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In vitro evidence for an antioxidant role of 3-hydroxykynurenine and 3-hydroxyanthranilic acid in the brain

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Abstract

We investigated the *in vitro* effect of 3-hydroxykynurenine (3HKyn), 3-hydroxyanthranilic acid (3HAA), kynurenine (Kyn) and anthranilic acid (AA) on various parameters of oxidative stress in rat cerebral cortex and in cultured C6 glioma cells. It was demonstrated that 3HKyn and 3HAA significantly reduced the thiobarbituric acid-reactive substances (TBA-RS) and chemiluminescence measurements in rat cerebral cortex, indicating that these metabolites prevent lipid peroxidation in the brain. In addition, GSH spontaneous oxidation was significantly prevented by 3HAA, but not by the other kynurenines in cerebral cortex. We also verified that 3HKyn and 3HAA significantly decreased the peroxyl radicals induced by the thermolysis of 2,2′-azo-bis-(2-amidinopropane)-derived peroxyl radicals, and to a higher degree than the classical peroxyl scavenger trolox. 2-Deoxy-D-ribose degradation was also significantly prevented by 3HKyn, implying that this metabolite was able to scavenge hydroxyl radicals. Furthermore, the total antioxidant reactivity of C6 glioma cells was significantly increased when these cells were exposed from 1 to 48 h to 3HKyn, being the effect more prominent at shorter incubation times. TBA-RS values in C6 cells were significantly reduced by 3HKyn when exposed from 1 to 6 h with this kynurenine. However, C6 cell morphology was not altered by 3HKyn. Finally, we tested whether 3HKyn could prevent the increased free radical production induced by glutaric acid (GA), the major metabolite accumulating in glutaric acidemia type I, by evaluating the isolated and combined effects of these compounds on TBA-RS levels and 2′,7′-dihydrodichlorofluorescein (DCFH) oxidation in rat brain. GA provoked a significant increase of TBA-RS values and of DCFH oxidation, effects that were attenuated and fully prevented, respectively, by 3HKyn. The results strongly indicate that 3HKyn and 3HAA behave as antioxidants in cerebral cortex and C6 glioma cells from rats.

Keywords: 3-Hydroxykynurenine; 3-Hydroxyanthranilic acid; Glutaric acid; Oxidative stress; Antioxidant properties

Activation of the kynurenine pathway (Fig. 1) has been postulated to be involved in the pathophysiology of common neurodegenerative disorders, such as Huntington's disease (Reynolds and Pearson, 1989; Pearson and Reynolds, 1992; Stone et al., 2003), Parkinson's disease (Ogawa et al., 1992), Alzheimer's disease (Guillemin and Brew, 2002), as well as in hepatic encephalopathy (Pearson and Reynolds, 1991) and glutaryl-CoA dehydrogenase deficiency (GAI) (Heyes, 1987; Varadkar and Surtees, 2004). In this particular, 3-hydroxykynurenine (3HKyn) and 3-hydroxyanthranilic acid (3HAA) concentrations were found to be increased in some of these disorders. Furthermore, it was demonstrated that 3HKyn and 3HAA cause pronounced cell death with apoptotic features in neuronal cultures (Okuda et al., 1996, 1998). Other investigators found that cortical and striatal neurons were particularly vulnerable to the toxic effects of 3HKyn, being this susceptibility dependent on 3HKyn cellular uptake by the neutral amino acid transporters (Eastman et al., 1992).

The mechanisms of toxicity of these kynurenines are poorly established. However, tryptophan, phenylalanine and leucine cellular uptake has been shown to be inhibited by 3HKyn (Okuda et al., 1996, 1998), and this may provoke an unbalance

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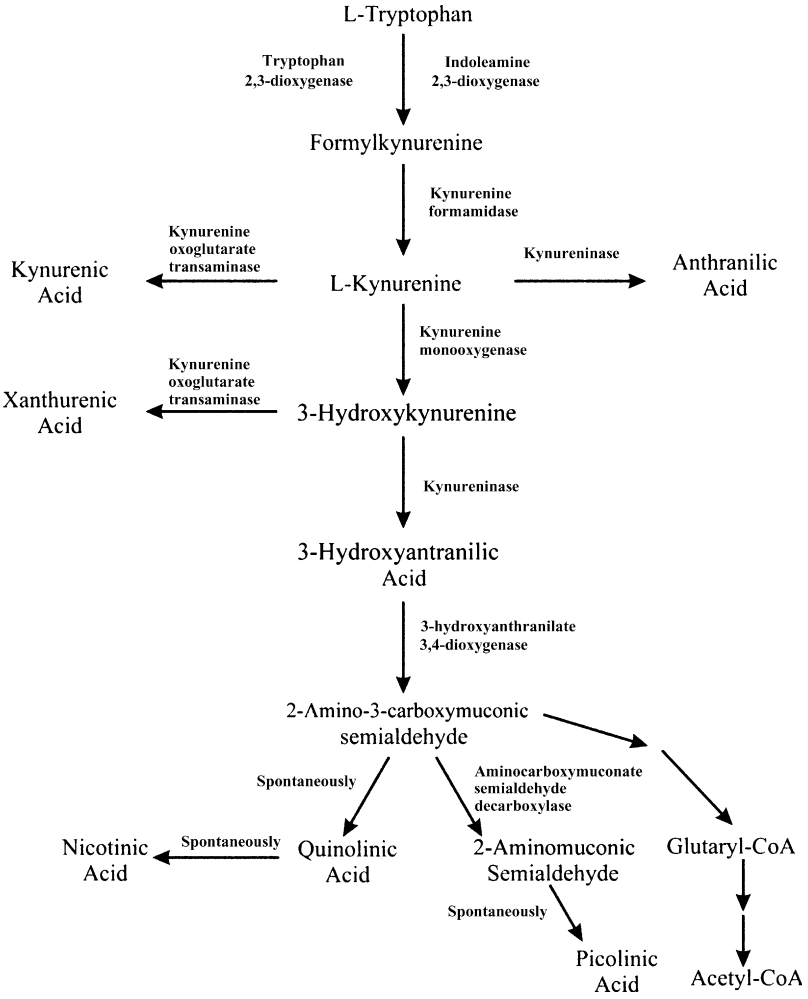

Fig. 1. The kynurenine pathway of tryptophan metabolism.

of the intracellular concentrations of these essential amino acids. Other studies demonstrated that 3HKyn and 3HAA are endogenous uncouplers of the mitochondrial respiration in rat heart mitochondria (Baran et al., 2003). Moreover, it has been shown that 3HKyn and 3HAA, at micromolar concentrations, induce reactive oxygen species generation, mainly hydrogen peroxide and hydroxyl radicals, causing neuronal death in primary striatal cultures, as well as in various neuronal cell lines (Okuda et al., 1996, 1998; Wei et al., 2000; Lee et al., 2001, 2004). 3HKyn and 3HAA was also shown to promote protein oxidative damage, which was dependent on metal ions in purified α-crystallin from calf lens (Goldstein et al., 2000). Finally, it was also shown that the auto-oxidation of 3HKyn and 3HAA gives rise to hydrogen peroxide and their respective organic radicals (Chevion et al., 1982; Ishii et al., 1992; Hiraku et al., 1995). Taken together, these observations indicate that 3HKyn and 3HAA induce oxidative stress particularly in neuronal cells.

In contrast, other reports demonstrated antioxidant properties of 3HKyn and 3HAA but not of kynurenine (Kyn) and anthranilic acid (AA) in the vascular system and in the eye lens (Christen et al., 1990, 1994; Luthra and Balasubramanian, 1992; Thomas et al., 1996). In this particular, 3HKyn and 3HAA were shown to be powerful scavengers of peroxyl radicals, even better than ascorbic acid and trolox (soluble vitamin E) at equimolar concentrations (Christen et al., 1990). It has also been observed that 3HAA acts as a co-antioxidant for the low-density lipoprotein (LDL), protecting it from lipid peroxidation. It was then postulated that 3HAA regenerates α-tocopherol, which is the endogenous antioxidant for LDL, by reducing the α-tocopheroxyl radical (Christen et al., 1994; Thomas et al., 1996).

Therefore, considering the controversial reports on the pro-oxidant and antioxidant properties of 3HKyn and 3HAA, the purpose of the present study was to investigate the effect of 3HKyn, Kyn, 3HAA and AA, at concentrations ranging from 1 to 100 μM, on various oxidative stress parameters in rat cerebral cortex, namely production of thiobarbituric acid-reactive substances (TBA-RS) in the presence or absence of Fe III and Fe II, spontaneous chemiluminescence and glutathione (GSH) levels. The effect of 3HKyn on total antioxidant reactivity (TAR), TBA-RS and cell morphology was also studied in C6 glioma cells. C6 glioma cells were used because they have similar properties to primary glial cell cultures (Mangoura et al., 1989; Vernadakis et al., 1991; Goya et al., 1996; Haghighat and McCandless, 1997; Bonini et al., 2004) and because previous studies investigating the role of these kynurenines on oxidative

stress were mainly carried out with neurons. Furthermore, we investigated whether these kynurenines could scavenge peroxyl and hydroxyl radicals by studying the reduction of the 2,2′-azo-bis-(2-amidinopropane)-derived peroxyl radicals (AAPH) and 2-deoxy-D-ribose degradation, respectively.

It has been recently hypothesized that the kynurenine pathway may be involved in the neuropathology of glutaric acidemia type I (GAI) (Varadkar and Surtees, 2004). Considering that glutaric acid (GA), the major metabolite accumulating in GAI was previously shown to induce oxidative stress *in vitro* (de Oliveira Marques et al., 2003), we also tested the combined effects of 3HKyn and GA on TBA-RS measurement and 2′,7′-dihydrodichlorofluorescein (DCFH) oxidation.

1. Experimental procedures

1.1. Animals and reagents

Male 30-day-old Wistar rats 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 ± 1 °C) colony room, with free access to water and a 20% (w/w) protein commercial chow (Supra, Porto Alegre, RS, Brazil). The "Principles of laboratory animal care" (NIH publication no 80-23, revised 1996) were followed in all experiments and our research protocol was approved by the Ethical Committee for Animal Experimentation of the Universidade Federal do Rio Grande do Sul. All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data. All chemicals were purchased from Sigma (St. Louis, MO, USA), except thiobarbituric acid, which was purchased from Merck (Darmstadt, Germany), Dulbecco's modified Eagle's medium (DMEM) purchased from Gibco BRL (Carlsbad, CA, USA) and fetal bovine serum (FBS) obtained from Cultilab (Campinas, SP, Brazil). 3HKyn, Kyn, 3HAA and AA were prepared on the day of the experiments in 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl.

1.2. Tissue preparation and incubation

On the day of the experiments the rats were sacrificed by decapitation without anesthesia, and the brain was rapidly excised on a Petri dish placed on ice. The olfactory bulbs, pons, medulla, cerebellum and striatum were discarded. 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 using a ground glass type Potter-Elvehjem homogenizer. Homogenates were centrifuged at 750 g for 10 min at 4 °C to discard nuclei and cell debris (Evelson et al., 2001). The pellet was discarded and the supernatant, a suspension of mixed and preserved organelles, including mitochondria, was separated and immediately used for measuring the oxidative stress parameters.

Cerebral cortex supernatants were incubated at 37 °C for 1 h with 3HKyn, Kyn, 3HAA or AA at concentrations ranging from 1 to 100 μM. In some experiments 1 mM GA was added to the supernatants. Controls did not contain any of these metabolites in the incubation medium. Immediately after incubation, aliquots were taken to measure TBA-RS levels, spontaneous chemiluminescence, DCFH oxidation and GSH levels. TBA-RS levels were also measured in the presence of 25 μM Fe III or Fe II.

1.3. Maintenance of C6 cell line

The C6 rat glioma cell line was obtained from American Type Culture Collection (Rockville, Maryland, USA). The cells were grown and maintained in DMEM (pH 7.4) containing 2.5 mg/ml Fungizone® and 100 U/l gentamycin, supplemented with 5% FBS. Cells were kept at 37 °C, a minimum relative humidity of 95%, and in an atmosphere of 5% CO_2/95% air.

1.4. Morphological studies

After cells reached confluence, the culture medium was removed by suction. The cells were incubated for 1, 6, 12, 24 or 48 h at 37 °C in an atmosphere of 5% CO_2/95% air in DMEM (pH 7.4) containing 0% FBS in the absence (controls) or presence of 100 μM 3HKyn. Morphological studies were performed using phase contrast optics.

1.5. Cell homogenate preparation

After incubation (1–48 h) in the absence or presence of 100 μM 3HKyn the culture medium was discarded and cells were rinsed and homogenized in cold 20 mM phosphate buffer containing 140 mM KCl, pH 7.4 using a ground glass type Potter-Elvehjem homogenizer. C6 cell homogenates were immediately used for TAR and TBA-RS measurements.

1.6. Thiobarbituric acid-reactive substances (TBA-RS) measurement

TBA-RS was determined according to the method of Esterbauer and Cheeseman (1990). Briefly, 300 μL of cold 10% trichloroacetic acid were added to 150 μL of brain supernatant and centrifuged at 300 × g for 10 min. Three hundred microlitres of the supernatant were transferred to a Pyrex tube and incubated with 300 μL of 0.67% thiobarbituric acid (TBA) in 7.1% sodium sulphate in boiling water bath for 25 min. The mixture was 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. Each curve point was subjected to the same treatment as supernatants. TBA-RS was calculated as nmol TBA-RS/mg protein.

1.7. Chemiluminescence

Samples were assayed for spontaneous chemiluminescence in a dark room by the method of Gonzalez-Flecha et al. (1991). Incubation flasks with 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 0.5 mL of brain supernatant was immediately added and chemiluminescence was measured for 30 min at room temperature. The background chemiluminescence was subtracted from the total value and the results were calculated as cpm/mg protein and represented as percentage of controls.

1.8. Glutathione levels (GSH) measurement

GSH levels were measured according to Browne and Armstrong (1998). Pre-treated cerebral cortex supernatants were diluted in 20 volumes (1:20, v/v) of 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 and 420 nm, respectively. Calibration standard curve was performed with GSH (0.01–1 mM). GSH concentrations were calculated as nmol/mg protein.

1.9. 2′,7′-dihydrodichlorofluorescein (DCFH) oxidation

Reactive species production was assessed according to LeBel et al. (1992) by using 2′,7′-dihydrodichlorofluorescein diacetate (DCF-DA). DCF-DA prepared in 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl, was incubated with pre-treated cerebral cortex supernatants during 30 min at 37 °C. DCF-DA is enzymatically hydrolyzed by intracellular esterases to form non-fluorescent DCFH, which is then rapidly oxidized to form highly fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of reactive species (RS). The DCF fluorescence intensity parallels to the amount of RS formed. Fluorescence was measured using excitation and emission wavelengths of 480 nm and 535 nm, respectively. Calibration curve was performed with standard DCF (0.25–10 μM) and the levels of RS were calculated as pmol DCF formed/mg protein.

AAPH-peroxyl derived radicals:

$$\text{AAPH} \longrightarrow 2R^{\cdot}$$

$$\text{(A)}\quad R^{\cdot} + O_2 \longrightarrow ROO^{\cdot}\ \text{(peroxyl radical)}$$

Hydroxyl radical formation:

$$\text{Fe III-EDTA} + H_2O_2 \longrightarrow \text{Fe II-EDTA} + O_2^{\cdot -} + H^+$$

$$\text{Fe III-EDTA} + O_2^{\cdot -} \longrightarrow \text{Fe II-EDTA} + O_2$$

$$\text{(B)}\quad \text{Fe II-EDTA} + H_2O_2 \longrightarrow \text{Fe III-EDTA} + OH^- + OH^{\cdot}\ \text{(hydroxyl radical)}$$

Fig. 2. Schematic representation of 2,2′-azo-bis-(2-amidinopropane) (AAPH)-peroxyl derived radicals (A) and hydroxyl radical (B) formation.

1.10. Total antioxidant reactivity (TAR)

TAR, which represents the reactivity or quality of the tissue antioxidants, was determined by measuring the luminol chemiluminescence intensity induced by 2,2′-azo-bis-(2-amidinopropane) (AAPH) according to the method of Lissi et al. (1995). Luminescence was measured after 1 min of addition of 4 mL of 2 mM AAPH (in 0.1 M glycine buffer, pH 8.6) and 10 μL of luminol into a glass scintillation vial (initial luminescence). One hundred microlitres of C6 cell homogenates or 10 μL of 10 to 100 μM trolox (calibration curve), which provokes a decrease of light intensity, were then added and the luminescence was measured after 1 min (final luminescence). The quantity of light quenched by trolox is then compared with the luminescence quenched by the cell homogenate. The ratio between the initial and the final luminescence values was used to calculate TAR measurement. TAR values were expressed as nmol trolox/mg of protein and represented as percentage of control.

1.11. Measurement of the reduction of AAPH-derived radicals

The reactivity of 3HKyn, 3HAA, Kyn and AA towards AAPH derived radicals was determined according to Lissi et al. (1992). The reaction mixture containing 4 mL of 10 mM AAPH in 0.1 M glycine buffer, pH 8.6, and 10 μL of 4 mM luminol generates, at room temperature, a constant light intensity corresponding to peroxyl radicals formation (Fig. 2A). This was considered to be the initial luminescence values. Ten microlitres of trolox (150, 300 or 600 μM), corresponding to the positive control, or the kynurenines 3HKyn, 3HAA, Kyn and AA (100 μM) were added to the reaction medium. Trolox addition provokes a marked reduction of light intensity, which is maintained for a certain period after which light intensity rapidly increases. This lag time is called induction time (IT), which is directly proportional to the antioxidant capacity of the compound. The IT of the kynurenines was compared with the IT of trolox.

1.12. Measurement of 2-deoxy-D-ribose degradation

The hydroxyl radical scavenging activity of the kynurenines was determined by assaying the malondialdehyde chromogen originated from 2-deoxy-D-ribose degradation (Halliwell and Gutteridge, 1981). The reaction medium contained 3 mM 2-deoxy-D-ribose, 20 μM $FeCl_3$, 100 μM EDTA, 500 μM H_2O_2, 100 μM ascorbate and the kynurenines (100 μM) in 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl. Fig. 2B displays hydroxyl radical formation. After 1 h incubation at room temperature, 10% trichloroacetic acid and 0.67% thiobarbituric acid were added, and followed by 25 min incubation in boiling water. The mixture was allowed to cool on running tap water for 5 min. The resulting pink-stained TBA-RS was determined in a spectrophotometer at 532 nm. The results were expressed in units of absorbance.

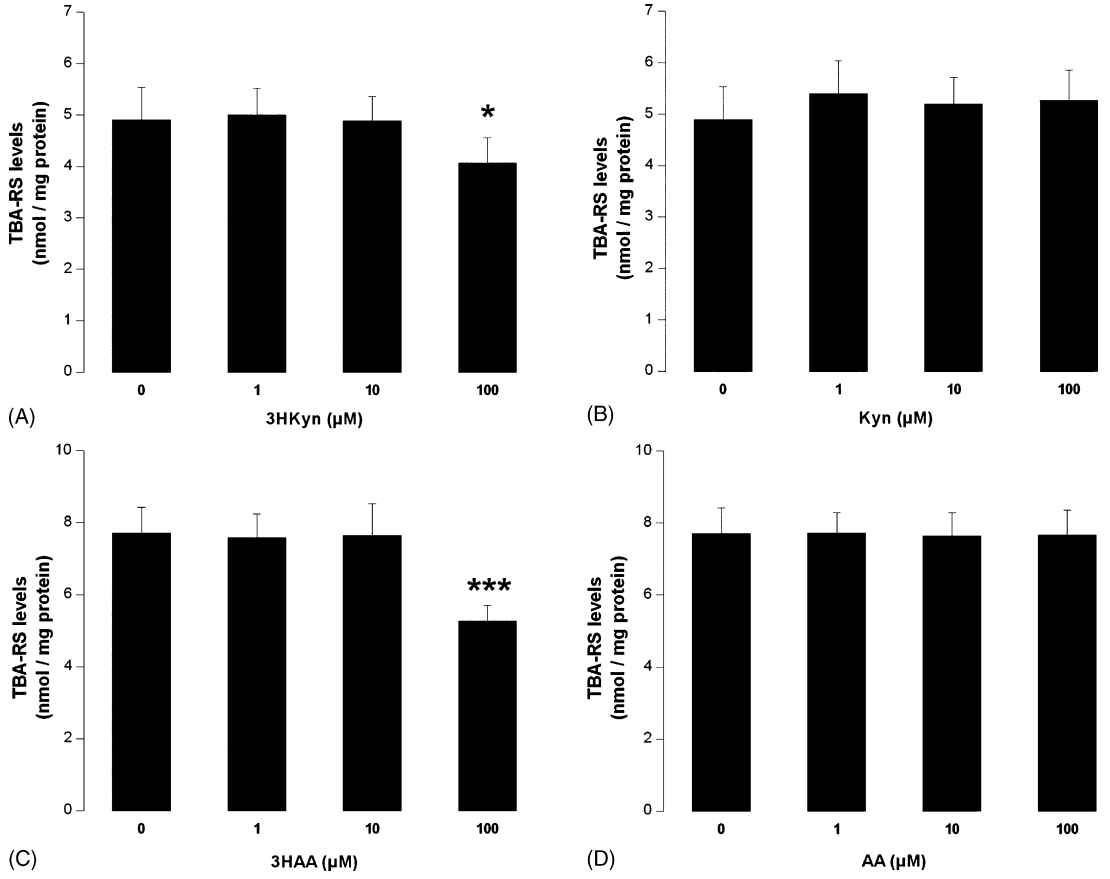

Fig. 3. Effect of 3-hydroxykynurenine (3HKyn) (A), kynurenine (Kyn) (B), 3-hydroxyanthranilic acid (3HAA) (C) and anthranilic acid (AA) (D) on thiobarbituric acid-reactive species (TBA-RS) in cerebral cortex supernatants from 30-day-old rats. Values are means ± S.D. for six independent experiments (animals) performed in triplicate and are expressed as nmol TBA-RS/mg of protein. $^{*}P < 0.05$, $^{***}P < 0.001$, compared to controls (Duncan multiple range test).

1.13. Protein determination

Protein content was determined in cerebral cortex supernatants and C6 cell homogenates according to Lowry et al. (1951) using bovine serum albumin as standard.

1.14. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by the Duncan multiple range test when the F-value was significant. Only significant F-values are given in the text. Linear regression analysis was also used to test dose-dependent effects. The Student's t-test for paired samples was also used to compare two means. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC-compatible computer. A value of $P < 0.05$ was considered to be significant.

2. Results

2.1. Effect of kynurenines on oxidative stress parameters in rat cerebral cortex

Fig. 3A and C shows that 3HKyn and 3HAA, at 100 μM concentration, significantly inhibited TBA-RS measurement in cortical homogenates [3HKyn: $F_{(3,\ 20)} = 4.03$; $P < 0.05$] [3HAA: $F_{(3,\ 20)} = 19.07$; $P < 0.001$], whereas Kyn and AA had no effect on this parameter (Fig. 3B and D). Chemiluminescence was also significantly diminished by 3HKyn in a dose-dependent manner [$\beta = -0.63$; $P < 0.01$] and by 3HAA at 100 μM concentration [3HKyn: $F_{(3,\ 12)} = 7.75$; $P < 0.01$] [3HAA: $F_{(3,\ 12)} = 8.43$; $P < 0.01$] (Fig. 4A and C). In contrast, Kyn and AA did not provoke any effect on chemiluminescence (Fig. 4B and D). The effect of 3HKyn and of 3HAA on TBA-RS formation was also studied in the presence of Fe III that favors the auto-oxidation of these kynurenines and in the presence of Fe II that activates the Fenton reaction. We observed similar inhibitions of TBA-RS levels caused by 3HKyn and 3HAA in the presence of ferric ion [3HKyn: $F_{(6,\ 34)} = 4.36$; $P < 0.01$] [3HAA: $F_{(6,\ 28)} = 10.73$; $P < 0.01$] (Fig. 5A) and ferrous ion [3HKyn: $F_{(6,\ 35)} = 1.67$; $P < 0.05$] [$F_{(6,\ 28)} = 60.84$; $P < 0.001$] (Fig. 5B). It can be also observed in Fig. 5B that 3HAA significantly diminished TBA-RS levels at the concentrations of 10 and 100 μM. In contrast, Kyn and AA did not affect this parameter.

Next we tested whether these kynurenines could alter the concentrations of GSH, the most abundant tissue endogenous antioxidant, in cerebral cortex. *N*-ethylmaleimide (NEM, 500 μM), a strong sulfhydryl groups oxidant, was used as a positive control in these experiments. Table 1 shows that GSH levels were not modified by 3HKyn, Kyn and AA, whereas 3HAA inhibited the spontaneous oxidation of GSH occurring

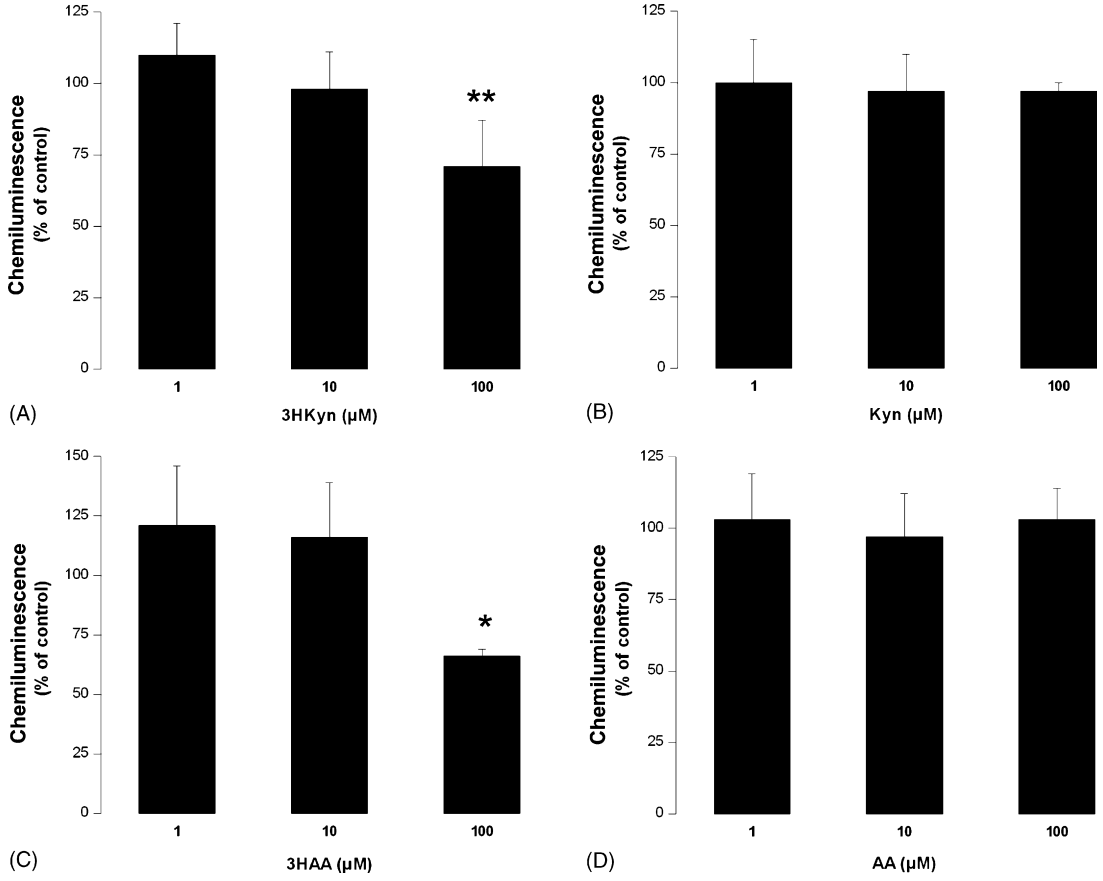

Fig. 4. Effect of 3-hydroxykynurenine (3HKyn) (A), kynurenine (Kyn) (B), 3-hydroxyanthranilic acid (3HAA) (C) and anthranilic acid (AA) (D) on chemiluminescence in cerebral cortex supernatants from 30-day-old rats. Values are means ± S.D. for four to six independent experiments (animals) performed in duplicate and are expressed as percentage of controls (control values: (A) 3336 ± 1423 cpm/mg of protein; (B) 3034 ± 1198 cpm/mg of protein; (C) 3035 ± 273 cpm/mg of protein; (D) 3006 ± 993 cpm/mg of protein). $^{*}P < 0.05$, $^{**}P < 0.01$, compared to controls (Duncan multiple range test).

along the *in vitro* incubation [$F_{(3,\ 26)} = 3.21$; $P < 0.05$]. Furthermore, it can be also seen in the table that NEM markedly reduced GSH levels [$t_{(3)} = 8.42$; $P < 0.01$].

2.2. Effect of 3HKyn on oxidative stress parameters and morphology of cultured C6 glioma cells

C6 glioma cells were exposed to 100 μM 3HKyn during 1–48 h and TAR, TBA-RS levels and cell morphology were evaluated. Fig. 6A shows that 3HKyn induced a significant increase of TAR values (up to 400%) (incubation times: 1 h, $t_{(5)} = 3.65$; $P < 0.01$; 6 h, $t_{(5)} = 4.66$; $P < 0.01$; 12 h, $t_{(5)} = 5.37$; $P < 0.01$; 24 h, $t_{(5)} = 3.65$; $P < 0.01$; 48 h, $t_{(5)} = 4.79$; $P < 0.01$) and that the values gradually decreased as incubation time advanced [$F = 37.01$; $P < 0.001$; $\beta = -0.83$; $P < 0.001$]. On the other hand, Fig. 6B shows that the TBA-RS levels were significantly decreased by 3HKyn when the cells were incubated for 1 and 6 h, but not at longer incubation times (incubation times: 1 h, $t_{(5)} = 4.61$; $P < 0.05$; 6 h, $t_{(5)} = 44.08$; $P < 0.001$). Finally, we verified that C6 cell morphology did not change in the presence of 3HKyn at all incubation times (Fig. 6C).

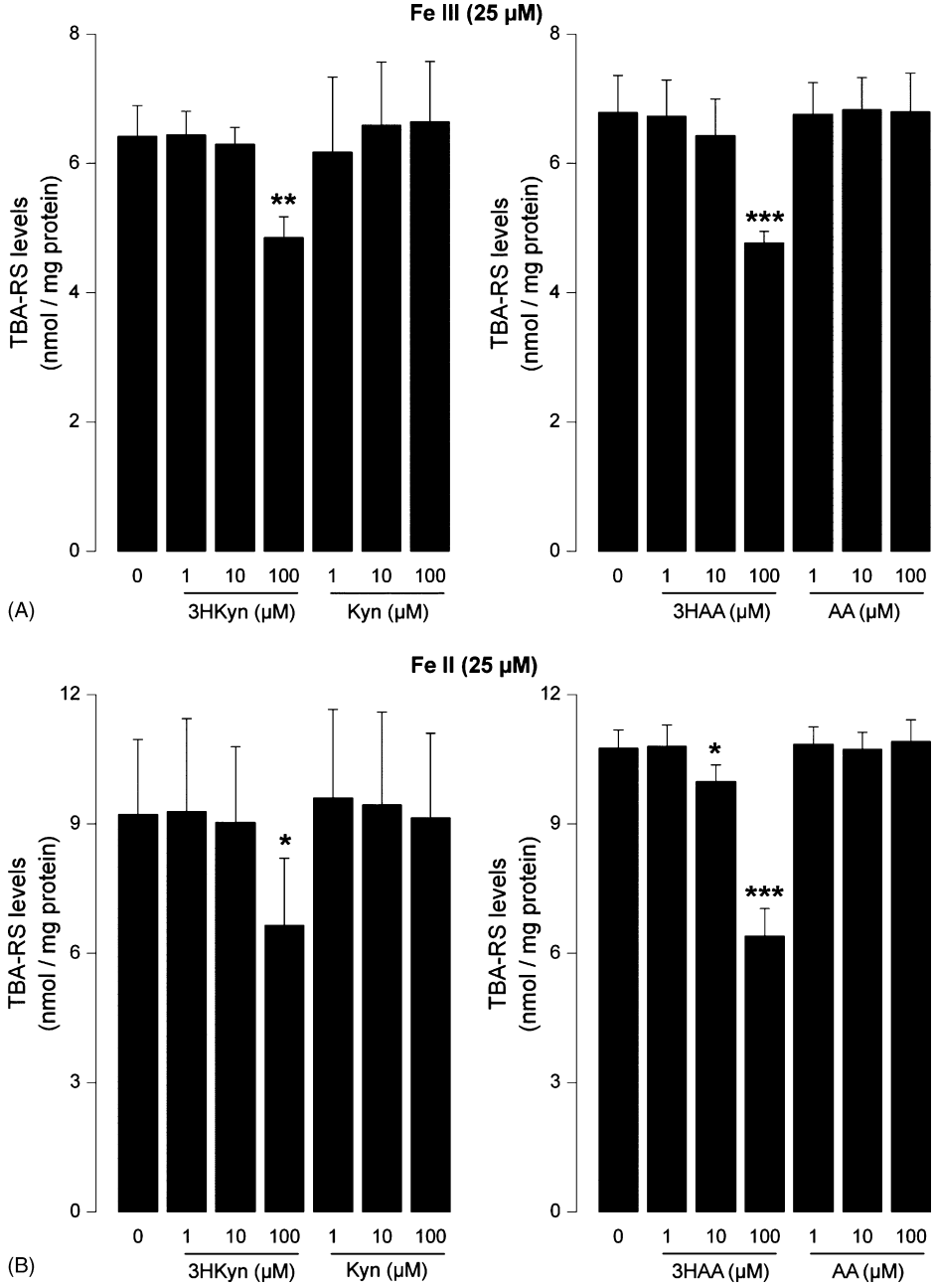

Fig. 5. Effect of 3-hydroxykynurenine (3HKyn), kynurenine (Kyn), 3-hydroxyanthranilic acid (3HAA) and anthranilic acid (AA) on thiobarbituric acid-reactive species (TBA-RS) in the presence of Fe III (A) or Fe II (B) in cerebral cortex supernatants from 30-day-old rats. Values are means ± S.D. for five to six independent experiments (animals) performed in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared to controls (Duncan multiple range test).

Table 1
Effect of 3-hydroxykynurenine (3HKyn), kynurenine (Kyn), 3-hydroxyanthranilic acid (3HAA), anthranilic acid (AA) and *N*-ethylmaleimide (NEM) on glutathione (GSH) levels in cerebral cortex supernatants from 30-day-old rats

Compounds	GSH levels (nmol/mg of protein)				
	0 μM	1 μM	10 μM	100 μM	500 μM
3HKyn	13.4 ± 2.0	12.6 ± 2.3	12.6 ± 2.6	15.5 ± 1.7	–
Kyn	13.4 ± 1.9	13.7 ± 4.2	14.3 ± 4.2	13.1 ± 2.8	–
3HAA	13.9 ± 2.0	13.6 ± 1.6	14.0 ± 1.9	16.1 ± 1.2*	–
AA	20.7 ± 3.6	20.3 ± 4.2	19.4 ± 3.3	22.3 ± 1.8	–
NEM	15.8 ± 3.2	–	–	–	4.5 ± 1.9#

Values are means ± S.D. for four to eight independent experiments (animals) performed in triplicate.
* $P < 0.05$, compared to controls (Duncan multiple range test).
# $P < 0.01$, compared to controls (Student's *t*-test for paired samples).

2.3. Targeting the reactive species scavenged by 3HKyn and 3HAA

In the next set of experiments we searched for the reactive species that could be scavenged by 3HKyn and 3HAA. These experiments were carried out in a system devoid of brain supernatants. The reduction of the AAPH-derived radicals, which reflects peroxyl radical scavenging, was first assayed in the presence of these kynurenines. Fig. 7A shows that 100 μM 3HKyn and 3HAA strongly reduced the peroxyl radicals induced by AAPH thermolysis and to a higher degree as that of trolox, the classical peroxyl scavenger. We also observed that Kyn and AA did not change this parameter, indicating that these compounds did not possess peroxyl scavenging activity (data not shown). Furthermore, we tested the ability of these kynurenines to scavenge hydroxyl radicals by measuring 2-deoxy-D-ribose. Fig. 7B shows that the degradation of 2-deoxy-D-ribose was significantly prevented by all concentrations of 3HKyn (1–100 μM) [$F_{(3,\ 8)} = 7.77$; $P < 0.01$], but not by 3HAA and the non-hydroxylated kynurenines. Furthermore, 3HKyn scavenging capacity was equivalent to that of 500 μM melatonine, a classical hydroxyl radical scavenger (data not shown). Taken together, these results indicate that 3HKyn and 3HAA are able to scavenge peroxyl radicals, whereas only 3HKyn has the ability to scavenge hydroxyl radicals.

2.4. Combined effects of 3HKyn and GA on TBA-RS and DCF oxidation measurements in rat cerebral cortex

We also tested whether the antioxidant properties of 3HKyn could prevent the increase of free radicals induced by GA (de Oliveira Marques et al., 2003), the major metabolite accumulating in the neurometabolic disorder GAI, by studying the isolated and combined effects of these compounds on TBA-RS levels and DCFH oxidation in rat cerebral cortex. We observed that the significant increase of TBA-RS levels induced by 1 mM GA was attenuated by 100 μM 3HKyn [$F_{(3,\ 14)} = 25.39$; $P < 0.001$] (Fig. 8A). Furthermore, 3HKyn fully prevented the significantly GA-induced DCFH oxidation [$F_{(3,\ 12)} = 15.12$; $P < 0.001$] (Fig. 8B).

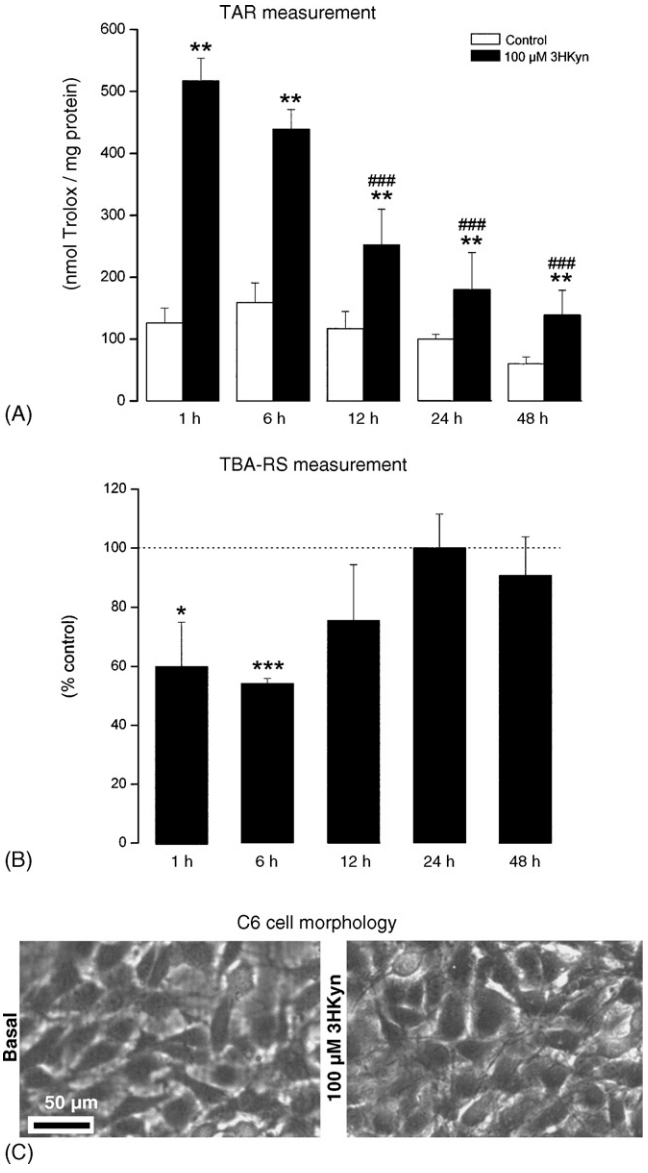

Fig. 6. Effect of 3-hydroxykynurenine (3HKyn) on total antioxidant reactivity (TAR) (A), thiobarbituric acid-reactive species (TBA-RS) (B) and morphology (C) of C6 glioma cells at different exposition times (1–48 h). (A) TAR values are mean ± S.D. for three independent experiments performed in triplicate and expressed as nmol trolox/mg of protein. (B) TBA-RS values are mean ± S.D. for three independent experiments performed in triplicate and expressed as percentage of controls (control values: 1 h, 0.075 ± 0.023 nmol TBA-RS/mg of protein; 6 h, 0.032 ± 0.007 nmol TBA-RS/mg of protein; 12 h, 0.585 ± 0.214 nmol TBA-RS/mg of protein; 24 h, 0.514 ± 0.196 nmol TBA-RS/mg of protein; 48 h, 0.320 ± 0.035 nmol TBA-RS/mg of protein). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, compared to controls; ###$P < 0.001$, compared to 1 h incubation (Student's *t*-test for paired samples and Duncan multiple range test).

3. Discussion

A considerable body of evidence indicates that oxidative damage is involved in the pathophysiology of the common neurodegenerative disorders Parkinson's disease, Huntington's disease and Alzheimer's disease. Accordingly, lipid, protein and DNA oxidative damage, as well as reduced concentrations of GSH and ascorbic acid and decreased activities of the antioxidant

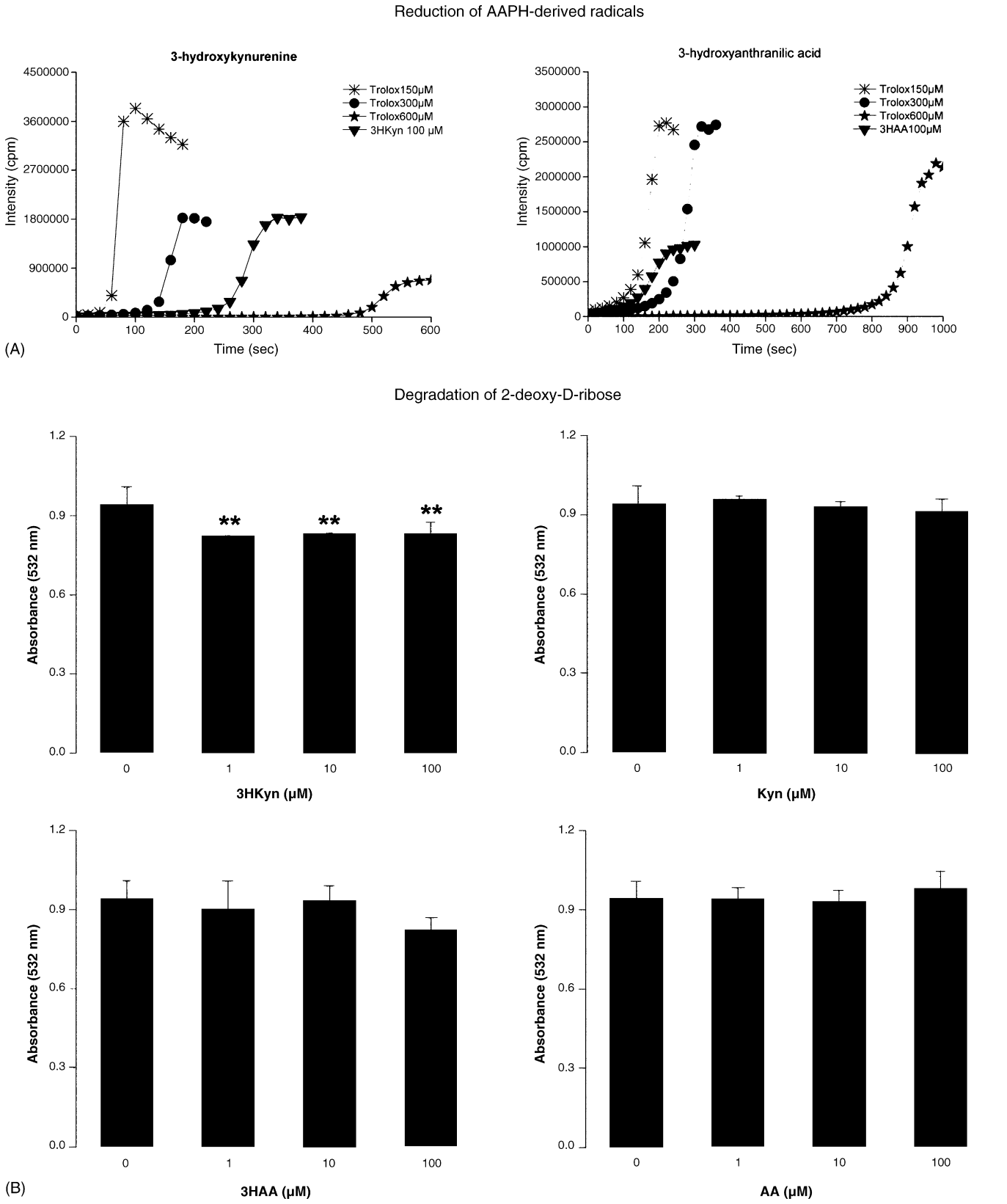

Fig. 7. Effect of kynurenines and trolox on 2,2′-azo-bis-(2-amidinopropane)-derived peroxyl radicals reduction (A) and 2-deoxy-D-ribose oxidation (B) in a system devoid of brain supernatants. Values are mean ± S.D. for three independent experiments performed in triplicate. $^{**}P < 0.01$, compared to controls (Duncan multiple range test). 3HKyn, 3-hydroxykynurenine; Kyn, kynurenine; 3HAA, 3-hydroxyanthranilic acid; AA, anthranilic acid.

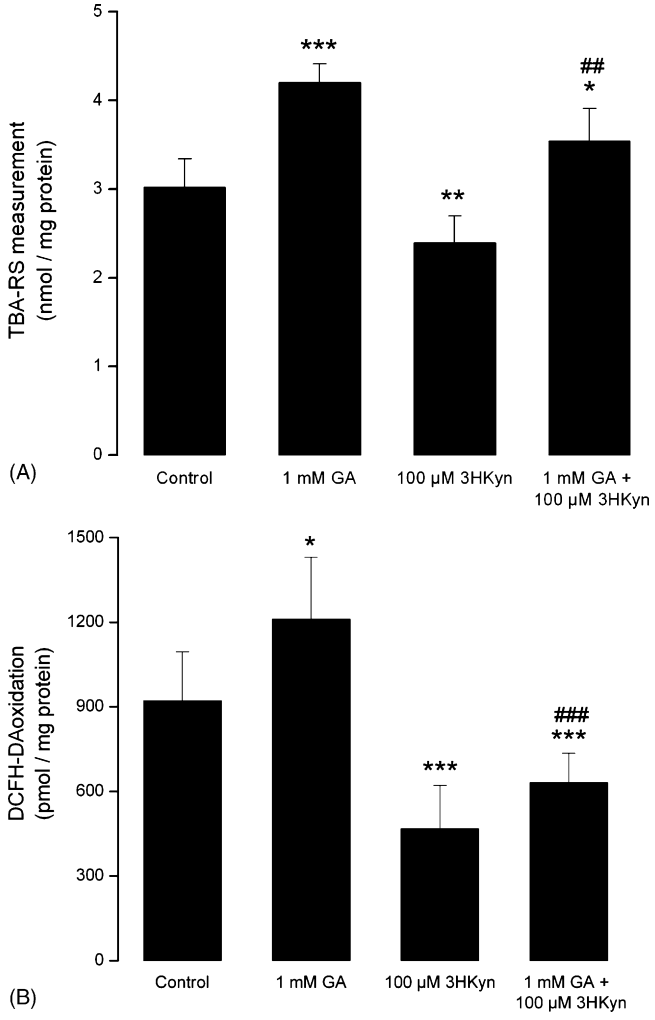

Fig. 8. Effect of 3-hydroxykynurenine (3HKyn; 100 μM) and glutaric acid (GA; 1 mM) alone or combined on thiobarbituric acid-reactive species (TBA-RS) measurement (A) and 2′,7′-dihydrodichlorofluorescein (DCFH) oxidation (B) in cerebral cortex supernatants from 30-day-old rats. Values are mean ± S.D. for five independent experiments (animals) performed in triplicate $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared to controls; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, compared to GA (Duncan multiple range test).

enzymes catalase and glutathione peroxidase have been demonstrated in patients affected by these disorders (Olanow, 1993; Coyle and Puttfarcken, 1993; Perry et al., 2003). Furthermore, it was also demonstrated that the concentrations of some intermediates of the kynurenine pathway, such as 3HKyn, are increased in post-mortem brain of individuals with these neurodegenerative disorders (Pearson and Reynolds, 1991, 1992; Ogawa et al., 1992; Sardar et al., 1995). On the other hand, it has been demonstrated that the *o*-aminophenolic kynurenines, 3HKyn and 3HAA, may lead to generation of reactive oxygen species probably by auto-oxidation (Chevion et al., 1982; Dykens et al., 1987; Ishii et al., 1992; Hiraku et al., 1995; Okuda et al., 1996, 1998; Goldstein et al., 2000). Toxic pro-oxidant effects of 3HKyn and 3HAA were mainly observed in neuronal cell cultures exposed for long periods and high concentrations (100–200 μM) of these compounds (Lee et al., 2001, 2004). However, other reports have demonstrated antioxidant properties for the same compounds (Christen et al., 1990, 1994; Luthra and Balasubramanian, 1992; Thomas et al., 1996). Therefore, because of the controversial findings on whether kynurenines induce oxidative stress or behave as antioxidants, in the present study we evaluated the effect of 3HKyn, Kyn, 3HAA and AA on various parameters of oxidative stress in rat cerebral cortex and in C6 glioma cells.

Initially, we observed that 3HKyn and 3HAA, at 100 μM concentration, significantly diminished chemiluminescence and TBA-RS levels, whereas Kyn and AA did not alter these lipid peroxidation parameters. Considering that light emitted in the chemiluminescence assay usually arises from peroxidizing lipids resulting from an increase in reactive oxygen or nitrogen species production and that TBA-RS reflects the amount of malondialdehyde formation, an end product of membrane fatty acid peroxidation (Halliwell and Gutteridge, 1999), our data indicate that 3HKyn and 3HAA inhibited spontaneous lipid peroxidation in the brain, protecting cerebral cortex against oxidative damage. Moreover, the inhibitory property of 3HKyn and 3HAA on *in vitro* lipid peroxidation remained even in the presence of Fe III or Fe II, which stimulate auto-oxidation of these metabolites and hydroxyl radical formation, respectively. Therefore, it is conceivable that under our conditions 3HKyn and 3HAA auto-oxidation or hydroxyl formation did not occur or was insufficient to overcome the antioxidant properties of these compounds.

We also found that 3HAA, but not 3HKyn, Kyn and AA, prevented the spontaneous oxidation of GSH, which occurs during incubation of cortical homogenates, reinforcing our previous findings showing an antioxidant activity for this compound.

Then, we verified that 100 μM 3HKyn and 3HAA were able to reduce AAPH-derived radicals (peroxyl radicals) in a system devoid of cortical or cell homogenates in a more efficient way than trolox (α-tocopherol). These results are in agreement with those previously published by Christen et al. (1990), showing that 3HKyn and 3HAA, at low micromolar concentrations, are able to scavenge peroxyl radicals, protecting phosphatidylcholine lipossomes and B-phycoerythrin from oxidation. In this particular, 3HKyn and 3HAA antioxidant action protecting B-phycoerythrin from peroxyl-mediated oxidative damage was more effective than that of equimolar amounts of the classical antioxidants ascorbate and trolox. The same authors reported in a further study that 3HAA reduced α-tocopheroxyl radical restoring the levels of α-tocopherol, preventing LDL lipid peroxidation. In contrast, the non-phenolic analogues Kyn and AA did not show these properties (Christen et al., 1994). We also observed in the present study that 3HKyn was able to scavenge hydroxyl radicals, since it reduced 2-deoxy-D-ribose oxidation. Therefore, it is presumed that 3HKyn and 3HAA are powerful peroxyl scavengers and that 3HKyn is also a scavenger of hydroxyl radicals.

Our present data are in apparent disagreement with other reports showing that 3HKyn and 3HAA induces reactive oxygen species generation, particularly hydrogen peroxide and hydroxyl radical, being also cytotoxic to primary neuronal cultures from rat striatum (Okuda et al., 1996, 1998). Similarly,

it was shown that 3HKyn and 3HAA provoke protein oxidative damage and induce apoptosis characterized by chromatin condensation and internucleosomal DNA cleavage in PC12, GT1-7 and SK-N-SH cells (Goldstein et al., 2000; Wei et al., 2000; Lee et al., 2001, 2004). Interestingly, most of the pro-oxidant results were achieved in neuronal cell cultures, in which cell exposure to these kynurenines was much longer (24–72 h) than that used in our assays (1 h), and the concentrations giving these pro-oxidant effects were obtained in general with 100–200 μM (Lee et al., 2001, 2004). Although the reasons for these apparently contradictory results are unknown, it is possible that they may have occurred due to different experimental conditions, tissues and/or cells and time exposure utilized in the assays to measure oxidative stress parameters. Therefore, in an attempt to verify whether exposure time could influence our results, we incubated C6 glioma cells for 1–48 h with 100 μM 3HKyn and tested its effects on TAR and TBA-RS measurement. We observed that 3HKyn treatment gave rise to highly significant increases (up to 400% increase) of TAR values at all incubation periods utilized, and that this effect was particularly evident at shorter incubation times (1–6 h) declining along incubation (12–48 h). Similarly, TBA-RS values were significantly reduced when C6 glioma cells were exposed only to short (1–6 h), but not to longer periods (12–48 h). These findings may suggest that the antioxidant activity exerted by 3HKyn diminished along time possibly due to auto-oxidation generating free radicals.

Another interesting aspect that should be discussed is the type of cells used in the experiments. Most studies demonstrating pro-oxidant activity of 3HKyn and 3HAA employed neuronal cell cultures, whereas we utilized a glial cell line (C6 glioma cells), as well as cortical homogenates, which contain predominantly glial cells. Taken together, it may be suggested that distinct neural cells may result in differential effects provoked by these kynurenines. In this context, it should be emphasized that the antioxidant defenses including GSH and catalase content are higher in astrocytes, and that these cells are known to protect neurons against oxidative damage and cell death (Sagara et al., 1993; Desagher et al., 1996; Dringen et al., 2000).

The morphological studies demonstrated that 3HKyn did not change C6 cell morphology, which is in agreement with the observations that 3HKyn is neurotoxic, rather than gliotoxic (Okuda et al., 1998). However, although the morphology of C6 cells was not changed, we cannot establish at the present time whether other key systems necessary to cell survival or functioning could be altered by 3HKyn.

We also found that the significant enhancement of TBA-RS levels provoked by GA, the major metabolite accumulated in GAI, was partially reduced by 100 μM 3HKyn and that this kynurenine fully prevented the significant increase of DCFH oxidation caused by GA. If these findings occur *in vivo*, it may be presumed that the simultaneous increase of GA and 3HKyn in the brain of GAI patients, as previously hypothesized (Varadkar and Surtees, 2004), may compensate the pro-oxidant and antioxidant activities of these compounds, respectively (de Oliveira Marques et al., 2003).

Finally, it is difficult to establish at this stage whether the antioxidant effects found for 3HKyn and 3HAA at relatively high concentrations may have biological significance in brain of patients affected by neurodegenerative processes. However, we cannot rule out that during neuroinflammatory processes, when the kynurenine pathway is activated in microglial cells and/or when invading macrophages infiltrate the brain, the concentrations of kynurenines may increase dramatically reaching the micromolar range within the brain. In this case, the *o*-aminophenolic compounds might represent a potential protective mechanism against pro-oxidants like quinolinic acid (Heyes et al., 1992; Schwarcz and Pellicciari, 2002). In this scenario, oxidative damage caused by quinolinic acid has been largely demonstrated (Rios and Santamaría, 1991; Santamaria and Rios, 1993; Perez-Severiano et al., 1998, 2004; Rodriguez-Martinez et al., 2000; Cabrera et al., 2000; Rossato et al., 2002; Santamaría et al., 2003; Leipnitz et al., 2005).

In conclusion, our present data show antioxidant activities for the *o*-aminophenolic kynurenines 3HKyn and 3HAA in cortical homogenates and C6 glioma cells. This is in line with previous reports postulating that 3HKyn and 3HAA could serve as endogenous natural antioxidants in a variety of inflammatory diseases in which oxidative stress is involved due to the activation of the rate-limiting enzyme of the kynurenine pathway indoleamine 2,3-dioxygenase by the pro-inflammatory cytokine interferon-γ (Christen et al., 1990, 1994; Thomas et al., 1996).

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References

Baran, H., Staniek, K., Kepplinger, B., Stur, J., Draxler, M., Nohl, H., 2003. Kynurenines and the respiratory parameters on rat heart mitochondria. Life Sci. 72, 1103–1115.

Bonini, P., Cicconi, S., Cardinale, A., Vitale, C., Serafino, A.L., Ciotti, M.T., Marlier, L.N.J.L., 2004. Oxidative stress induces p53-mediated apoptosis in glia: p53 transcription-independent way to die. J. Neurosci. Res. 75, 83–95.

Browne, R.W., Armstrong, D., 1998. Reduced glutathione and glutathione disulfide. Methods Mol. Biol. 108, 347–352.

Cabrera, J., Reiter, R.J., Tan, D.X., Qi, W., Sainz, R.M., Mayo, J.C., Garcia, J.J., Kim, S.J., El-Sokkary, G., 2000. Melatonin reduces oxidative neurotoxicity due to quinolinic acid: in vitro and in vivo findings. Neuropharmacology 39, 507–514.

Chevion, M., Navok, T., Glaser, G., Mager, J., 1982. The chemistry of favism-inducing compounds. The properties of isouramil and divicine and their reaction with glutathione. Eur. J. Biochem. 127, 405–409.

Christen, S., Peterhans, E., Stocker, R., 1990. Antioxidant activities of some tryptophan metabolites: possible implication for inflammatory diseases. Proc. Natl. Acad. Sci. U.S.A. 87, 2506–2510.

Christen, S., Thomas, S.R., Garner, B., Stocker, R., 1994. Inhibition by interferon-gamma of human mononuclear cell-mediated low density lipoprotein oxidation. Participation of tryptophan metabolism along the kynurenine pathway. J. Clin. Invest. 93, 2149–2158.

Coyle, J.T., Puttfarcken, P., 1993. Oxidative stress, glutamate, and neurodegenerative disorders. Science 363, 689–695.

de Oliveira Marques, F., Hagen, M.E., Pederzolli, C.D., Sgaravatti, A.M., Durigon, K., Testa, C.G., Wannmacher, C.M., de Souza Wyse, A.T., Wajner,

M., Dutra-Filho, C.S., 2003. Glutaric acid induces oxidative stress in brain of young rats. Brain Res. 964, 153–158.

Desagher, S., Glowinski, J., Premont, J., 1996. Astrocytes protect neurons from hydrogen peroxide toxicity. J. Neurosci. 16, 2553–2562.

Dringen, R., Gutterer, J.M., Hirrlinger, J., 2000. Glutathione metabolism in brain metabolic interaction between astrocytes and neurons in the defense against reactive oxygen species. Eur. J. Biochem. 267 (16), 4912–4916.

Dykens, J.A., Sullivan, S.G., Stern, A., 1987. Oxidative reactivity of the tryptophan metabolites 3-hydroxyanthranilate, cinnabarinate, quinolinate and picolinate. Biochem. Pharmacol. 36, 211–217.

Eastman, C.L., Guilarte, T.R., Lever, J.R., 1992. Uptake of 3-hydroxykynurenine measured in rat brain slices and in a neuronal cell line. Brain Res. 584, 110–116.

Esterbauer, H., Cheeseman, K.H., 1990. Determination of aldehydic lipid peroxidation products: malonaldehyde and 4-hydroxynonenal. Methods Enzymol. 186, 407–421.

Evelson, P., Travacio, M., Repetto, M., Escobar, J., Llesuy, S., Lissi, E., 2001. Evaluation of total reactive antioxidant potential (TRAP) of tissue homogenates and their cytosols. Arch. Biochem. Biophys. 388, 261–266.

Goldstein, L.E., Leopold, M.C., Huang, X., Atwood, C.S., Saunders, A.J., Hartshorn, M., Lim, J.T., Faget, K.Y., Muffat, J.A., Scarpa, R.C., Chylack Jr., L.T., Bowden, E.F., Tanzi, R.E., Bush, A.I., 2000. 3-Hydroxykynurenine and 3-hydroxyanthranilic acid generate hydrogen peroxide and promote alpha-crystallin cross-linking by metal ion reduction. Biochemistry 39, 7266–7275.

Gonzalez-Flecha, B., Llesuy, S., Boveris, A., 1991. Hydroperoxide-initiated chemiluminescence: an assay for oxidative stress in biopsies of heart, liver, and muscle. Free Rad. Biol. Med. 10, 93–100.

Goya, L., Feng, P.T., Aliabadi, S., Timiras, P.S., 1996. Effect of growth factors on the in vitro growth and differentiation of early and late passages of C6 glioma cells. Int. J. Dev. Neurosci. 14, 409–417.

Guillemin, G.J., Brew, B.J., 2002. Implications of the kynurenine pathway and quinolinic acid in Alzheimer's disease. Redox Rep. 7, 199–206.

Halliwell, B., Gutteridge, J.M., 1981. Formation of thiobarbituric-acid-reactive substance from deoxyribose in the presence of iron salts: the role of superoxide and hydroxyl radicals. FEBS Lett. 128, 347–352.

Halliwell, B., Gutteridge, J.M.C., 1999. Detection of free radicals and others reactive species: trapping and fingerprinting. In: Halliwell, B., Gutteridge, J.M.C. (Eds.), Free Radicals in Biology and Medicine. 3rd ed. Oxford University Press, Oxford, pp. 351–425.

Heyes, M.P., 1987. Hypothesis: a role for quinolinic acid in the neuropathology of glutaric aciduria type I. Can. J. Neurol. Sci. 14, 441–443.

Heyes, M.P., Saito, K., Crowley, J.S., Davis, L.E., Demitrack, M.A., Der, M., Dilling, L.A., Elia, J., Kruesi, M.J., Lackner, A., Larsen, S.A., Lee, K., Leonard, H.L., Markey, S.P., Martin, A., Milstein, S., Mouradian, M.M., Pranzatelli, M.R., Quearry, B.J., Salazar, A., Smith, M., Strauss, S.E., Suderland, T., Swedo, S.E., Tourtellotte, W.W., 1992. Quinolinic acid and kynurenine pathway metabolism in inflammatory and non-inflammatory neurological disease. Brain 115, 1249–1273.

Hiraku, Y., Inoue, S., Oikawa, S., Yamamoto, K., Tada, S., Nishino, K., Kawanishi, S., 1995. Metal-mediated oxidative damage to cellular and isolated DNA by certain tryptophan metabolites. Carcinogenesis 16, 349–356.

Ishii, T., Iwahashi, H., Sugata, R., Kido, R., 1992. Formation of hydroxanthommatin-derived radical in the oxidation of 3-hydroxykynurenine. Arch. Biochem. Biophys. 294, 616–622.

LeBel, C.P., Ischiropoulos, H., Bondy, S.C., 1992. Evaluation of the probe 2′,7′-dichlorofluorescein as an indicator of reactive oxygen species formation and oxidative stress. Chem. Res. Toxicol. 5, 227–231.

Lee, M.W., Park, S.C., Chae, H.S., Bach, J.H., Lee, H.J., Lee, S.H., Kang, Y.K., Kim, K.Y., Lee, W.B., Kim, S.S., 2001. The protective role of HSP90 against 3-hydroxykynurenine-induced neuronal apoptosis. Biochem. Biophys. Res. Commun. 284, 261–267.

Lee, H.J., Bach, J.H., Chae, H.S., Lee, S.H., Joo, W.S., Choi, S.H., Kim, K.Y., Lee, W.B., Kim, S.S., 2004. Mitogen-activated protein kinase/extracellular signal-regulated kinase attenuates 3-hydroxykynurenine-induced neuronal cell death. J. Neurochem. 88, 647–656.

Leipnitz, G., Schumacher, C., Scussiato, K., Dalcin, K.B., Wannmacher, C.M., Wyse, A.T., Dutra-Filho, C.S., Wajner, M., Latini, A., 2005. Quinolinic acid reduces the antioxidant defenses in cerebral cortex of young rats. Int. J. Dev. Neurosci. 23, 695–701.

Lissi, E., Pascual, C., del Castillo, M.D., 1992. Luminol luminescence induced by 2,2′-azo-bis(2-amidinopropane) thermolysis. Free Rad. Res. Commun. 17, 299–311.

Lissi, E., Salim-Hanna, M., Pascual, C., del Castillo, M.D., 1995. Evaluation of total antioxidant potential (TRAP) and total antioxidant reactivity from luminol-enhanced chemiluminescence measurements. Free Rad. Biol. Med. 18, 153–158.

Lowry, O.H., Rosebrough, N.J., Lewis-Farr, A., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Luthra, M., Balasubramanian, D., 1992. 3-Hydroxykynurenine and 3-hydroxyanthranilic acid may act as endogenous antioxidants in the eye lens. Exp. Eye Res. 55, 641–643.

Mangoura, D., Sakellaridis, N., Jones, J., Vernadakis, A., 1989. Early and late passage C6 glial cell growth: similarities with primary glial cells in culture. Neurochem. Res. 14, 941–947.

Ogawa, T., Matson, W.R., Beal, M.F., Myers, R.H., Bird, E.D., Milbury, P., Saso, S., 1992. Kynurenine pathway abnormalities in Parkinson's disease. Neurology 42, 1702–1706.

Okuda, S., Nishiyama, N., Saito, H., Katsuki, H., 1996. Hydrogen peroxide-mediated neuronal cell death induced by an endogenous neurotoxin, 3-hydroxykynurenine. Proc. Natl. Acad. Sci. U.S.A. 93, 12553–12558.

Okuda, S., Nishiyama, N., Saito, H., Katsuki, H., 1998. 3-Hydroxykynurenine, an endogenous oxidative stress generator, causes neuronal cell death with apoptotic features and region selectivity. J. Neurochem. 70, 299–307.

Olanow, C.W., 1993. A radical hypothesis for neurodegeneration. Trends Neurosci. 16, 439–444.

Pearson, S.J., Reynolds, G.P., 1991. Determination of 3-hydroxykynurenine in human brain and plasma by high-performance liquid chromatography with electrochemical detection. Increased concentrations in hepatic encephalopathy. J. Chromatogr. 565, 436–440.

Pearson, S.J., Reynolds, G.P., 1992. Increased brain concentrations of a neurotoxin, 3-hydroxykynurenine, in Huntington's disease. Neurosci. Lett. 144, 199–201.

Perez-Severiano, F., Escalante, B., Rios, C., 1998. Nitric oxide synthase inhibition prevents acute quinolinate-induced striatal neurotoxicity. Neurochem. Res. 23, 1297–1302.

Perez-Severiano, F., Rodriguez-Perez, M., Pedraza-Chaverri, J., Maldonado, P.D., Medina-Campos, O.N., Ortiz-Plata, A., Sanchez-Garcia, A., Villeda-Hernandez, J., Galvan-Arzate, S., Aguilera, P., Santamaría, A., 2004. *S*-Allylcysteine, a garlic-derived antioxidant, ameliorates quinolinic acid-induced neurotoxicity and oxidative damage in rats. Neurochem. Int. 45, 1175–1183.

Perry, G., Taddeo, M.A., Petersen, R.B., Castellani, R.J., Harris, P.L., Siedlak, S.L., Cash, A.D., Liu, Q., Nunomura, A., Atwood, C.S., Smith, M.A., 2003. Oxidative damage in Alzheimer disease. BioMetals 16, 77–81.

Reynolds, G.P., Pearson, S.J., 1989. Increased brain 3-hydroxykynurenine in Huntington's disease. Lancet 2, 979–980.

Rios, C., Santamaría, A., 1991. Quinolinic acid is a potent lipid peroxidant in rat brain homogenates. Neurochem. Res. 16, 1139–1143.

Rodriguez-Martinez, E., Camacho, A., Maldonado, P.D., Pedraza-Chaverri, J., Santamaría, D., Galvan-Arzate, S., Santamaría, A., 2000. Effect of quinolinic acid on endogenous antioxidants in rat corpus striatum. Brain Res. 858, 436–439.

Rossato, J.I., Zeni, G., Mello, C.F., Rubin, M.A., Rocha, J.B., 2002. Ebselen blocks the quinolinic acid-induced production of thiobarbituric acid reactive species but does not prevent the behavioral alterations produced by intrastriatal quinolinic acid administration in the rat. Neurosci. Lett. 318, 137–140.

Santamaria, A., Rios, C., 1993. MK-801, an *N*-methyl-D-aspartate receptor antagonist, blocks quinolinic acid-induced lipid peroxidation in rat corpus striatum. Neurosci. Lett. 159, 51–54.

Santamaría, A., Flores-Escartin, A., Martinez, J.C., Osório, L., Galvan-Arzate, S., Pedraza-Chaverri, J., Maldonado, P.D., Medina-Campos, O.N., Jimenez-Capdeville, M.E., Manjarrez, J., Rios, C., 2003. Copper blocks quinolinic

acid neurotoxicity in rats: contribution of antioxidant systems. Free Rad. Biol. Med. 35, 418–427.

Sagara, J.I., Miura, K., Bannai, S., 1993. Maintenance of neuronal glutathione by glial cells. J. Neurochem. 61, 1672–1676.

Sardar, A.M., Bell, J.E., Reynolds, G.P., 1995. Increased concentrations of the neurotoxin 3-hydroxykynurenine in the frontal cortex of HIV-1-positive patients. J. Neurochem. 64, 932–935.

Schwarcz, R., Pellicciari, R., 2002. Manipulation of brain kynurenines: glial targets, neuronal effects, and clinical opportunities. J. Pharmacol. Exp. Ther. 303, 1–10.

Stone, T.W., Mackay, G.M., Forrest, C.M., Clark, C.J., Darlington, L.G., 2003. Tryptophan metabolites and brain disorders. Clin. Chem. Lab. Med. 41, 852–859.

Thomas, S.R., Witting, P.K., Stocker, R., 1996. 3-Hydroxyanthranilic acid is an efficient, cell-derived co-antioxidant for alpha-tocopherol, inhibiting human low density lipoprotein and plasma lipid peroxidation. J. Biol. Chem. 271, 32714–32721.

Varadkar, S., Surtees, R., 2004. Glutaric aciduria type I and kynurenine pathway metabolites: a modified hypothesis. J. Inherit. Metab. Dis. 27, 835–842.

Vernadakis, A., Kentroti, S., Brodie, C., Mangoura, D., Sakellaridis, N., 1991. C6 glioma cells of early passage have progenitor properties in culture. Adv. Exp. Med. Biol. 296, 181–195.

Wei, H., Leeds, P., Chen, R.W., Wei, W., Leng, Y., Bredesen, D.E., Chuang, D.M., 2000. Neuronal apoptosis induced by pharmacological concentrations of 3-hydroxykynurenine: characterization and protection by dantrolene and Bcl-2 overexpression. J. Neurochem. 75, 81–90.