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TOXIC SYNERGISM BETWEEN QUINOLINIC ACID AND ORGANIC ACIDS ACCUMULATING IN GLUTARIC ACIDEMIA TYPE I AND IN DISORDERS OF PROPIONATE METABOLISM IN RAT BRAIN SYNAPTOSOMES: RELEVANCE FOR METABOLIC ACIDEMIAS

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Abstract—The brain of children affected by organic acidemias develop acute neurodegeneration linked to accumulation of endogenous toxic metabolites like glutaric (GA), 3-hydroxyglutaric (3-OHGA), methylmalonic (MMA) and propionic (PA) acids. Excitotoxic and oxidative events are involved in the toxic patterns elicited by these organic acids, although their single actions cannot explain the extent of brain damage observed in organic acidemias. The characterization of co-adjuvant factors involved in the magnification of early toxic processes evoked by these metabolites is essential to infer their actions in the human brain. Alterations in the kynurenine pathway (KP) – a metabolic route devoted to degrade tryptophan to form NAD^+ – produce increased levels of the excitotoxic metabolite quinolinic acid (QUIN), which has been involved in neurodegenerative disorders. Herein we investigated the effects of subtoxic concentrations of GA, 3-OHGA, MMA and PA, either alone or in combination with QUIN, on early toxic endpoints in rat brain synaptosomes. To establish specific mechanisms, we pre-incubated synaptosomes with different protective agents, including the endogenous N-methyl-D-aspartate (NMDA) receptor antagonist kynurenic acid (KA), the antioxidant S-allylcysteine (SAC) and the nitric oxide synthase (NOS) inhibitor nitro-L-arginine methyl ester (L-NAME). While the incubation of synaptosomes with toxic metabolites at subtoxic concentrations produced no effects, their co-incubation (QUIN + GA, +3-OHGA, +MMA or +PA) decreased the mitochondrial function and increased reactive oxygen species (ROS) formation and lipid peroxidation. For all cases, this effect was partially prevented by KA and L-NAME, and completely avoided by SAC. These findings suggest that early damaging events elicited by organic acids involved in metabolic acidemias can be magnified by toxic synergism with QUIN, and this process is mostly mediated by oxidative stress, and in a lesser extent by excitotoxicity and nitrosative stress. Therefore, QUIN can be hypothesized to contribute to the pathophysiology of brain degeneration in children with metabolic acidemias.

Key words: organic acidemias, excitotoxicity, oxidative stress, mitochondrial dysfunction, cell damage, toxic synergism.

INTRODUCTION

The hereditary metabolic disorders known as organic acidemias (OA) are characterized by a blockage of the aberrant catabolism of amino acids and lipids due to a deficient activity of specific enzymes. These alterations are responsible for the accumulation and high urinary excretion of potentially toxic organic acids (Bodamer et al., 2006). Neurological symptoms and brain abnormalities are seen in patients suffering from OA. Glutaric acidemia type I (GA I), methylmalonic acidemia (MMAcidemia) and propionic acidemia (PAcidemia) have a relatively high prevalence in the population, all with a severe clinical presentation in the neonatal period.

GA I is known to be caused by a deficiency of glutaryl-CoA dehydrogenase (GDD, McKusick 23167; OMIM # 231670) activity, resulting in the accumulation of glutaric (GA, 500–5000 μmol/L) and 3-hydroxyglutaric (3-OHGA, 40–200 μmol/L) acids in the CNS (Kölker et al., 2004; Sauer et al., 2006). Among its pathological features are

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Abbreviations: GA, glutaric acid; GA I, glutaric acidemia type I; GDD, glutaryl-CoA dehydrogenase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; KA, kynurenic acid; KP, kynurenine pathway; L-NAME, L-nitro-L-arginine methyl ester; MDA, Malondialdehyde; MMA, methylmalonic acid; MMAcidemia, methylmalonic acidemia; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NMDA, N-methyl-D-aspartate; NMDAr, NMDA receptors; NOS, nitric oxide synthase; OA, organic acidemias; PA, propionic acid; PAcidemia, propionic acidemia; QUIN, quinolinic acid; RNS, reactive nitrogen species; ROS, reactive oxygen species; SAC, S-allylcysteine; TBA, thiobarbituric acid; 3-OHGA, 3-hydroxyglutaric acid.

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a fronto-temporal cortical atrophy at birth, progressive spongy formation, leukoencephalopathy and acute damage of the caudate/putamen occurring between 6 months and 4 years of age (Amir et al., 1987; Hoffmann and Zschocke, 1999). Experimental evidence suggests that accumulating organic acids induce excitotoxicity, oxidative stress and energy metabolism impairment (Flott-Rahmel et al., 1997; Latini et al., 2002; de Oliveira Marques et al., 2003; Kölker et al., 2004; Wajner et al., 2004; Sauer et al., 2005; Latini et al., 2005a,b; Ferreira et al., 2007; Rosa et al., 2007), although the precise pathogenic mechanisms occurring in GA I have not been fully described.

In turn, MMAcidemia and PAcidemia are caused by severe deficiencies of methylmalonyl-CoA mutase (EC 5.4.99.2) and propionyl-CoA carboxylase (EC 6.4.1.3) activities, respectively. MMAcidemia is biochemically characterized by accumulation of methylmalonic acid (MMA) (1-2.5 mmol/L), whereas PAcidemia by propionic acid (PA) (5 mmol/L) in blood. Clinical manifestations of these two OA comprise lethargy, psychomotor delay/mental retardation, focal and generalized convulsions, vomiting, dehydration, hepatomegaly, hypotonia, and encephalopathy further leading to coma and death (Deodato et al., 2006; Hauser et al., 2011). Disrupted myelination revealing progressive cortical atrophy, as well as histopathological injury of the basal ganglia can be observed (Brismar and Ozand, 1994; Chemelli et al., 2000; Harting et al., 2008). For both acidemias, brain damage has been related to the toxic actions produced by their corresponding accumulating metabolites. This suggestion is based on experimental evidence demonstrating that MMA can cause brain mitochondrial energy metabolism disruption, as well as redox status and glutamatergic transmission alterations (Kölker et al., 2006; Sauer et al., 2006, 2010; Stellmer et al., 2007), whereas PA has also been shown to exert toxic effects in the rat brain (Wyse et al., 1998; Brusque et al., 1999; de Mattos-Dutra et al., 2000; Fontella et al., 2000; Pettenuzzo et al., 2002; Trindade et al., 2002; Rigo et al., 2006; Ribas et al., 2010a,b).

Tryptophan catabolism and NAD^+ synthesis occur in cells from different tissues through the kynurenine pathway (KP). This metabolic route is relevant for biomedical research as neuroactive intermediary metabolites are synthesized throughout (reviewed by Pérez-De La Cruz et al. (2007)), some of which are involved in pathogenic processes of neurological disorders, including Huntington's disease (HD) (reviewed by Schwarcz et al., 2010, 2012). One of these KP metabolites, quinolinic acid (QUIN or 2,3-pyridine dicarboxylic acid) is an endogenous N-methyl-D-aspartate receptor (NMDAr) agonist (Stone et al., 2003). QUIN induces excitotoxicity in animal models and cell cultures, provoking enhanced intracellular $[Ca^{2+}]$, augmented levels of extracellular glutamate, increased reactive oxygen species (ROS) and reactive nitrogen species (RNS) formation, decreased activity and expression of antioxidant systems, oxidative stress, stimulated protease activity and cell death (Rios and Santamaría, 1991; Rodríguez-Martínez et al., 2000; Tavares et al., 2000; Braidy et al., 2009, 2010; Pérez-De La Cruz et al., 2010). Moreover, QUIN could exert a pathogenic role in different neurodegenerative disorders since increased levels of this metabolite have been described in these pathological conditions (Schwarcz et al., 2010).

When considered separately, the toxic profiles characterized at the experimental level for the organic acids accumulating in OA and for QUIN in human neurological disorders could be not sufficient to explain the extent of cell and tissue damage produced by them *per se*, yielding the assumption that additional and additive mechanisms could account for the toxic profiles of these metabolites. Therefore, the aim of this work was to investigate whether GA, 3-OHGA, MMA or PA can exert synergic toxic effects with QUIN when tested in rat brain synaptosomes at subtoxic concentrations, upon the hypothesis that QUIN might eventually contribute to neurodegenerative processes in OA.

EXPERIMENTAL PROCEDURES

Reagents

GA, MMA, PA, QUIN, HEPES, thiobarbituric acid (TBA), kynurenic acid (KA), L-nitro-L-arginine methyl ester (L-NAME), malondialdehyde (MDA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and other reagents were obtained from Sigma–Aldrich Chemical Co. (St Louis, MO, USA). Dr. Ernesto Brunet (Universidad Autónoma de Madrid, Spain) kindly supplied 3-OHGA. Other reagents were obtained from other well-known commercial sources. S-allylcysteine (SAC) was synthesized according to previous reports (García et al., 2008, 2014).

Animals

Male Wistar adult (250–300 g) rats were used throughout the study. Animals ($N = 40$) were obtained from the vivarium of the Universidad Nacional Autónoma de México. All rats were housed five per cage and provided with food and water *ad libitum* under constant conditions of temperature (25 ± 3 °C), humidity and light (12:12-h light:dark schedule). All animal manipulations were carried out following the "Guidelines for the Use of Animals in Neuroscience Research" from the Society of Neuroscience, the local Ethics Committees, and in compliance with the ARRIVE guidelines.

Isolation of brain synaptosomal P2 fractions and treatments

Isolation of synaptosomal P2 fractions from rat brains was carried out according to Lopachin et al. (2009), with modifications (Rangel-López et al., 2015). All brains (without cerebellum) were surgically removed, weighted, transferred to ice-cooled PBS (pH 7.4), and homogenized in 10 volumes (g/ml) of sucrose (0.32 M). The cerebellum was excluded because this brain region is generally not altered in GA I, MMAcidemia and PAcidemia, whose accumulating metabolites were tested in our work. Homogenates were centrifuged for 10 min at 1073 × *g* (4 °C) and

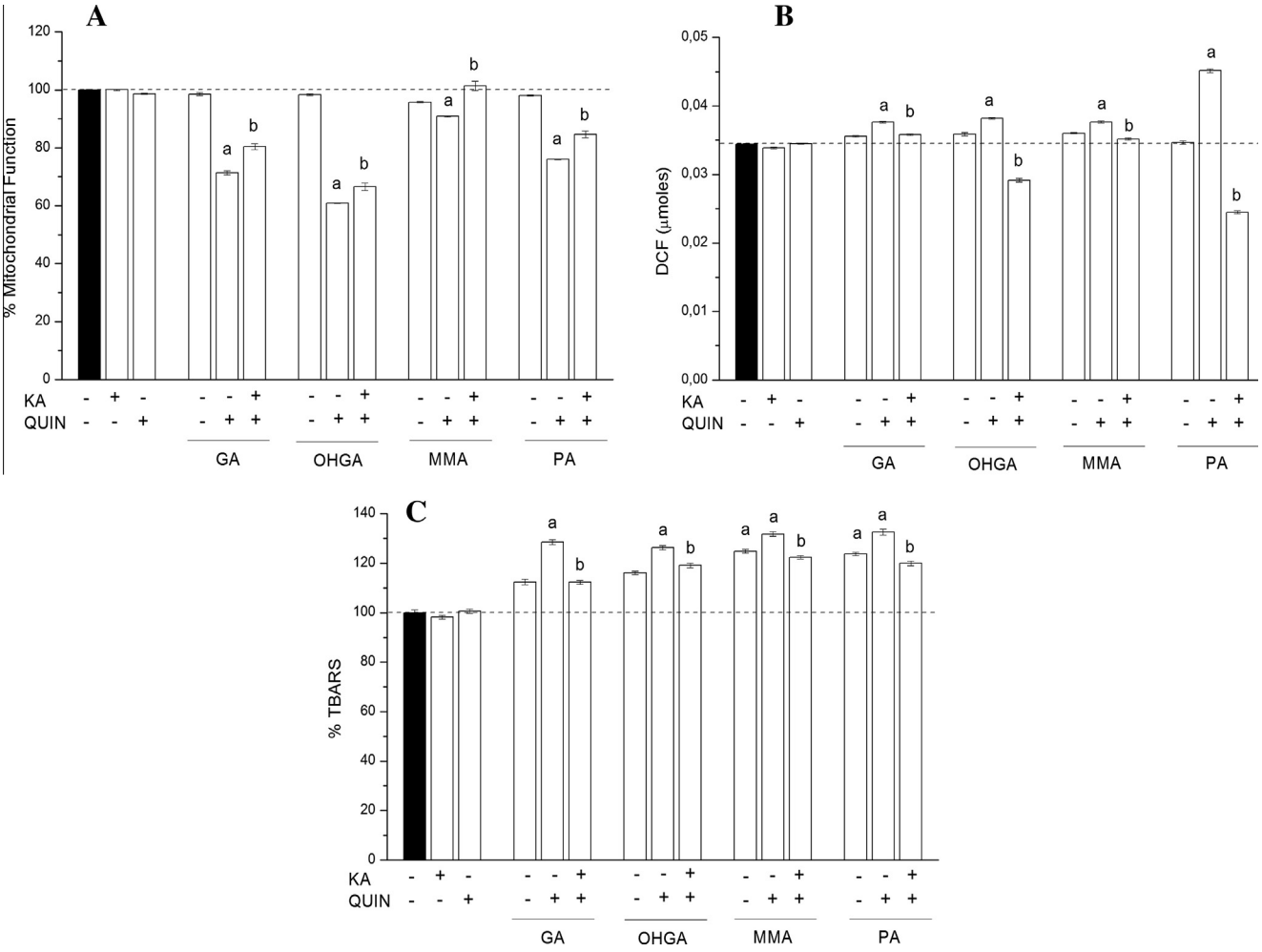

Fig. 1. Effects of kynurenic acid (KA, 50 μM) on the quinolinic acid (QUIN, 50 μM) plus glutaric acid (GA, 500 μM)-, 3-hydroxyglutaric acid (3-OHGA)-, methylmalonic acid (MMA)- or propionic acid (PA)-induced changes in mitochondrial function (A), reactive oxygen species (ROS) formation (B) and lipid peroxidation (C) in rat brain synaptosomal fractions. KA was added to synaptosomes in incubation 30 min before the simultaneous addition of QUIN and/or organic acids. The total incubation time was 60 min. All data are expressed as mean values ± S.E.M. of $n = 6–8$ experiments per group. One-way ANOVA followed by *post hoc* Duncan's test for comparisons among treatments. Symbols denote statistical differences vs. control ($^{a}P < 0.05$), and vs. QUIN plus each organic acid ($^{b}P < 0.05$).

the supernatants were re-centrifuged for 15 min at 17,000×*g* (4 °C). The resulting pellets were resuspended in 40 volumes (ml) of HEPES-buffer containing 0.1 M NaCl, 0.001 M NaH_2PO_4, 0.005 M $NaHCO_3$, 0.001 M $CaCl_2$, 0.006 M glucose, and 0.01 M HEPES (pH 7.4). Aliquots were briefly stored at −70 °C until employed for the experiments. Total protein quantifications were estimated using the technique reported by Bradford (1976).

Synaptosomal fractions were pre-incubated for 30 min at 37 °C with O_2 supply, and immediately thereafter co-incubated for 30 min with GA, 3-OHGA, MMA or PA (all at 500 μM) plus QUIN (50 μM). These concentrations were assumed to be subtoxic as revealed by the estimation of toxic endpoints with the different agents added separately. Effective concentrations of other agents (50 μM KA, an endogenous NMDAr antagonist; 100 μM SAC, a potent antioxidant; 100 μM L-NAME, a nitric oxide synthase (NOS) inhibitor; 0.05 U/mL catalase (CAT), an antioxidant and detoxifying enzyme; and 500 μM creatine (CREAT), a well-known metabolic precursor) were added as pretreatments 30 min before the addition of toxic metabolites in order to explore possible mechanisms involved in these models since a pharmacological perspective. Data of 6–8 experiments per group (three probes per condition per experiment) were collected for each endpoint evaluated. After exposed to the mentioned treatments, synaptosomal fractions were assigned to the different analytical procedures described as follows.

The assay of ROS formation

The formation of ROS was estimated according to previous reports (Santamaría et al., 2001; Rangel-López et al., 2015). After incubating in the presence of the different treatments, the synaptosomal fractions were diluted in nine volumes of 40 mM Tris plus HEPES buffer. Then, samples were incubated with 5 μM 2′,7′-dichlorofluorescein diacetate (DCFH-DA) for 60 min at 37 °C. Fluorescent signals were recorded at 488 nm of excitation and

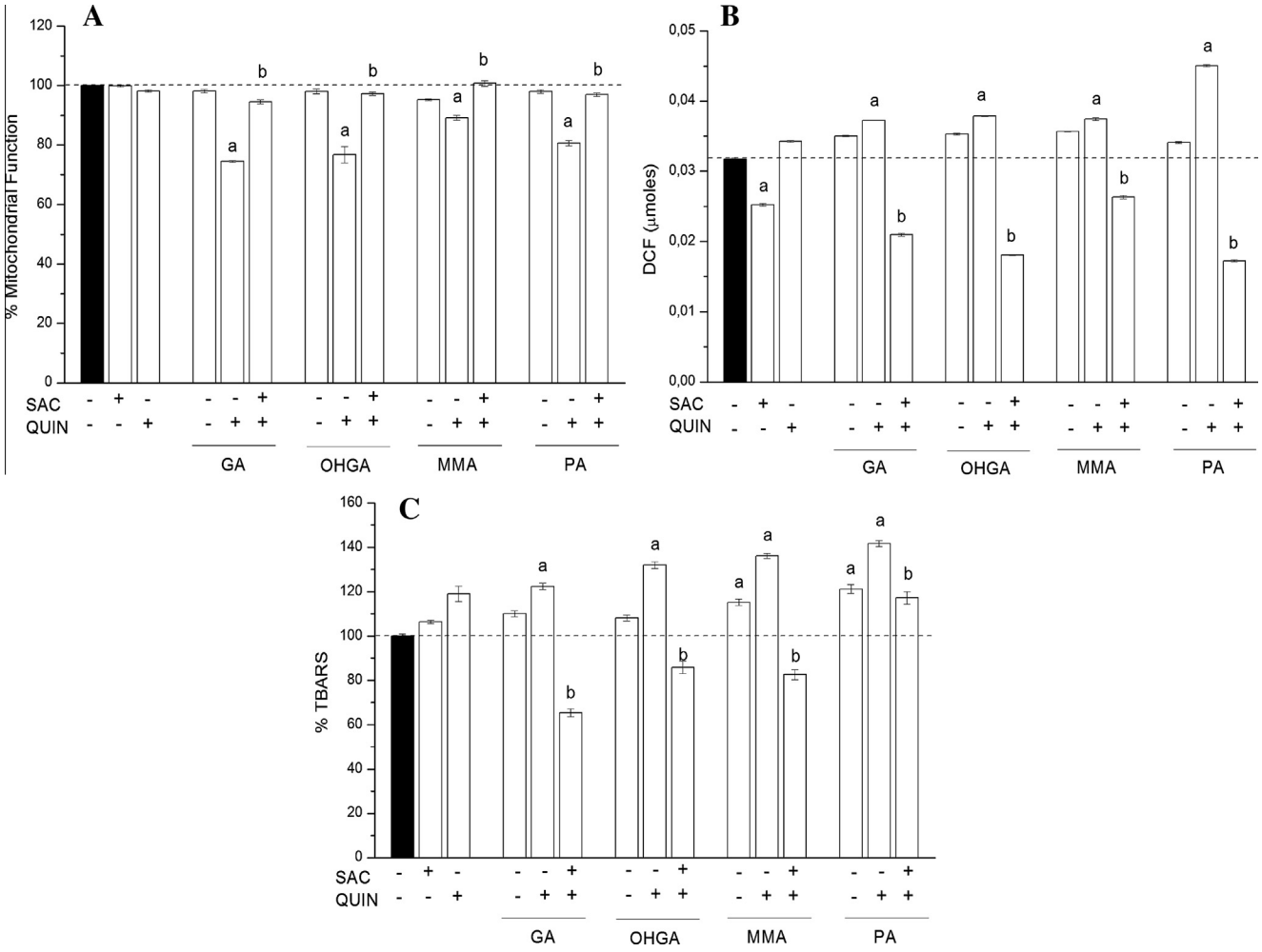

Fig. 2. Effects of S-allyl cysteine (SAC, 100 μM) on the quinolinic acid (QUIN, 50 μM) plus glutaric acid (GA, 500 μM)-, 3-hydroxyglutaric acid (3-OHGA)-, methylmalonic acid (MMA)- or propionic acid (PA)-induced changes in mitochondrial function (A), reactive oxygen species (ROS) formation (B) and lipid peroxidation (C) in rat brain synaptosomal fractions. SAC was added to synaptosomes in incubation 30 min before the simultaneous addition of QUIN and/or organic acids. The total incubation time was 60 min. All data are expressed as mean values ± S.E.M. of $n = 6–8$ experiments per group. One-way ANOVA followed by *post hoc* Duncan's test for comparisons among treatments. Symbols denote statistical differences vs. control ($^{a}P < 0.05$), and vs. QUIN plus each organic acid ($^{b}P < 0.05$).

525 nm of emission wavelengths in a CYT3MV Biotek Cytation 3 Imaging Reader. Results were expressed as micromoles of 2′,7′-dichlorofluorescein (DCF)/g wet tissue.

Assay of lipid peroxidation

The formation of thiobarbituric acid-reactive substances (TBARS) in synaptosomes was used as an index of lipid peroxidation, according to a previous report (García et al., 2008). After homogenized, the synaptosomal fractions (200 μL) were added with 500 μL of the TBA reagent containing 0.75 g of TBA + 15 g of trichloroacetic acid + 2.54 mL of HCl. The pink chromophore indicating the amount of peroxidized lipids was formed in samples after incubated in a water bath at 100 °C for 30 min. To stop the reaction, samples were kept on ice for 5 min and further centrifuged at 3000×*g* for 15 min. A CYT3MV Biotek Cytation 3 Imaging Reader was used to estimate the optical density of the supernatants at 532 nm. A standard curve was constructed in parallel with tetramethoxypropane and served for interpolation and calculation of the amounts of TBARS – mostly MDA – formed in samples. Final results were estimated as nanomoles of MDA per mg protein, and finally expressed as the percent of lipid peroxidation vs. control.

The MTT reduction assay for functional assessment of synaptosomes

The functional status of the respiratory chain and mitochondrial function was estimated in synaptosomes by the MTT reduction assay, according to a method previously described (Rangel-López et al., 2015). Briefly, the synaptosomes were added with 8 μl of MTT (5 mg/ml) and re-incubated for 60 min at 37 °C. Samples were centrifuged at 15,300×*g* for 15 min and the pellets resuspended in 1 ml of isopropanol. The aqueous phase was discarded after the first centrifugation. The second centrifugation was performed at 1700×*g* for 3 min. A CYT3MV Biotek Cytation 3 Imaging Reader was used to estimate the content of formazan at a 570 nm wavelength.

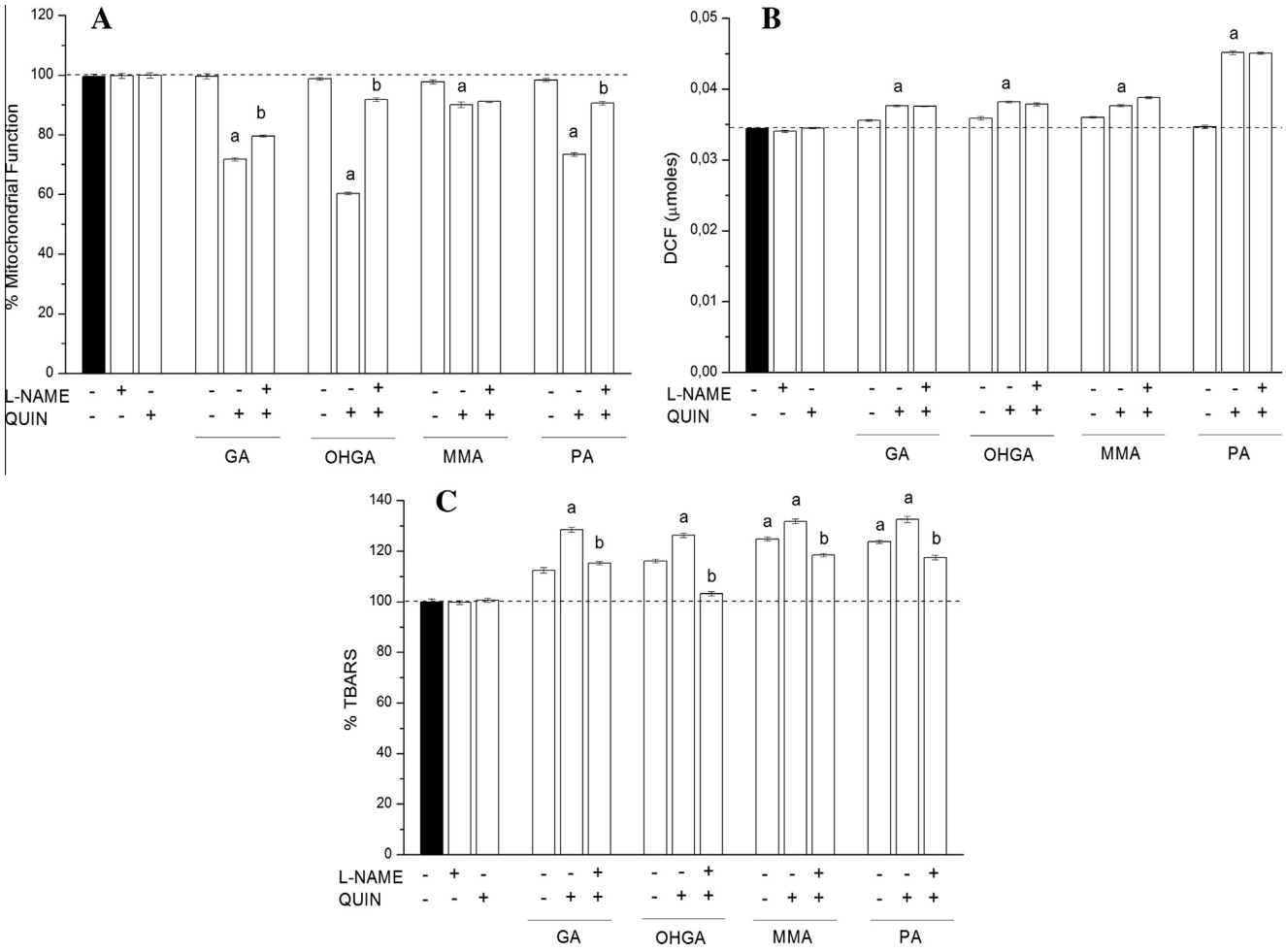

Fig. 3. Effects of L-nitro-arginine methyl ester (L-NAME, 100 μM) on the quinolinic acid (QUIN, 50 μM) plus glutaric acid (GA, 500 μM)-, 3-hydroxyglutaric acid (3-OHGA)-, methylmalonic acid (MMA)- or propionic acid (PA)-induced changes in mitochondrial function (A), reactive oxygen species (ROS) formation (B) and lipid peroxidation (C) in rat brain synaptosomal fractions. L-NAME was added to synaptosomes in incubation 30 min before the simultaneous addition of QUIN and/or organic acids. The total incubation time was 60 min. All data are expressed as mean values ± S.E.M. of $n = 6–8$ experiments per group. One-way ANOVA followed by *post hoc* Duncan's test for comparisons among treatments. Symbols denote statistical differences vs. control ($^{a}P < 0.05$), and vs. QUIN plus each organic acid ($^{b}P < 0.05$).

Results were expressed as the percent of MTT reduction vs. control values.

Statistical analysis

Data are expressed as mean values ± S.E.M. All results were statistically analyzed by a one-way analysis of variance (ANOVA), followed by *post hoc* Duncan's test. Statistical significance was assigned to comparison of treatments reaching values of $P < 0.05$. The statistical procedures were performed using the scientific statistic software GraphPad Prism 5 (GraphPad Scientific, San Diego, CA, USA).

RESULTS

GA, 3-OHGA, MMA and PA exerted a synergism with QUIN on different toxic endpoints

As above mentioned, in this study we used concentrations of organic acids that could be considered as subtoxic on the basis of previous studies and our own experience. The 500 μM concentration of GA, 3-OHGA, MMA and PA used herein represents the half of their toxic concentration (1 mM) previously used in synaptosomal fractions in a recent study (Colín-González et al., 2015).

While most of the toxic metabolites tested (QUIN, GA, 3-OHGA, MMA and PA) did not produce *per se* significant changes in mitochondrial function (3% below the control for QUIN, 6% below the control for GA, 5% below the control for 3-OHGA, 8% below the control for MMA, and 3% below the control for PA), ROS formation (2% below the control for QUIN, 3% above the control for GA, 4% above the control for 3-OHGA, 3% above the control for MMA, and 1% above the control for PA) or lipid peroxidation (2% above the control for QUIN, 10% above the control for GA, 14% above the control for 3-OHGA, 22% above the control for MMA [$P < 0.05$], and 20% above the control for PA [$P < 0.05$]) (Figs. 1–3), the simultaneous incubation of synaptosomes with QUIN and the organic acids produced a toxic synergism evidenced by decreased levels of MTT reduction (30% below the control for QUIN + GA [$P < 0.05$], 42% below the control for QUIN + 3-OHGA [$P < 0.05$], 9%

below the control for QUIN + MMA [$P < 0.05$], and 22% below the control for QUIN + PA [$P < 0.05$]), moderate but still significant changes in ROS formation (8% above the control for QUIN + GA [$P < 0.05$], 9% above the control for QUIN + 3-OHGA [$P < 0.05$], 7% above the control for QUIN + MMA [$P < 0.05$] and 29% above the control for QUIN + PA [$P < 0.05$]), and more prominent increases in lipid peroxidation (32% above the control for QUIN + GA [$P < 0.05$], 28% above the control for QUIN + 3-OHGA [$P < 0.05$], 34% above the control for QUIN + MMA [$P < 0.05$], and 31% above the control for QUIN + PA [$P < 0.05$]) (Figs. 1–3).

The toxic markers stimulated by the co-incubation of synaptosomes with QUIN + organic acids were partially sensitive to KA

The pre-conditioning of synaptosomal fractions for 30 min with the NMDAr antagonist KA resulted in partial and moderate but still significant prevention of mitochondrial dysfunction induced by QUIN + GA (10% above the toxic treatment and 20% below the control [$P < 0.05$]), QUIN + 3-OHGA (6% above the toxic treatment and 36% below the control [$P < 0.05$]), QUIN + MMA (13% above the toxic treatment and 3% above the control [$P < 0.05$]), and QUIN + PA (7% above the toxic treatment and 15% below the control [$P < 0.05$]). When tested *per se* or when challenging each toxic metabolite separately, KA had no effect on MTT reduction (Fig. 1A).

KA also moderately but significantly reduced the ROS formation induced by QUIN + GA (8% below the toxic treatment [$P < 0.05$] and 6% above the control), QUIN + 3-OHGA (26% below the toxic treatment and 17% below the control [$P < 0.05$]), QUIN + MMA (5% below the toxic treatment [$P < 0.05$] and 3% above the control), and in a more prominent manner by QUIN + PA (53% below the toxic treatment and 37% below the control [$P < 0.05$]) (Fig. 1B).

In regard to oxidative damage to lipids, this marker was moderately but still significantly reduced by KA (19% below, comparing KA + QUIN + GA vs. QUIN + GA [$P < 0.05$]; 5% below, comparing KA + QUIN + 3-OHGA vs. QUIN + 3-OHGA [$P < 0.05$]; 15% below, comparing KA + QUIN + MMA vs. QUIN + MMA [$P < 0.05$]; 14% below, comparing KA + QUIN + PA vs. QUIN + PA [$P < 0.05$]) (Fig. 1C). In addition, KA moderately reduced the levels of lipoperoxidation induced by the metabolic acids *per se* (data not shown).

The effects exerted by the combination of QUIN + organic acids were prevented by SAC

The antioxidant SAC, added as pretreatment to synaptosomes, completely prevented the mitochondrial dysfunction induced by the combination of QUIN plus all organic acids (30% above and 5% below when comparing SAC + QUIN + GA vs. QUIN + GA [$P < 0.05$] and Control, respectively; 23% above and 3% below when comparing SAC + QUIN + 3-OHGA vs. QUIN + 3-OHGA [$P < 0.05$] and Control, respectively; 17% above and 2% above when comparing SAC + QUIN + 3-MMA vs. QUIN + MMA [$P < 0.05$] and Control, respectively; 14% above and 3% below when comparing SAC + QUIN + 3-PA vs. QUIN + PA [$P < 0.05$] and Control, respectively) (Fig. 2A).

When SAC was tested in the ROS formation assay, the antioxidant was able to significantly reduce the QUIN + organic acids-induced DCFH oxidation even below the control values (44% below and 38% below when comparing SAC + QUIN + GA vs. QUIN + GA and Control, respectively [$P < 0.05$]; 55% below and 47% below when comparing SAC + QUIN + 3-OHGA vs. QUIN + 3-OHGA and Control, respectively [$P < 0.05$]; 28% below and 19% above when comparing SAC + QUIN + 3-MMA vs. QUIN + MMA and Control, respectively; 72% below and 59% below when comparing SAC + QUIN + 3-PA vs. QUIN + PA and Control, respectively [$P < 0.05$]) (Fig. 2B).

The TBARS formation induced by QUIN + organic acids was decreased by SAC in all cases (50% below and 39% below when comparing SAC + QUIN + GA vs. QUIN + GA and Control, respectively [$P < 0.05$]; 37% below and 15% below when comparing SAC + QUIN + 3-OHGA vs. QUIN + 3-OHGA and Control, respectively [$P < 0.05$]; 45% below and 21% below when comparing SAC + QUIN + 3-MMA vs. QUIN + MMA and Control, respectively [$P < 0.05$]; 21% below and 15% above when comparing SAC + QUIN + 3-PA vs. QUIN + PA and Control, respectively [$P < 0.05$]) (Fig. 2C).

L-NAME exerted differential effects on the toxic endpoints stimulated by QUIN + organic acids

The NOS inhibitor L-NAME slightly improved the QUIN + GA- and QUIN + PA-induced MTT reduction (14% and 20% above, respectively [$P < 0.05$]). More prominent prevention of mitochondrial dysfunction was produced by L-NAME on QUIN + 3-OHGA (55% above [$P < 0.05$]) (Fig. 3A). No effect of L-NAME was found on QUIN + MMA. L-NAME *per se* had no effect on MTT reduction. L-NAME *per se* did not induce any effect on MTT reduction.

Despite that L-NAME did not prevent the levels of ROS formation induced by none of the QUIN + organic acids conditions (Fig. 3B), this agent was able to significantly reduce the levels of DCFH oxidation induced by all toxic metabolites *per se* (data not shown).

Regarding lipid peroxidation (Fig. 3C), L-NAME reduced the oxidative damage induced by QUIN + GA (13% below, [$P < 0.05$]), QUIN + 3-OHGA (18% below [$P < 0.05$]), QUIN + MMA (21% below [$P < 0.05$]), and QUIN + PA (20% below [$P < 0.05$]). Finally, L-NAME *per se* did not induce any effect on TBARS production (data not shown).

Neither CAT, nor CREAT, was able to prevent the mitochondrial dysfunction induced by QUIN + organic acids

The antioxidant and metabolic modulators CAT and CREAT were tested in synaptosomes challenged with the combination of QUIN + organic acids. The only endpoint evaluated was MTT reduction capacity. None

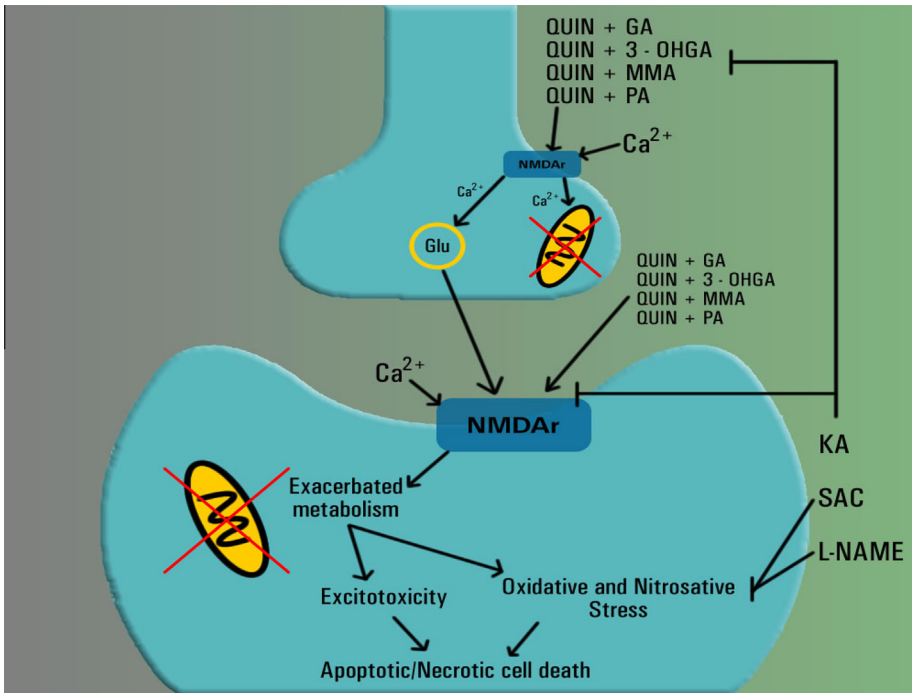

Fig. 4. Schematic representation of the toxic events stimulated by the synergic action of glutaric acid (GA), 3-hydroxyglutaric acid (3-OHGA), methylmalonic acid (MMA) or propionic acid (PA) plus quinolinic acid (QUIN) in the rat brain synaptosomes, and their prevention by agents with different protective profiles like kynurenic acid (KA, endogenous NMDAr antagonist), S-allyl cysteine (SAC, antioxidant and redox modulatory agent) and L-nitro-arginine methyl ester (L-NAME, NOS inhibitor). All toxic metabolites *per se* can stimulate excitotoxic processes, reactive oxygen/nitrogen species (ROS/RNS) formation and mitochondrial respiratory chain disruption, further leading to cell death via apoptotic/necrotic events. When simultaneously present at subtoxic concentrations, metabolic acids and QUIN might potentiate toxic events by synergic actions, as demonstrated herein with synaptosomal fractions. The efficacy that the different protective agents tested exerted on these models suggests that these synergic patterns can be interrupted at different levels, thus revealing the active role of several toxic mechanisms in these models.

of these agents prevented at all the alterations produced by the toxic combinations (data not shown). In light of this lack of effects, no further endpoints were tested for these agents.(see Fig. 4).

DISCUSSION

To date, the precise mechanisms underlying neurodegeneration in GA I remain poorly unknown, despite the many studies available that have been published so far investigating the pathophysiology of this disorder.

Based on a previously proposed hypothesis (Varadkar and Surtees, 2004), our group has recently addressed the issue of a possible active role of the KP in the neuropathology of GA I through the concerted action of two pathological events comprising the *in vivo* knockout of GDD (Gcdh, the key degradation enzyme for GA) followed by an intrastriatal infusion of QUIN in mice (Seminotti et al., 2015). These animals ($Gcdh^{-/-}$) were fed a high lysine diet to stimulate GA production. QUIN exerted an acute synergic action with the stimulated $Gcdh^{-/-}$ condition to decrease energetic markers like creatine kinase activity and the respiratory chain complex IV, while it increased oxidative stress endpoints like oxidative damage to lipids, nitrite/nitrate formation and depleted levels of reduced glutathione in striatal tissue. Despite that this first contribution clearly establishes hard basis to support the hypothesis that the KP (through QUIN) may be involved in the neuronal damage observed in GA I and other acidemias, specific toxic mechanisms underlying neuronal degeneration and occurring at the level of synaptic structures (the nerve communication functional unit) are still needed. Therefore, in the present study we aimed to challenge synaptic terminals with QUIN and different toxic organic acids (GA, 3-OHGA, MMA and PA) accumulating in common OA, and evaluated endpoints of oxidative toxicity and mitochondrial dysfunction to provide more specific information on the role of synergic actions between these molecules as initiators of deleterious events in the brain. The sensitivity of the markers evaluated to different pharmacological agents acting at different mechanistic levels was also assessed to complement the *in vitro* acute toxic paradigms developed.

Our results revealed that there is an acute toxic synergism exerted by QUIN + organic acids in all the three toxic endpoints evaluated herein, but this effect was differentially expressed among the organic metabolite tested: in the case of mitochondrial dysfunction, the order of magnitude was QUIN + 3-OHGA > QUIN + GA = QUIN + PA > QUIN + MMA. This result clearly suggests that the first three models recruit in a more prominent manner mitochondrial alterations than that produced by the combination of QUIN + MMA. In contrast, regarding ROS formation, the order of magnitude for this effect was as follows: QUIN + PA > QUIN + 3-OHGA = QUIN + GA = QUIN + MMA. These tendencies suggest that the toxic model exerted by QUIN + PA is more prompt to oxidative

damage; however, when the biological consequence of ROS formation was estimated (oxidative damage to lipids), the order of magnitude tells another story: QUIN + GA = QUIN + MMA = QUIN + PA = QUIN + 3-OHGA. Therefore, it seems evident that, although all models involved the active formation of precursors for oxidative damage in a differential manner, the levels of oxidative damage in all of them are more or less similar, thus supporting the concept that oxidative stress is a major component of the toxic pattern induced by these synergic models.

We also found a moderate, but still significant prevention of mitochondrial dysfunction induced by KA in all the toxic models tested, which contrasted with a potent reduction of ROS formation for all cases – including the toxins *per se* – reaching values even below the basal levels. Once again, the response represented by ROS formation seems to be disproportional when compared to its biological consequence, lipid peroxidation, which for the case of KA, was modestly but still significantly reduced in all models. Altogether, these results clearly suggest that there is a glutamatergic component involved in the initiation of the toxic events produced in all the synergic models tested, but the precise contribution of this component remains to be characterized in further studies.

While KA was employed in these models to evidence the degree of participation of the glutamatergic component (as above mentioned), SAC was used as a tool to emphasize the oxidative component involved in these models. In contrast to KA, the effect of SAC on the mitochondrial dysfunction induced by QUIN + organic acids was more intense. The order of magnitude of protection by SAC on the toxic models was QUIN + GA = QUIN + 3-OHGA = QUIN + PA > QUIN + MMA. In addition, SAC was a potent inhibitor of ROS formation, not only in all synergic models, but also when tested against the toxins *per se*, as well as in the basal condition. The order of magnitude of protection induced by SAC in the synergic models was QUIN + PA > QUIN + 3-OHGA = QUIN + GA > QUIN + MMA. This effect was confirmed through the action of SAC on the QUIN + organic acids-induced lipid peroxidation, in which SAC not only reduced this marker even below the control levels in all synergic models, but also when tested against the toxins *per se*. The order of magnitude of SAC efficacy among the synergic models was QUIN + GA > QUIN + MMA = QUIN + 3-OHGA > QUIN + PA. The relevance of these findings is laying in the evidence collected that oxidative stress is playing a major role in the alterations evoked by the combined actions of QUIN + all organic acids, as evidenced by the robust inhibitory effects that SAC exerted on all toxic endpoints, which in magnitude were more prominent than those of KA, thus leaving the glutamatergic component as an convergent mechanism to the already-in-progress damage induced by oxidative stress.

Moreover, as part of the oxidative damage in course, another converging subordinated mechanism is nitrosative stress. The characterization of the actions of nitric oxide and other nitrogen species on diverse markers of cell dysfunction and oxidative damage is crucial for the estimation of the degree of participation of nitrosative stress in these models. We made this approach through the use of the NOS inhibitor L-NAME, which produced partial prevention of mitochondrial dysfunction in three of the four synergic models. The order of magnitude of the preventive action of L-NAME among the models was QUIN + 3-OHGA > QUIN + PA > QUIN + GA. Noteworthy, despite L-NAME reduced the levels of ROS/RNS formation induced by all the toxins *per se* even below the control levels, it was unable to reduce this endpoint at all in the synergic models tested. In contrast, L-NAME partially reduced lipid peroxidation in all the synergic models. The order of magnitude of this effect among the models was QUIN + 3-OHGA > QUIN + GA = QUIN + MMA = QUIN + PA. In addition, this agent reduced the oxidative damage to lipids when tested against all organic acids *per se*. Therefore, the effects of L-NAME on these markers and models suggest that, as expected, nitrosative stress is also participating in the acute pattern of toxicity elicited by the synergic condition produced by QUIN and the organic acids, being responsible of part of the mitochondrial dysfunction and oxidative damage to lipids, but not in ROS formation.

QUIN-induced neurodegeneration has been shown to involve the activation of different signaling pathways and transcription factors. So far, the most relevant mechanism recruited by QUIN in the CNS is excitotoxicity, an overstimulation of glutamatergic NMDAr mainly acting on subunits NR2A and NR2B, thus leading to an increased Ca^{2+} influx through and the consequent pathological cascade resulting in neuronal death (Pérez-De La Cruz et al., 2012). Recent data of our group show that QUIN stimulates the mitochondrial dysfunction *in vivo* in GDD knockout ($Gcdh^{-/-}$) mice subjected to a high Lys dietary intake, which is probably occurring because of the additive effect of QUIN, GA and 3-OHGA (Seminotti et al., 2015). Therefore, although the precise mechanisms by which QUIN and the metabolic acids exerted a synergic action in this study remain to be solved in further studies, in our previous work we found support to our present findings since $Gcdh^{-/-}$ mice under a high Lys dietary intake were more susceptible to the effects of QUIN. It can be hypothesized that increased amounts of the accumulating organic acids GA and 3-OHGA may be participating in these effects. A clue to solve this issue appeared recently in a report showing that NMDAr, specifically NR2A and NR2B subunits, are highly expressed in $Gcdh^{-/-}$ animals receiving a high Lys overload (Lagranha et al., 2014). Given that QUIN stimulates these NMDA receptor subunits, as some of the organic metabolites are supposed to do, it can be suggested that the increased stimulation of these receptors by these neurotoxic metabolites in a synergic action may play a crucial role in both mutant mice and synaptosomes.

Of major consideration is the fact that the concept of synergism is not new at all for metabolic acids. Recently, a toxic interaction between azaspiracid (50 nM) – a toxin found in shellfish harvested in Ireland

– and GA (1 mM) was shown to produce a significant inhibition of sodium channels in *in vitro* experiments, while when added separately, these two compounds had no effects on these channels (Chevallier et al., 2015). Moreover, GA was found as a component of the same shellfish used in the study. Furthermore, the synergic action of QUIN and glutamate to induce and exert neurotoxicity has been well documented through the stimulation of glutamate release from the presynaptic terminals and the inhibition of reuptake by astrocytes, both leading to a recruited excitotoxicity (Tavares et al., 2002). It can be then assumed that chemical or biological interactions like those described above can potentiate the toxic features of endogenous toxic agents, thus giving support to our hypothesis that an interaction of QUIN and the organic acids involved in acidurias at the biological, chemical and/or molecular levels may contribute to the understanding of toxic mechanisms occurring in these and other neurological disorders coursing with neurodegeneration. In addition, our study might also have implications for cancer research and could bring an additional dimension of translational relevance since it has been shown that both GA metabolism (Quincozes-Santos et al., 2010; Vissers et al., 2011) and kynurenine metabolism (Sahm et al., 2013; Adams et al., 2014) through events like QUIN uptake by tumor cells (Saito et al., 1993; Müller and Schwarz, 2007), have been demonstrated to be involved partially in tumor growth in gliomas.

CONCLUDING REMARKS

Our present results show that the combination of QUIN plus GA, 3-OHDA PA or MMA impairs mitochondrial function and enhances oxidative damage in rat brain synaptic terminals. In addition, we found that this damage was primarily linked to ROS formation, and to a lesser extent, to RNS formation and acute excitotoxicity. As proposed in a previous study of our group (Seminotti et al., 2015), our present results also support the hypothesis that increased concentrations of QUIN produced after the KP activation occurring during inflammatory and other toxic events, could play a crucial role in the magnification of the striatal degeneration that follows alterations in patients affected by GA I (Varadkar and Surtees, 2004), MMAcidemia and PAacidemia through a synergic action with the accumulating metabolites GA, 3-OHGA, MMA or PA, which may turn the striatum more vulnerable during pathologic episodes commonly occurring in OA. Hence, the identification of new toxic mechanisms recruiting the deleterious actions produced by neurotoxins with different profiles represent an alternative with potential application for the design of more effective therapeutic approaches.

CONFLICT OF INTEREST DECLARATION

The authors declare no conflict of interest.

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