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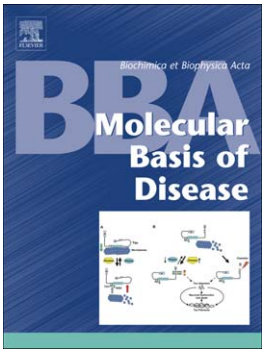

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Evidence that the inhibitory effects of guanidinoacetate on the activities of the respiratory chain, Na^+,K^+-ATPase and creatine kinase can be differentially prevented by taurine and vitamins E and C administration in rat striatum *in vivo*

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Abstract

Guanidinoacetate methyltransferase (GAMT) deficiency is an inherited neurometabolic disorder biochemically characterized by tissue accumulation of guanidinoacetate (GAA) and depletion of creatine. Affected patients present epilepsy and mental retardation whose etiopathogeny is unclear. In a previous study we showed that instrastriatal administration of GAA caused a reduction of Na^+,K^+-ATPase and creatine kinase (CK) activities, as well as an increase in TBARS (an index of lipid peroxidation). In the present study we investigated the *in vitro* and *in vivo* effects of GAA on glucose uptake from [U-^{14}C] acetate (citric acid cycle activity) and on the activities of complexes II, II-III, III and IV of the respiratory chain in striatum of rats. Results showed that 50 and 100 µM GAA (*in vitro* studies) and GAA administration (*in vivo* studies) significantly inhibited complexes II and II-III, respectively, but did not alter complexes III and IV, as well as CO_2 production. We also studied the influence of taurine or vitamins E and C on the inhibitory effects caused by intrastriatal administration of GAA on complexes II and II-III, Na^+,K^+-ATPase and CK activities, and on TBARS in rat striatum. Pre-treatment with taurine and vitamins E and C revealed that taurine prevents the effects of intrastriatal adminitration of GAA on the inhibition of complex II, complex II-III, and Na^+,K^+-ATPase activities. Vitamins E and C prevent the effects of intrastriatal administration of GAA on the inhibition of CK and Na^+,K^+-ATPase activities, and on the increase of TBARS. The data suggest that GAA *in vivo and in vitro* treatment disturbs important parameters of striatum energy metabolism and that oxidative damage may be mediating these effects. It is presumed that defects in striatum bioenergetics might

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be involved in the pathophysiology of striatum damage characteristic of patients with GAMT-deficiency.

Key words: GAMT-deficiency, metabolic disease, guanidinoacetate, respiratory chain, taurine, vitamins E and C.

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1. Introduction

Guanidinoacetate (GAA) is the immediate precursor of creatine, being mainly synthesized in the kidney from arginine and glycine by glycine amidinotransferase. It is then transported by creatine transporters to various tissues, including brain and liver, where it is methylated to creatine by guanidinoacetate methyltransferase (GAMT). GAMT-deficiency is an inherited metabolic disease biochemically characterized by tissue accumulation of GAA and depletion of creatine. Affected patients present neurological symptoms, including muscular hypotonia, involuntary extrapiramidal movements and epilepsy [1]. The diagnosis is based on excessive levels of GAA in body fluids of the affected patients, which is a pathognomonic feature of GAMT-deficiency [1].

Neurological dysfunction is commonly found in human GAMT-deficiency, but the exact mechanisms underlying brain damage in this disease are not well established. However, at the present the neurological symptoms of the affected patients have been mainly attributed to creatine deficiency, although GAA accumulation may play a role in this process. This is in accordance with the findings of Schulze and colleagues [2] showing that GAA is probably epileptogenic in man. In this context, it was demonstrated that GAA alters neurotransmission [3] and decreases membrane fluidity [4]. It should be however emphasized that creatine deprivation is highly neurotoxic *per se*, as evidenced by the serious neurological dysfunction affecting patients with AGAT deficiency and creatine transporter deficiency in which there is no accumulation of GAA [5,6]

Tissues with high energy requirement, such as the brain, are more susceptible to damage under situations of reduced aerobic metabolism [7]. In this context,

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impairment of energy metabolism caused by mitochondrial dysfunction has been implicated in the pathogenesis of a number of neurological conditions, including dementia, cerebral ischemia, Alzheimer's disease and Parkinson's disease [8,9,10,12,14]. To the best of our knowledge, there is little information associating GAA and energy metabolism. Schmidt and colleagues [13] recently showed that the knockout mice for GAMT presented an increase of complex V activity, suggesting that this effect was a compensation mechanism for energy impairment. In addition, recent reports from our laboratory showed that intrastriatal administration of GAA inhibits Na^+,K^+-ATPase and creatine kinase (CK) activities in rat striatum [14].

On the other hand, the central nervous system (CNS) is highly sensitive to oxidative stress due to its high oxygen consumption, its high iron and lipid contents, especially polyunsaturated fatty acids, and the low activity of antioxidant defenses [15]. In this context, we have previously demonstrated that GAA induced lipid peroxidation in the brain [14].

Therefore, in the present study the main objective was to investigate the *in vitro* and *in vivo* effects of GAA on CO_2 production from [U-^{14}C] acetate and on the activities of complexes II, II-III, III and IV of the respiratory chain in rat striatum. Since vitamins E and C and taurine have antioxidant properties [16,17,18] and taurine is neuroprotective [19,20] and has an antiepileptic action in experimental models [21] and in humans [22], we also evaluated the *in vivo* influence of taurine and vitamins E and C on the inhibition of the activities of some complexes of the respiratory chain caused by GAA, as well as of Na^+,K^+-ATPase and CK, which were previously shown to be inhibited by GAA. Striatum was used because patients with GAMT-deficiency may present basal ganglia abnormalities [1].

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2. Material and Methods

2.1. Animals and Reagents

Sixty-day-old Wistar rats were obtained from the Central Animal House of the Department of Biochemistry, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil. The animals were maintained on a 12:12 h light/dark cycle in an air-conditioned constant temperature (22°C ± 1°C) colony room, with food and water *ad libitum*. All reagents used were of analytical grade and purchased from Sigma Co. (St Louis, MO, USA).

2.2. In vivo studies

2.2.1. Guanidinoacetate Administration

For the *in vivo* studies, surgery and intrastriatal infusion were performed as described in Zugno et al. [14]. Sixty day-old rats were anesthetized with an intraperitoneal injection of ketamine plus xylazine (100 mg/ kg and 14 mg/ kg, respectively). The head of the animals was fixed in a stereotaxic apparatus, the skin of the skull was removed and a 27-gauge 9-mm guide cannula was then placed above the striatum (AP: -0.5mm; L: -2.5mm; DV: -2.5mm) [23]. The cannula was fixed with jeweler acrylic cement. Experiments were performed 48 h after surgery. A 30-gauge cannula was fitted into the guide cannula and connected by polyethylene tube to a 5 µL Hamilton microsyringe. The tip of the infusion cannula protruded 1.0 mm beyond the guide cannula towards the striatum. The animals were divided into two groups: group 1 (control group), rats that received saline solution; group 2 (GAA-treated), rats that received 10 µM of GAA solution (0.02 nmol/striatum). Two µL of saline or GAA solution was administered intrastriatally during 2 min. The needle was

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left in place for another 1 min before being softly removed, so that the total procedure lasted 3 min. The correct position of the needle was tested by injecting 0.5 µL of methylene blue (4% in saline solution). The animals were killed by decapitation without anesthesia 30 min after injection. The experimental protocol was approved by the Ethics Committee for animal research of the Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil and followed the NIH *Guide for the Care and Use of Laboratory Animals* (NIH publication 85-23, revised 1985). All efforts were made to minimize the number of animals used and their suffering.

2.2.2. Taurine and vitamins E and C administration

In some experiments the animals were pretreated during 7 days with daily intraperitoneal administration of a-tocopherol (40 mg/kg) and ascorbic acid (100 mg/kg) or taurine (50 mg/kg). Controls animals received saline. Doses of vitamins E and C and taurine were chosen according to Wyse et al. [24] and Sener [18], respectively. Animals were sacrificed by decapitation without anesthesia 30 minutes after the intrastriatal injection of GAA.

2.2.3. Tissue Preparation

The brain was rapidly excised on a Petri dish placed on ice and the striatum was dissected, weighed and kept chilled until homogenization with a ground glass type Potter-Elvehjem homogenizer in the specific buffer used for each technique.

For the determination of the electron transfer chain complexes and CK activities, the striatum was homogenized in 20 volumes of SETH buffer, pH 7.4 (250 mM sucrose, 2 mM EDTA, 10 mM Trizma base, and 50 IU/mL heparin). The homogenates were centrifuged at 800 X g for 10 min at 4ºC. The pellet was

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discarded and the supernatants, a suspension of mixed and preserved organelles, including mitochondria, kept at -70°C until determination of the enzyme activities. The maximal period between supernatant preparation and enzyme analysis was always less than 7 days.

For Na^+,K^+-ATPase activity measurement, synaptic plasma membranes were prepared according to the method of Jones and Matus [25], with some modifications [26]. The homogenate was centrifuged at 1,000 x g for 20 min and the supernatant removed and centrifuged at 12,000 x g for a further 20 min. The pellet was then resuspended in hypotonic buffer (5.0 mM Tris-HCl buffer, pH 8.1), incubated at 0 ^{0}C for 30 min, and applied on a discontinuous sucrose density gradient consisting of successive layers of 0.3, 0.8 and 1.0 M. After centrifugation at 69,000 x *g* for 2 h, the fraction at the 0.8–1.0 M sucrose interface was taken as the membrane enzyme preparation.

For the $^{14}CO_2$ production assay, the striatum was homogenized (1:10, w/v) in Krebs-Ringer bicarbonate buffer pH 7.4 using an ice-chilled glass homogenizing vessel at 900 rpm. Total homogenates were used in these experiments.

2.3. In vitro studies

For the *in vitro* studies, striatum from 60-day-old non-treated rats was dissected and homogenized in a similar manner as that for the *in vivo* experiments to obtain supernatants. The supernatants were then incubated in the presence of 10, 30, 50 or 100 µM GAA.

2.4.Determination of the Respiratory Chain Complexes Activities

Mitochondrial respiratory chain enzyme activities (complexes II, III, II-III and

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IV) were measured in striatum supernatants 30 minutes after GAA or NaCl intrastriatal injection. The protein concentration in the supernatants varied from 1.5-4.0 mg protein/mL in all experiments. The activities of succinate: DCIP-oxidoreductase (complex II) and succinate: cytochrome c oxidoreductase (complex II-III) were determined according to the method of Fischer *et al* [27]. The activity of ubiquinol: cytochrome *c* oxidoreductase (complex III) was assayed according to the method described by Birch-Machin *et al*. [28] and that of cytochrome c oxidase (complex IV) according to Rustin *et al*. [29]. The methods described to measure these activities were slightly modified, as described in details in previous reports [30,31]. The activities of the respiratory chain complexes were expressed as nmol/min mg protein.

For the *in vitro* studies, 10, 30, 50 or 100 µM GAA was supplemented to the incubation medium, whereas the control group did not contain the metabolite.

2.5. Creatine Kinase (CK) activity assay

CK activity was measured in striatum supernatants 30 minutes after GAA or NaCl intrastriatal injection in a reaction mixture consisting of 60 mM Tris-HCl, pH 7.5, containing 7 mM phosphocreatine, 9 mM $MgSO_4$, 0.625 mM lauryl maltoside and approximately 0.4-1.2 µg protein in a final volume of 100 µL. For the *in vitro* studies, 10, 30, 50 or 100 µM GAA was supplemented to the incubation medium, whereas the control group did not contain the metabolite. After 10 min of pre-incubation at 37ºC, the reaction was started by the addition of 0.3 µmol ADP. The reaction was stopped after 10 min by addition of 1 µmol of *p*-hydroxymercuribenzoic acid. The creatine formed was estimated according to the colorimetric method of Hughes [32] with slight modifications as described previously [33]. The color was developed by

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the addition of 100 µL 2% α-naphtol and 100 µL 0.05% diacetyl in a final volume of 1 mL and read spectrophotometrically at 540 nm after 20 min. Results were expressed as µmol creatine/min/mg protein.

2.6. Na^+,K^+-ATPase activity assay

The reaction mixture for Na^+, K^+-ATPase assay contained 5.0 mM $MgCl_2$, 80.0 mM NaCl, 20.0 mM KCl and 40.0 mM Tris-HCl, pH 7.4, in a final volume of 200 µL. The reaction was initiated by addition of ATP to a final concentration of 3.0 mM. Controls were carried out under the same conditions with the addition of 1.0 mM ouabain. Na^+, K^+-ATPase activity was calculated by the difference between the two assays according to the method of Wyse and colleagues [34]. Released inorganic phosphate (Pi) was measured by the method of Chan et al. [35]. Specific activity of the enzyme was expressed as nmol Pi released per min per mg of protein.

2.7. $^{14}CO_2$ production from [1-^{14}C]acetate

For the experiments designed to evaluate $^{14}CO_2$ production, the animals were sacrificed 30 minutes after intrastriatal GAA or NaCl injection. The striatum homogenates containing 1.5-2 mg protein were added to small flasks (11 cm^3). Flasks were pre-incubated in a metabolic shaker at 37°C for 15 min. After pre-incubation, 0.1 µCi [1-^{14}C]-acetate and 1.0 mM of unlabeled acetate were added to the incubation medium. The flasks were gassed with a O_2:CO_2 (95:5) mixture and sealed with rubber stoppers and Parafilm M. Glass center wells containing a folded 65 mm/5 mm piece of Whatman 3 filter paper were hung from the stoppers. After 60 min of incubation at 37°C, 0.1 mL of 50% trichloroacetic acid was added to the

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medium and 0.1 mL of benzethonium hydroxide was added to the center wells with a needle introduced through the rubber stopper. The flasks were left to stand for 30 min to complete $^{14}CO_2$ trapping and then opened. The filter papers were removed and added to vials containing scintillation fluid, and radioactivity was measured [36]. Results correspond to pmol $^{14}CO_2$/h/mg protein and were expressed as percentage of controls.

2.8. Protein determination

Protein content was measured by the method of Lowry *et al* [37], using bovine serum albumin as standard.

2.9. Statistical analysis

Data were analyzed by the Student's t-test or by one-way analysis of variance (ANOVA) followed by the Duncan multiple range test when the F-test was significant. Pearson linear regression coupled to ANOVA was also used to verify dose-dependent effects. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software. A value of $p<0.05$ was considered to be significant.

3. Results

First, we tested the effect of intrastriatal administration of GAA on CO_2 production from acetate (citric acid cycle activity) and on complexes II, II-III, III and IV activities of the respiratory chain in striatum of 60-day-old rats. Figure 1 shows that GAA significantly inhibited (20-25%) complex II [$t(8)=6.74$, $p<0.01$] (A) and complex II-III [$t(8)=3.22$, $p<0.01$] (B), but did not alter complexes III (C) and IV activities (D),

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neither CO_2 production (E). We also investigated the *in vitro* effects of GAA on the same parameters studied *in vivo*. Figure 2 shows that 50-100 µM of GAA significantly inhibited complex II (around 25%) [$F(4,20)=4.73$, $p<0.01$] (A), but did not alter complexes II-III (B), III (C) and IV activities (D), neither CO_2 production (E).

A previous work from our group has shown that GAA inhibits Na^+,K^+-ATPase and CK activities in rat striatum [14]. Therefore, since GAA induces oxidative stress [38, 39], we also examined the influence of taurine and vitamins E and C, which have antioxidant properties, on the inhibitory effects caused by intrastriatal administration of GAA on complexes II and complex II-III activities, as well as on Na^+,K^+-ATPase and CK activities. Results showed that taurine and vitamins E and C administration did not alter *per se* the parameters studied, but only taurine prevented the inhibition of the activities of complexes II [$F(5,36)= 3.98$, $p<0.01$] (A) and II-III [$F(5,30)=6.04$, $p<0.01$] (B) caused by GAA (Figure 3). Furthermore, taurine and vitamins E plus C prevented the inhibitory effects caused by GAA on Na^+,K^+-ATPase activity [$F(5,24)=7.30$, $p<0.01$] (Figure 4A), whereas vitamins E plus C prevented the GAA-induced inhibition on CK [$F(5,30)=3.97$, $p<0.01$] (Figure 4B) activity.

Next, we evaluated the effect of taurine or vitamins E and C administration on GAA-induced *in vivo* increase of the thiobarbituric acid reactive substances (TBARS) measurement, an index of lipid peroxidation in rat striatum. Figure 5 shows that GAA significantly increased TBARS and vitamins E and C, but not taurine, prevented this effect [$F(5,22)=7.821$, $p<0.001$].

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4. Discussion

GAMT-deficiency is an inherited metabolic disease biochemically characterized by tissue accumulation of GAA. Affected patients present neurological damage whose underlying mechanisms are poorly known [1]. In a previous work we demonstrated that GAA inhibits Na^+,K^+-ATPase and CK activities in rat striatum. In the present study we investigated the *in vivo and in vitro* effects of GAA on some parameters of energy metabolism, including CO_2 production from [U-^{14}C] acetate (citric acid cycle activity) and the activities of complexes II, II-III, III and IV of the respiratory chain in rat striatum. We verified that intrastriatal administration of GAA significantly reduced complexes II and II-III and did not affect complexes III and IV. We also observed that complex II, but not the other complexes analyzed, was inhibited when exposed to 50 and 100 µM of GAA *in vitro.* These results indicate that GAA is a selective inhibitor of complex II of the respiratory chain. Furthermore, CO_2 production was also not affected *in vivo and in vitro* by GAA, indicating that the activity of the citric acid cycle was not disturbed by this organic acid.

Considering that GAA is a compound that induces oxidative stress [20,39] and that complex II, Na^+,K^+-ATPase and CK activities are vulnerable to free radical attack [40,41,42], it is possible that the reduction of the enzymatic activities caused by GAA administration, as demonstrated in a previous work for Na^+,K^+-ATPase and CK [14] and in the present study for complex II, could occur through oxidative damage. To test this hypothesis, we pretreated the animals during a week with taurine or vitamins E and C and injected GAA afterwards. Taurine was previously demonstrated to possess antioxidant activities [18,19] and to be a neuroprotector [43]. In this context, we have recently demonstrated that taurine prevents the inhibition of Na^+,K^+-ATPase

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activity caused by GAA *in vitro* [20]. Furthermore, vitamins E and C are classic antioxidants [18]. Vitamin E is a lipid-soluble vitamin that interacts with cell membranes, traps free radicals and interrupts the oxidative chain that damages cells [44, 45], preventing the uncontrolled propagation of lipid peroxidation by free radicals [45]. The resultant tocopheroxil radical requires ascorbate (vitamin C) for its regeneration back to reduced tocopherol [46,47].

Our results showed that taurine prevented the inhibitory effect caused by intrastriatal administration of GAA on the activities of complexes II and II-III. Furthermore, the inhibition of Na^+,K^+ATPase activity caused by intrastriatal administration of GAA was prevented by both taurine and vitamins E and C. Finally, we found that the inhibition of the activity of CK as well as the increase of TBARS caused by GAA was prevented by pre-treatment with vitamins E and C, but not by taurine. The exact mechanisms underlying taurine effects are not clearly understood. However, strong evidences show that taurine acts as a reactive oxygen species and nitrogen radical scavenger [48] and also as a membrane stabilizer [49]. Vitamins E and C are known to prevent the propagation of lipid peroxidation by free radicals [45].

Taken together, the present data indicate that disturbances of bioenergetics and oxidative stress are interrelated mechanisms underlying the neurological dysfunction characteristic of GAMT-deficient patients. However, it should be emphasized that since we did not measure brain GAA concentrations after GAA administration, we cannot establish precisely whether our present results of altered oxidative metabolism are of relevance for the GAMT deficient patients.

In conclusion, we demonstrated here that the respiratory chain activity is compromised in vivo and in vitro by GAA probably via oxidative attack. Considering previous reports demonstrating that other important enzyme activities of the CNS, i.e.

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Na^+,K^+-ATPase and CK, were also inhibited by GAA, it is conceivable that brain accumulation of GAA may disturb CNS functioning. However, it should be emphasized that creatine deficiency is neurotoxic *per se*, since patients with AGAT deficiency and creatine transporter deficiency in which there is no accumulation of GAA present serious neurological dysfunction [5,6]**.** The present findings may explain, at least in part the neurotoxicity caused by GAA in patients affected by GAMT-deficiency. Our results also suggest that the administration of antioxidants should be considered as an adjuvant therapy to specific diets or to other pharmacological interventions. However, more studies must be conducted before administration of antioxidants is given to GAMT-deficient patients.

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Figure 1. Effect of intrastriatal administration of guanidinoacetate on complex II (A), complex II-III (B), complex III (C) and complex IV (D) activities and on CO_2 production (E) in striatum from 60-day-old rats. Values are mean ± standard deviation for five to six independent experiments per group. *Different from control, p<0.01 (Duncan´s multiple range test).

Figure 2. Effect of guanidinoacetate addition on the assays of complex II (A), complex II-III (B), complex III (C) and complex IV (D) activities and CO_2 production (E) in striatum from 60-day-old rats. Values are mean ± standard deviation for five to six independent experiments per group. *Different from control, p<0.01 (Duncan´s multiple range test).

Figure 3. Effect of pretreatment with taurine and vitamins E and C on the inhibition caused by intrastriatal administration of guanidinoacetate on complex II (A) and complex II-III (B) activities in striatum from 60-day-old rats. Values are mean ± standard deviation for five to seven independent experiments per group. *Different from control, p<0.01 (Duncan´s multiple range test). GAA=guanidinoacetate; Tau=taurine; Vits=vitamins E and C; GAA + Tau= guanidinoacetate plus Taurine; GAA + Vits= guanidinoacetate plus vitamins E and C.

Figure 4. Effect of pretreatment of administration with taurine and vitamins E and C on the inhibition caused by intrastriatal administration of guanidinoacetate on Na^+,K^+-

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ATPase (A) and creatine kinase (B) activities in striatum from 60-day-old rats. Values are mean ± standard deviation for five to seven independent experiments per group. *Different from control, p<0.01 (Duncan´s multiple range test). GAA=guanidinoacetate; Tau=taurine; Vits=vitamins E and C; GAA + Tau= guanidinoacetate plus Taurine; GAA + Vits= guanidinoacetate plus vitamins E and C.

Figure 5. Effect of pretreatment with administration of taurine and vitamins E and C on the increase of TBARS caused by intrastriatal administration of guanidinoacetate in striatum from 60-day-old rats. Values are mean ± standard deviation for four to six independent experiments per group. *Different from control, p<0.01 (Duncan´s multiple range test). GAA=guanidinoacetate; Tau=taurine; Vits=vitamins E and C; GAA + Tau= guanidinoacetate plus Taurine; GAA + Vits= guanidinoacetate plus vitamins E and C.

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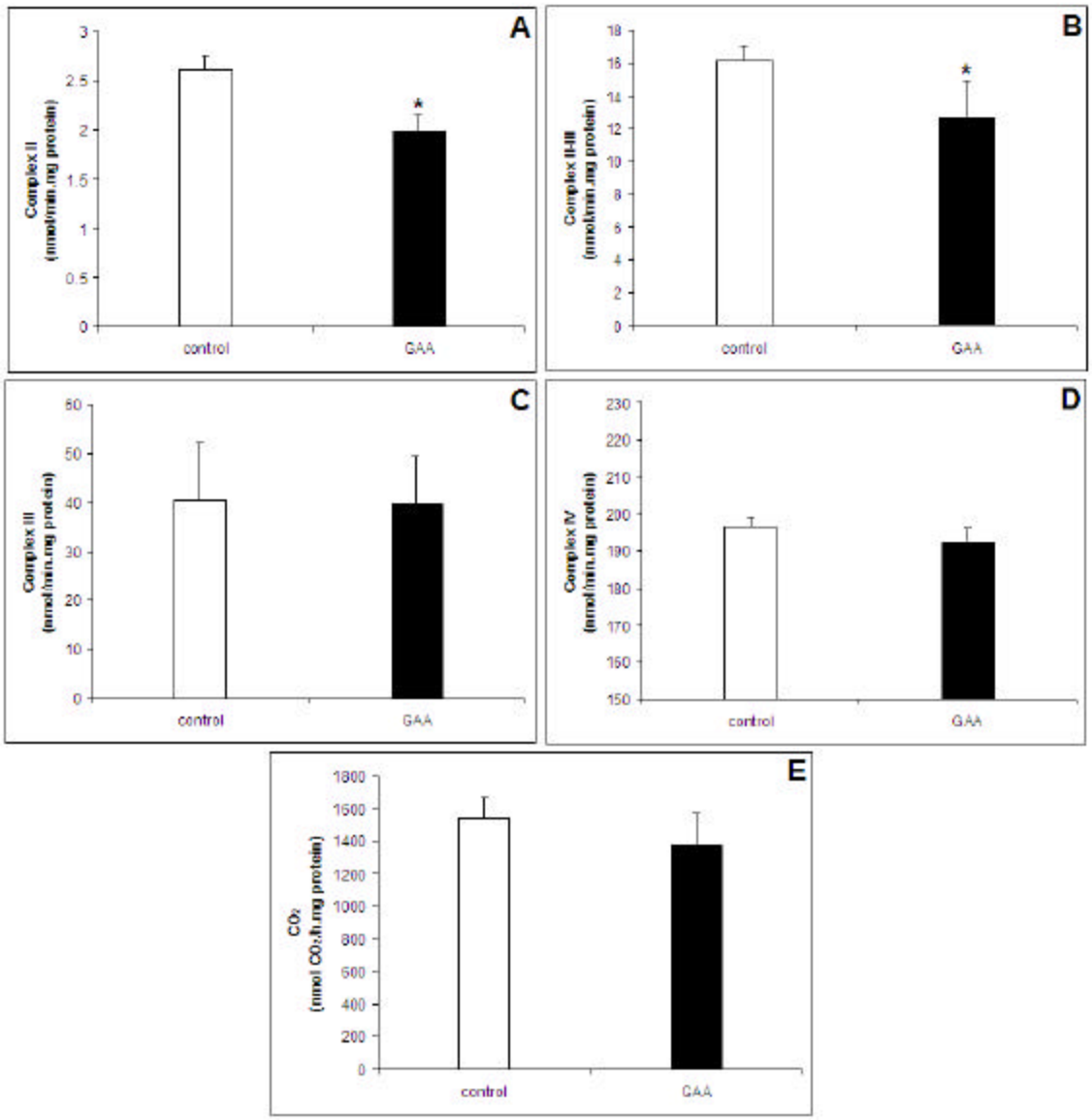

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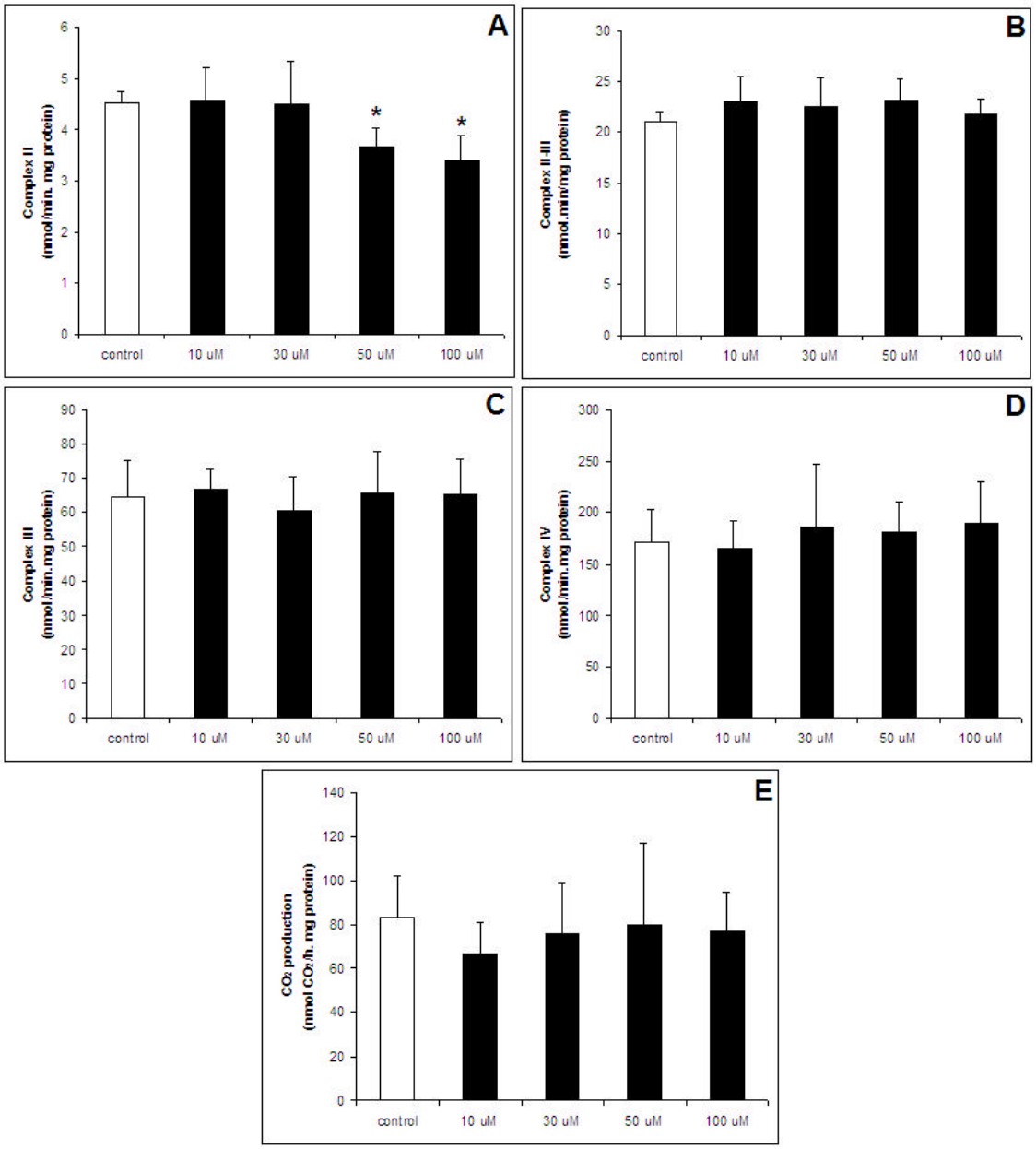

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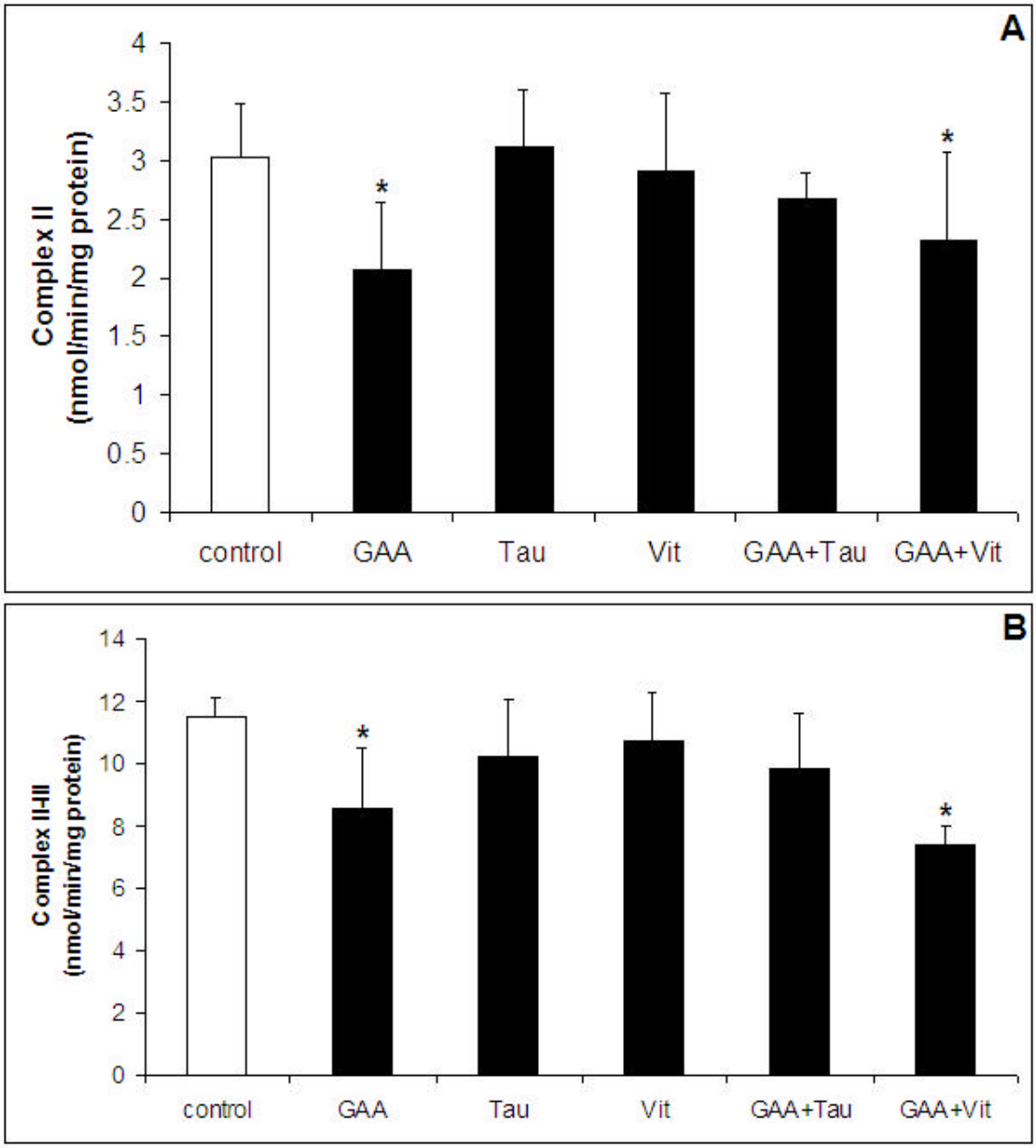

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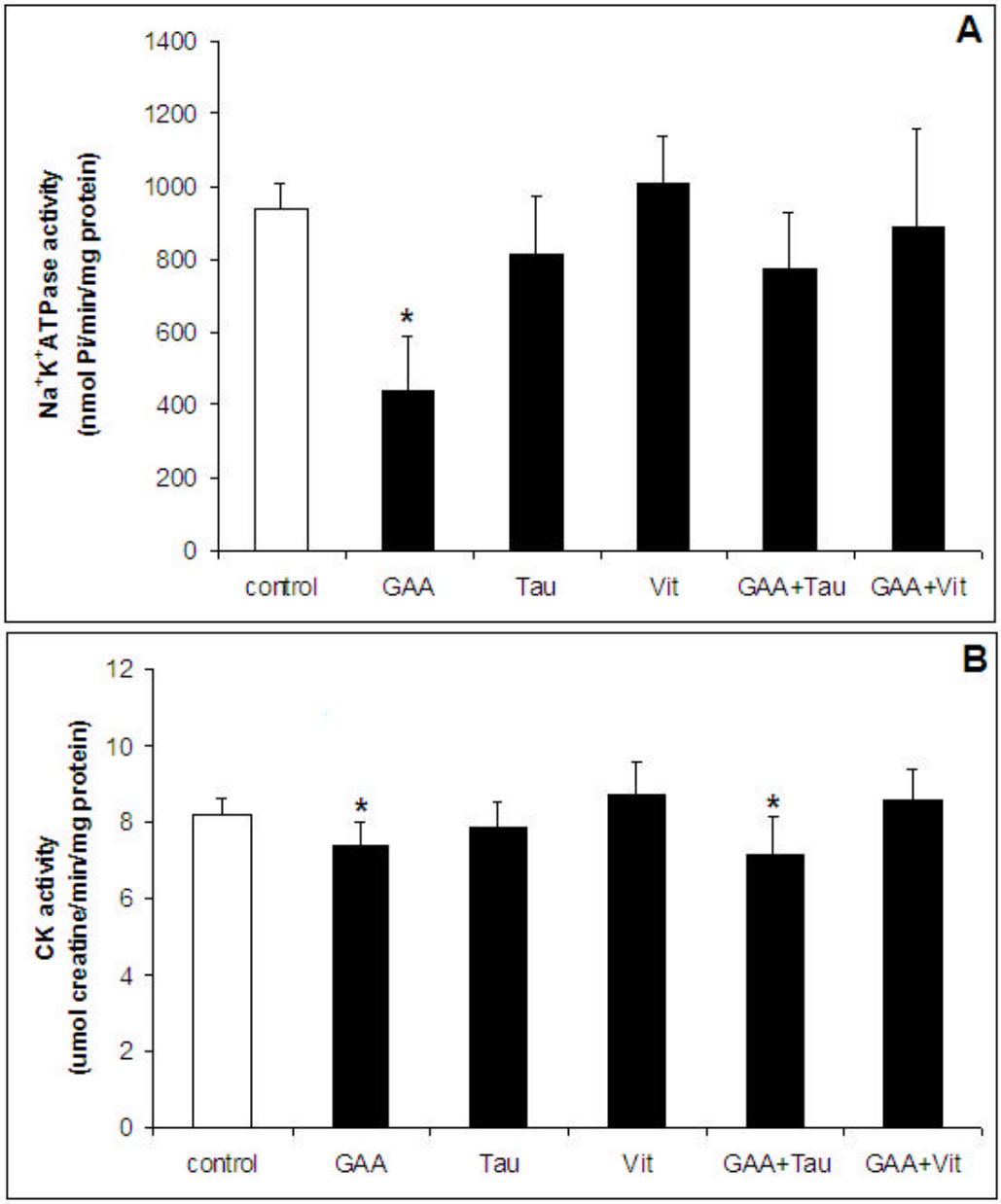

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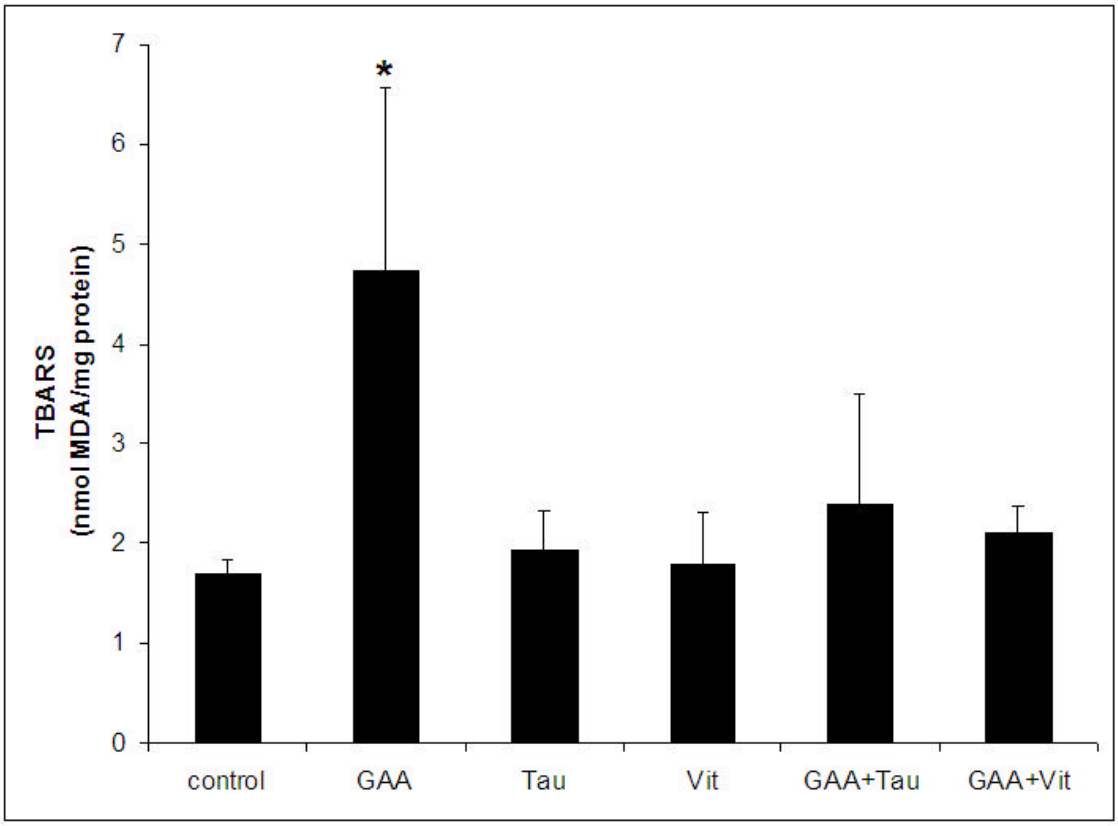

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