) and isovalerylglycine (IVG) on thiobarbituric acid-reactive substances (TBA-RS) in rat cerebral cortex supernatants. Cortical supernatants were incubated during 60 min in the presence of IVA or IVG at concentrations ranging from 0.01–10 mM (A) and 0.01–1.0 mM (B), respectively. Values are means ± standard deviation for four-to-eight independent experiments performed in triplicate and are expressed as percentage of controls (Controls: A: 4.48 ± 0.25 nmol/mg of protein; B: 3.72 ± 0.66 nmol/mg of protein. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared to controls (Duncan multiple range test).

$[F_{(3,16)} = 5.109; p < 0.05]$ in a dose-dependent profile $[\beta = 0.68; p < 0.01]$, whereas IVA did not alter this parameter (Figure 2). The results indicate that IVG induced lipid oxidative damage in rat brain. We then evaluated the role of antioxidants on IVG-induced increase on TBA-RS levels. Cortical supernatants were co-incubated with the antioxidants TRO (α-tocopherol; 1.5 μM), MEL (200 μM), GSH (100 μM), L-NAME (500 μM), NAC (1 mM), the combination of SOD plus CAT (2.5 mU/mL each) or Cr (3 mM) and 1.0 mm IVG. Our results show that only NAC was able to fully prevent IVG-induced TBA-RS levels increase at these doses of the antioxidants $[F_{(3,12)} = 15.310; p < 0.001]$ (Figure 3).

We also tested whether higher doses of TRO (10 μM), MEL (1 mM), GSH (750 μM) or the combination of SOD plus CAT (15 mU/mL each) could prevent the augmented levels of TBA-RS induced by IVG in cortical supernatants. Figure 4 shows that, at these doses, TRO, MEL and GSH totally prevented the lipid peroxidation induced by IVG $[F_{(5,26)} = 17.136; p < 0.001]$.

In contrast, IVG and IVA did not change these lipid oxidative parameters in brain mitochondrial preparations (results not shown).

We also tested whether glycine (GLY, 0.1–5.0 mM), which is a component of IVG chemical structure, could induce lipid peroxidation in brain cortical supernatants. We observed that GLY significantly increased TBA-RS $[F_{(4,15)} = 12.88; p < 0.001]$ and chemiluminescence $[F_{(3,12)} = 4.503; p < 0.05]$ in a dose-dependent manner [TBA-RS: $\beta = 0.856$;

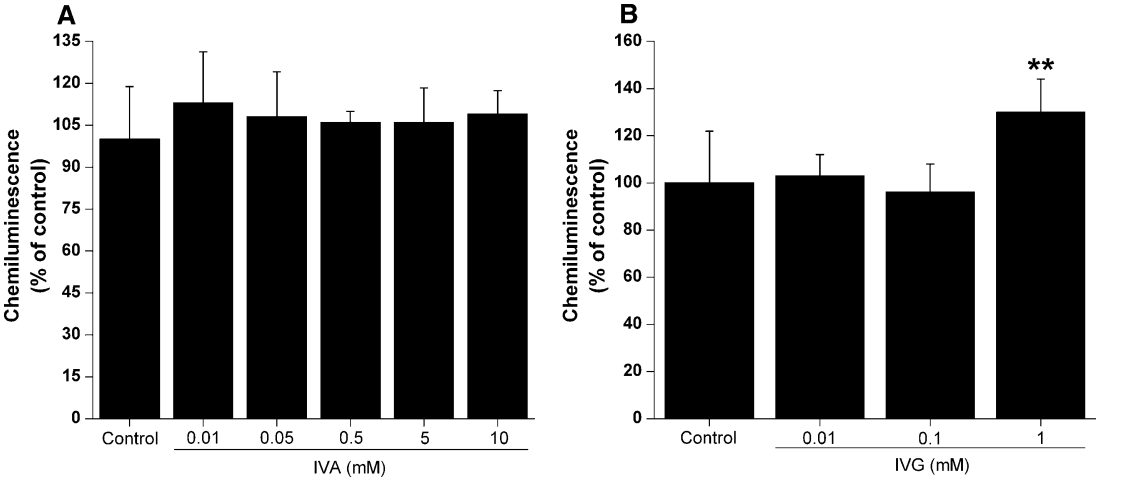

Figure 2. *In vitro* effect of isovaleric acid (IVA) and isovalerylglycine (IVG) on chemiluminescence in rat cerebral cortex supernatants. Cortical supernatants were incubated during 60 min in the presence of IVA or IVG at concentrations ranging from 0.01–10 mM (A) and 0.01–1.0 mM (B), respectively. Values are means ± standard deviation for four-to-five independent experiments performed in triplicate and are expressed as percentage of controls (Controls: A: 3326 ± 627 cpm/mg of protein; B: 3318 ± 764 cpm/mg of protein. ** $p < 0.01$, compared to controls (Duncan multiple range test).

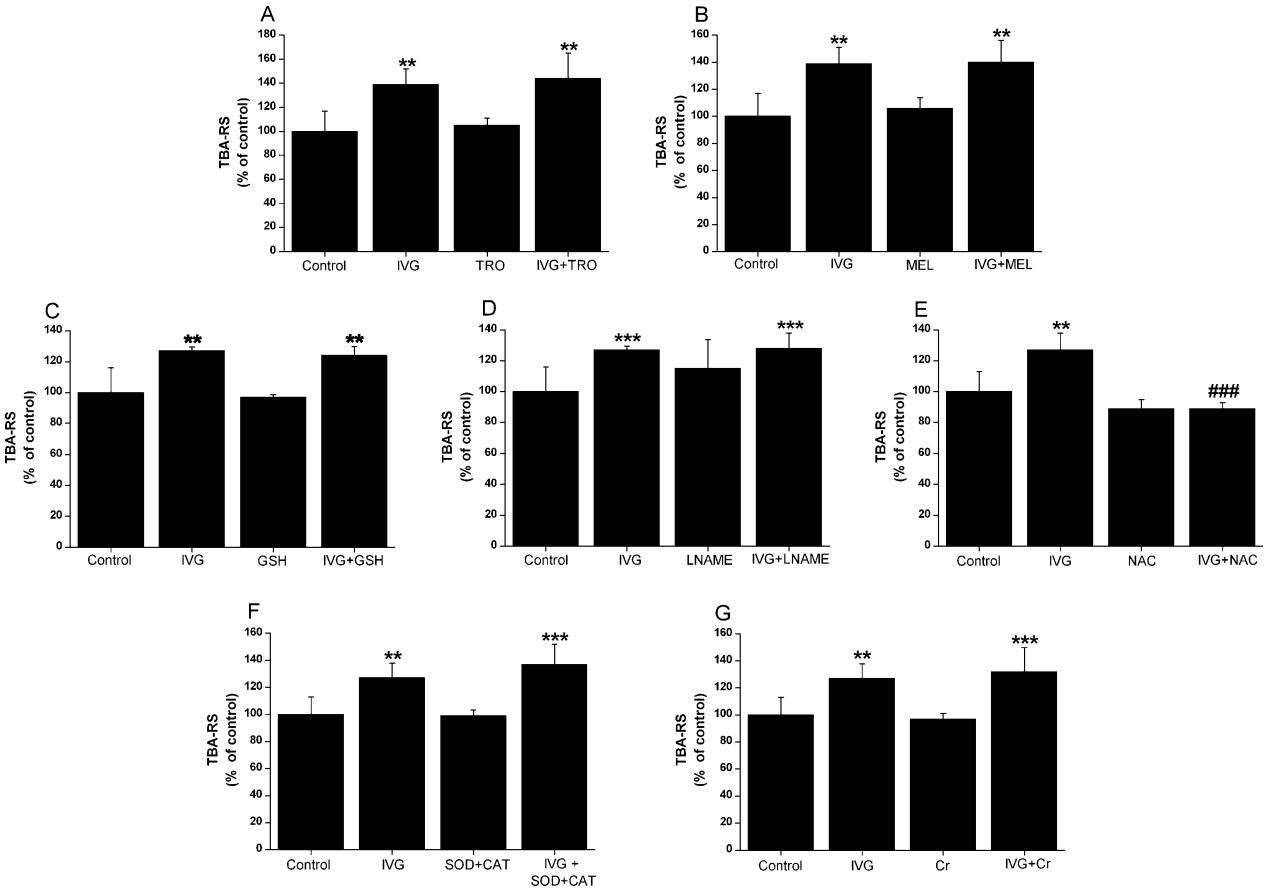

Figure 3. Effect of antioxidants on isovalerylglycine (IVG)-induced *in vitro* lipid peroxidation (TBA-RS) in rat cerebral cortex supernatants. Cortical supernatants were co-incubated with 1.5 μM α-tocopherol (Trolox) (A), melatonin (MEL) (B), 100 μM glutathione (GSH) (C), 500 μM N^{ω}-nitro-L-arginine methyl ester (L-NAME) (D), 1 mM N-acetylcysteine (NAC) (E), combination of 2.5 mU/mL catalase (CAT) plus 2.5 mU/mL superoxide dismutase (SOD) (F) or 3 mM creatine (Cr) (G) and 1 mM IVG. Values are means ± standard deviation for four independent experiments performed in triplicate and are expressed as percentage of controls (Controls co-incubation [nmol/mg of protein]: A: 2.61 ± 0.45; B: 2.61 ± 0.45; C: 3.18 ± 0.4; D: 3.67 ± 0.62; E: 2.61 ± 0.45; F: 3.68 ± 0.48; G: 3.18 ± 0.4). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, compared to controls; $^{\#\#\#}p < 0.001$, compared to 1.0 mM IVG (Duncan multiple range test).

$p < 0.001$] [Chemiluminescence: $\beta = 0.717$; $p < 0.01$] in supernatants from brain cortex (Figure 5).

IVG reduces the non-enzymatic antioxidant defenses in rat cortical supernatants

Since NAC, a precursor of GSH, prevented IVG-induced increase of lipid peroxidation at low concentrations, it was presumed that GSH levels were probably reduced by this accumulating metabolite. Therefore, the cortical non-enzymatic antioxidant defenses were also investigated by assessing GSH levels. We verified that only IVG significantly decreased GSH concentrations [$F_{(5,18)} = 4.373$; $p < 0.01$] in a dose-dependent profile in supernatants [$\beta = 0.703$; $p < 0.001$] (Figure 6), but not in mitochondrial preparations from rat brain (results not shown).

IVA induces protein oxidation in rat cortical supernatants and mitochondrial preparations

The next set of experiments was carried out to evaluate the effect of IVA and IVG on carbonyl formation in cerebral cortex supernatants and purified mitochondrial preparations. As it can be observed in Figure 7, IVA provoked a significant increase in carbonyl formation in both preparations (Figure 7A: cortical supernatants [$F_{(3,23)} = 7.186$; $p < 0.01$]; Figure 7B: mitochondrial preparations [$F_{(4,15)} = 19.270$; $p < 0.001$], whereas IVG did not alter this parameter in both preparations (results not shown). These results indicate that IVA causes protein oxidative damage.

IVG per se does not behave as a direct oxidant

Finally, we investigated whether IVG-induced decrease of brain GSH levels was due to a direct oxidative attack rather than due to promotion of free radical generation. We therefore exposed a commercial GSH solution (200 μM) to 1.0 mM IVG in the absence of brain supernatants. Figure 8 shows that IVG *per se* did not oxidize free GSH, whereas *N*-ethylmaleimide (NEM, 150 μM) (positive control) markedly oxidized GSH [$F_{(2,6)} = 621.12$; $p < 0.001$].

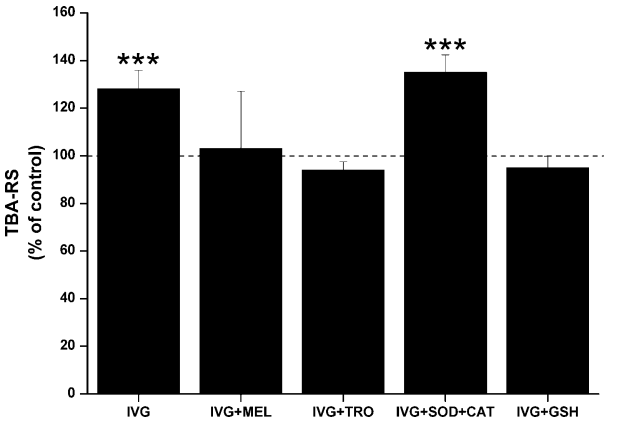

Figure 4. Effect of increasing concentrations of antioxidants on isovalerylglycine (IVG)-induced *in vitro* lipid peroxidation (TBA-RS) in rat cerebral cortex supernatants. Cortical supernatants were co-incubated with the antioxidants Trolox (TRO, α-tocopherol; 10 μM), melatonin (MEL; 1000 μM), the combination of catalase (CAT) plus superoxide dismutase (SOD) (15 mU/mL of each enzyme) or glutathione (750 μM) and 1.0 mM IVG. Values are means ± standard deviation of four-to-eight independent experiments performed in triplicate and are expressed as percentage of controls (Control: 4.46 ± 0.26 nmol/mg of protein). ***$p < 0.001$, compared to controls; $^{\#}p < 0.01$, $^{\#\#\#}p < 0.001$, compared to 1.0 mM IVG (Duncan multiple range test).

The data indicate that IVG itself does not behave as an oxidant agent.

Discussion

Patients affected by IVAcidemia, an autosomal recessive disorder, commonly present severe neurological dysfunction. They develop intermittent acute episodes with metabolic acidosis and metabolic decompensation following infectious or other physiological catabolic stress situations [5,21,22]. The biochemical hallmark of this disorder is the accumulation of IVA and their carnitine (isovalerylcarnitine) and glycine (IVG) derivatives, as well as of 3-OHIVA. The amount of free IVA during episodes of acute decompensation can achieve several hundred times in the blood, while isovalerylcarnitine and IVG are identified at high amounts in plasma and urine, respectively.

Although severe cerebral damage characterized by marked lesions in globi pallidi and corticospinal tracts is seen in the affected patients, the underlying mechanisms involved in the neuropathology of this disease are poorly known. Recent reports indicate that oxidative stress may be acting in this disorder since 3-OHIVA, which also accumulates in 3-methylglutaconic aciduria, was shown to cause lipid oxidative damage [13]. Furthermore, IVA caused inhibition of Na^+, K^+-ATPase activity in rat cerebral cortex probably by oxidation of critical groups on the enzyme [5]. Thus, in the present study we investigated whether the major accumulating metabolites in this disorder, namely IVA and IVG, cause lipid and protein oxidative damage and alter the major non-enzymatic antioxidant defense (GSH) in brain cortex of young rats. The maximal dose of IVA utilized in the *in vitro* assays was 10 mM, whereas for IVG, which accumulate in lower amounts, we used up to 1 mM concentration.

We first verified that IVG, but not IVA, at concentrations as low as 0.5 mM, significantly increased chemiluminescence and TBA-RS levels in cerebral cortical supernatants. Light in the chemiluminescence assay arises mainly from excited carbonyls, $O_2^{\bullet -}$, $ONOO^-$ and from peroxidizing lipids [23]. On the other hand, TBA-RS measurement reflects the amount of malondialdehyde formation, an end product of membrane fatty acid peroxidation [23]. Therefore, the increased values of these parameters elicited by relatively low concentrations of

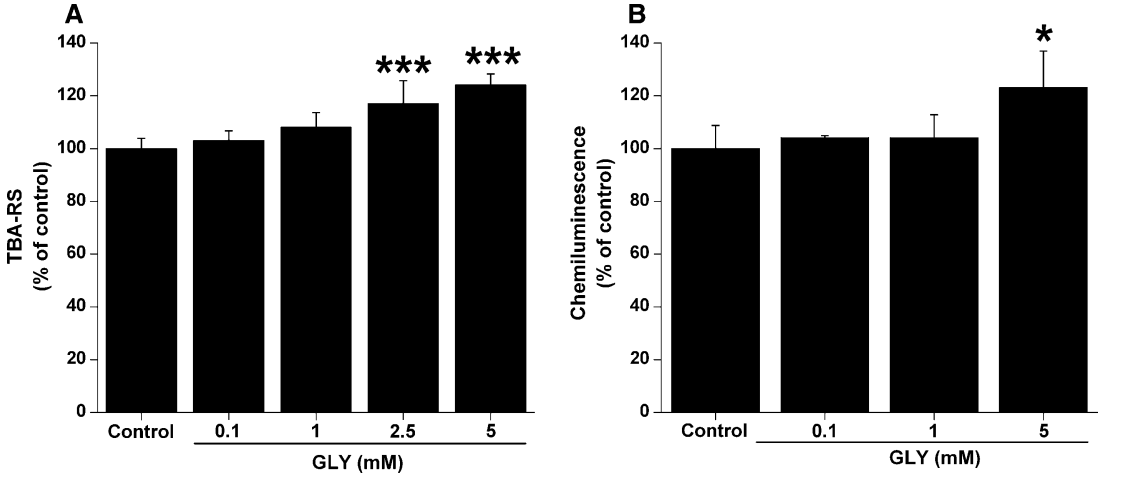

Figure 5. *In vitro* effect of glycine (GLY) on the lipid peroxidation parameters thiobarbituric acid-reactive substances (TBA-RS) (A) and chemiluminescence (B) in rat cerebral cortex supernatants. Cortical supernatants were incubated during 60 min in the presence of GLY at concentrations ranging from 0.1–5.0 mM. Values are means ± standard deviation for five-to-six independent experiments performed in triplicate and are expressed as percentage of controls (Controls: A: 4.48 ± 0.25 nmol/mg of protein, B: 2154 ± 187 cpm/mg of protein). *$p < 0.05$, ***$p < 0.001$, compared to controls (Duncan multiple range test).

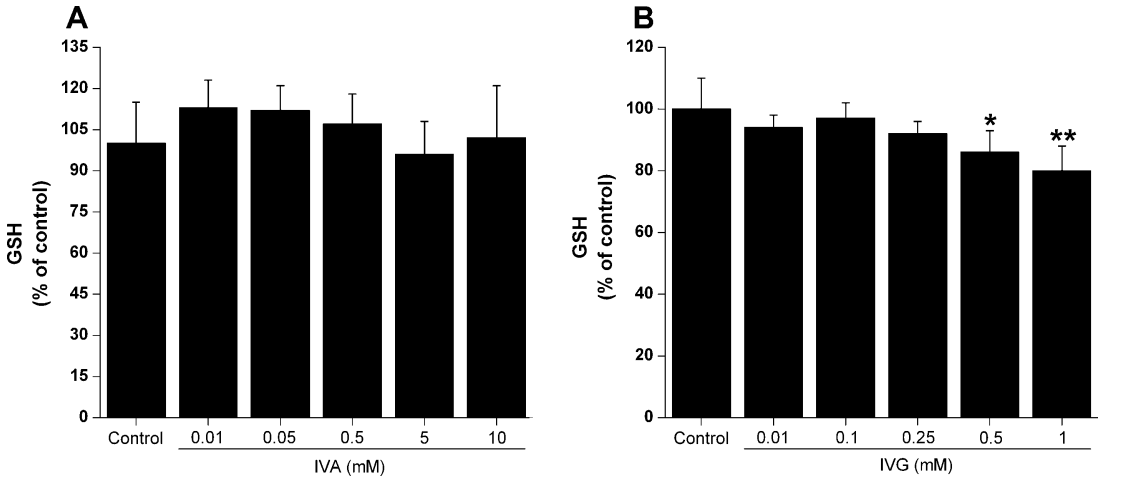

Figure 6. *In vitro* effect of isovaleric acid (IVA) and isovalerylglycine (IVG) on glutathione (GSH) levels in rat cerebral cortex supernatants. Cortical supernatants were incubated during 60 min in the presence of IVA or IVG at concentrations ranging from 0.01–10 mM (A) and 0.01–1.0 mM (B), respectively. Values are means ± standard deviation for four independent experiments performed in triplicate and are expressed as percentage of controls (Controls: A: 4.54 ± 0.69 nmol/mg of protein, B: 5.77 ± 1.05 nmol/mg of protein). $^{*}p < 0.05$, $^{**}p < 0.01$, compared to controls (Duncan multiple range test).

IVG strongly indicate that this compound caused lipid peroxidation *in vitro* in cerebral cortex.

We also verified that relatively low concentrations of NAC, precursor of GSH, fully prevented the increase of TBA-RS measurement provoked by IVG, indicating a reduction of GSH levels by IVG. Furthermore, the lipid oxidative damage induced by IVG was totally prevented by high doses of the free radical scavengers TRO (α-tocopherol), MEL and GSH, but not by the combination of SOD plus CAT. However, lower doses of these antioxidants and also of L-NAME and Cr were not able to change the IVG-induced increase of TBA-RS values. Overall, the data indicate that IVG provoked a strong pro-oxidant effect on membrane lipids from cerebral cortex probably mediated by reactive oxygen species generation. We also found that IVG did not change TBA-RS levels in disrupted mitochondrial preparations (post-mitochondrial supernatants), indicating that the mechanisms involved in IVG-mediated generation of oxidants leading to lipid damage depends on the cytosolic machinery.

Interestingly, glycine, which conjugates with IVA to give rise to IVG, was also able to induce lipid peroxidation, indicating that IVG-induction of lipid oxidative damage might be at least in part due to glycine. Further studies are therefore necessary to evaluate in more detail the role of glycine on oxidative stress in the brain. This would possibly contribute to unravel the exact neurotoxic mechanisms of this amino acid, particularly in non-ketotic hyperglycinemia.

We also found that carbonyl formation, an index of protein oxidative damage, was induced by a wide range of IVA concentrations (0.5–10 mM), but not by IVG, in supernatants and mitochondrial preparations from rat cerebral cortex, indicating that

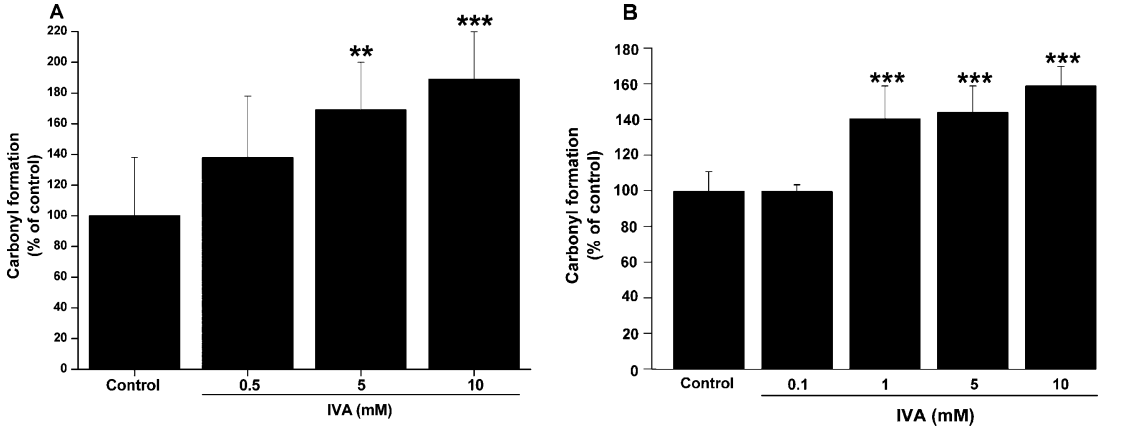

Figure 7. *In vitro* effect of IVA on carbonyl levels in rat cerebral cortex supernatants (A) and brain purified mitochondrial fractions (B). Cortical supernatants and mitochondrial fractions were incubated in the presence of IVA ranging from 0.01–10 mM. Values are means ± standard deviation for four-to-six independent experiments performed in triplicate and are expressed as percentage of controls (Controls: A: 0.41 ± 0.16 nmol/mg of protein; B: 2.70 ± 0.3 nmol/mg of protein). $^{**}p < 0.01$, $^{***}p < 0.001$, compared to controls (Duncan multiple range test).

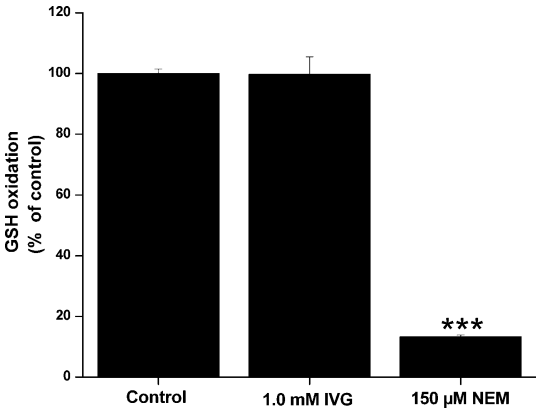

Figure 8. Effect of isovalerylglycine (IVG) on commercial reduced glutathione (GSH). Values are means ± standard deviation for three independent experiments performed in triplicate and are expressed as percentage of controls. GSH (200 μM) oxidation was measured in the absence of brain tissue. Control: 1834 ± 27 fluorescence units. ***$p < 0.001$, compared to controls (Duncan multiple range test). NEM = *N*-ethylmaleimide (positive control).

this organic acid provokes protein oxidation. Carbonyl groups (aldehydes and ketones) are mainly produced by oxidation of protein side chains (especially of Pro, Arg, Lys and Thr), by oxidative cleavage of proteins or from the reaction of reducing sugars or their oxidation products with lysine protein residues [24]. Although the exact mechanisms by which IVA caused protein oxidative damage are at the present unknown, it is conceivable that IVA or its generated reactive species may interact with these protein groups better than IVG, a structurally distinct compound, finally leading to protein oxidation.

With regard to the antioxidant defense system, IVG markedly reduced the total content of GSH, the main naturally-occurring antioxidant in rat cortical supernatants, and this is in line with our findings showing that NAC prevented IVG induction of lipid peroxidation. Since this parameter is used to evaluate the non-enzymatic antioxidant capacity of a tissue to prevent the damage associated to free radical processes, it can be concluded that the rat cortical non-enzymatic antioxidant defenses were compromised by IVG [23]. Considering that IVG was not able to directly oxidize thiol groups from commercial GSH, it is presumed that IVG itself is not a direct acting pro-oxidant agent, but possibly induced oxidative damage via reactive species generation [25,26].

Since oxidative stress results from an imbalance between the total antioxidant defenses and the reactive species generated in a tissue, our present data strongly indicate that IVG induces oxidative stress in rat cerebral cortex, a deleterious cell condition [23]. At this point, it should be emphasized that the brain has low cerebral antioxidant defenses compared with other tissues [27], a fact that makes this tissue more vulnerable to increased levels of reactive species. In fact, oxidative stress has been implicated in the pathophysiology of common neurodegenerative disorders, such as Parkinson's disease and Alzheimer's disease, as well as in epileptic seizures and demyelination [28–35].

We cannot at the present establish the exact signal transduction cascades by which IVG and IVA induced lipid and protein oxidation, respectively. However, it may be presumed that free radicals elicited by these acidic compounds could initiate the classical cascades leading to lipid and protein oxidative damage [23].

On the other hand, it is difficult to determine the pathophysiological relevance of our data since to our knowledge brain concentrations of IVA and IVG in IVAcidemia are not yet established. However, it should be noted that the significant alterations of the oxidative stress parameters evaluated occurred with relatively low doses of IVG (0.5 mM and higher) and at 0.5 mM IVA, a concentration that is found in CSF of patients affected by IVAcidemia. Furthermore, severe neurological symptoms in these patients occur especially during metabolic crises, in which the concentrations of the accumulating metabolites dramatically increase.

In conclusion, this is the first report showing that IVA and IVG, the major metabolites accumulating in IVAcidemia, elicit oxidative damage. In case the present *in vitro* data are confirmed *in vivo* in animal experiments and also in tissues from patients affected by IVAcidemia, it is tempting to speculate that reactive species may contribute, at least in part, to the neurological damage found in this disorder. Finally, it may be proposed that the administration of antioxidants should be considered as an adjuvant therapy to specific diets or to other pharmacological agents for these patients, especially during metabolic crises.

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