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Research Article

Protective Effects of Caffeic Acid on Quinolinic Acid-Induced Behavioral and Oxidative Alterations in Rats

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Abstract *Study design.* The protective effects of the natural antioxidant caffeic acid (CA) on behavioral tasks and lipid peroxidation were tested in an excitotoxic model produced by unilateral intrastriatal injection of quinolinic acid (QUIN), and in striatal slices incubated in the presence of the same toxin. CA (20 mg/kg) was administered intraperitoneally to rats every day for five days; then, rats received QUIN (240 nmol/μL). Six days later, motor asymmetry was quantified by the preferential use of forelimbs and the circling behavior tests. Rat striatal slices (300 μm thick) were incubated in the presence of CA (30–300 μM) and/or QUIN (100 μM) to estimate oxidative stress. *Results.* QUIN induced motor asymmetry in lesioned rats and increased lipid peroxidation in striatal slices when compared to control values. CA prevented the QUIN-induced toxic endpoints in a concentration-dependent manner. *Conclusion.* Our results support the neuroprotective role of CA in neurotoxic paradigms recruiting excitotoxic events.

Keywords motor asymmetry; antioxidant defense; excitotoxicity; oxidative stress; corpus striatum; caffeic acid; quinolinic acid

1. Introduction

The kynurenine pathway for tryptophan degradation is responsible for the formation of neuroactive metabolites in the CNS [1]. The alteration in the levels of these metabolites is involved in different neurological disorders [2]. One of these metabolites is quinolinic acid (QUIN), a glutamate agonist acting on NMDA receptors (NMDAr) [3]. Through excitotoxic events, QUIN induces oxidative stress, increased intracellular Ca^{2+} levels, enhanced levels of extracellular glutamate, augmented protease activity, and stimulated deadly cascades under different experimental conditions [4, 5]. In turn, excitotoxicity can be defined as a toxic mechanism affecting neurons that are continuously stimulated via overactivation of NMDAr and further increased intracellular Ca^{2+} levels triggering deadly cascades [6].

Caffeic acid (3,4-dihydroxycinnamic acid or CA) is a natural phenolic compound that has been shown to exert neuroprotective actions against different neurotoxic insults, including ischemia and excitotoxic damage in rodents, and these effects have been related with its antioxidant and anti-inflammatory properties [7, 8, 9, 10]. Although some positive effects of CA on endpoints of behavioral (motor activity) and redox status (reduced glutathione/oxidized glutathione) alterations in the excitotoxic model induced by QUIN in rats have already been reported [9], key behavioral and oxidative stress markers denoting neuroprotection are still needed as complementary evidence. In particular for this toxic model, motor asymmetry and lipid peroxidation as an index of oxidative damage in the striatum are required. Therefore, the present study aims to evaluate the effects of CA on QUIN-induced behavioral and biochemical alterations in the rat brain. For comparative purposes, the effect of CA was also tested in the 3-nitropropionic acid (3-NP) model of striatal toxicity. The chemical structures of both CA and QUIN are represented in Figure 1.

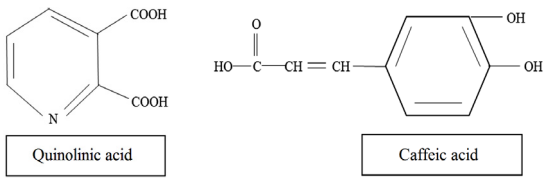

Figure 1: Schematic representation of the chemical structures of QUIN (a) and CA (b).

2. Materials and methods

2.1. Reagents

Apomorphine, CA, QUIN, 3-NP, thiobarbituric acid (TBA), and other reagents were obtained from Sigma-Aldrich

(St. Louis, MO, USA). Other chemicals, including buffers, were obtained from other commercial sources.

2.2. Animals and treatments

Twenty eight bred-in-house male Wistar rats (280–320 g) were randomly separated into four groups (seven animals per group). Animals were placed into acrylic cages and provided with Rodent Chow (Purina, St. Louis, MO, USA) and water ad libitum. Constant conditions of temperature (25 °± 1 °C), humidity (50 ± 10%), and lighting (12:12 light-dark cycle) were maintained throughout the experiments. All experimental procedures with animals were strictly carried out according to the "Guidelines for the Use of Animals in Neuroscience Research" from the Society for Neuroscience, the local ethical committees, and in compliance of the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.

The dose employed for CA was close to that used in a previous report [9]. The experimental groups were injected intraperitoneally (IP) with sterile water as vehicle or CA (20 mg/kg) every day for five consecutive days. Shortly after the last CA administration (30 min), the animals were anesthetized with sodium pentobarbital (50 mg/kg, IP) and 30 min later infused for 2 min with a single intrastriatal injection of QUIN (240 nmol/μL). The dose employed for QUIN has been previously reported [11]. The striatal infusion was performed at the following stereotaxic coordinates: 0.5 mm anterior to bregma, −2.6 mm lateral to bregma, and 4.5 mm ventral to the dura [12]. Control rats received sterile saline intrastriatally and sterile water (pH 7.4) IP. Six days after the striatal lesion, animals from all groups were subjected to behavioral tests.

2.3. Behavioral tests

2.3.1. Circling behavior test

Rotation behavior was evaluated in rats from all groups, following a protocol previously reported by us [13]. Six days after the intrastriatal QUIN infusion, all rats were administered with apomorphine subcutaneously (1 mg/kg, SC) and placed in individual acrylic cages. The number of ipsilateral rotations (complete 360 ° turns) to the lesioned side was recorded for 60 min in periods of 5 min. Results are expressed as the total number of ipsilateral turns per 60 min. Largely known as a morphine decomposition product, apomorphine is a nonselective dopamine agonist that activates both D1 and D2 dopamine receptors.

2.3.2. Cylinder test

The cylinder test is a behavioral method providing accurate information on locomotor asymmetry in rodent models. Animals were placed into an open-top, clear plastic cylinder, and monitored in regard to their forelimb activity while rearing against the wall. The use of forelimb was defined as the preferential placement of the whole palm of a given limb on the wall of the device, therefore indicating its use for body support while rearing. The number of ipsilateral and contralateral forelimb contacts was calculated and expressed as the count in 5 min.

2.4. Isolation of striatal slices

Rat brains were collected after animal decapitation and placed in ice-cold Krebs-bicarbonate buffer pH 7.4 (120 mM NaCl, 2 mM KCl, 0.5 mM $CaCl_2$, 26 mM $NaHCO_3$, 10 mM $MgSO_4$, 1.18 mM KH_2PO_4, 11 mM glucose, and 200 mM sucrose). Striatal slices were dissected, glued down against agar blocks in small chambers, submerged in cold oxygenated dissection buffer, and sectioned in 300 μm thick transverse slices using a vibratome (TS1000 Leica; Heidelberg, Germany). Thereafter, the slices were transferred to a sucrose-free dissection buffer, and bubbled with 95% O_2/5% CO_2 at room temperature for 30 min to recover from the slicing procedure. Then, slices were incubated for 30 min, exposed to different concentrations of CA (30, 100 or 300 μM) or vehicle for 30 min more, and added with QUIN (100 μM) for 3 h in a shaking water bath at 37 °C. Immediately after incubation, slices were placed on ice, collected, and scheduled for measurement of oxidative damage to lipids. For comparative purposes, a positive control consisting of slices incubated with the mycotoxin 3-nitropropionic acid (3-NP, 1 mM) and preincubated with CA (100 μM) was ran in parallel.

2.5. Assay of lipid peroxidation

The formation of thiobarbituric acid-reactive substances (TBARS) as an index of lipid peroxidation was determined in striatal slices, according to a previous report [14]. Briefly, 50 μL aliquots of the homogenates were added to 100 μL of the TBA reagent (0.75 g of TBA + 15 g of trichloroacetic acid + 2.54 mL of HCl) and incubated at 100 °C for 20 min. The pink chromophore produced after this reaction denoted the amount of peroxidized lipid products. Samples were kept on ice for 5 min and centrifuged at $3,000 \times$ g for 15 min. The optical density was measured in the supernatants in a CYT3MV Biotek Cytation 3 Imaging Reader at 532 nm. The amount of TBA-RS was calculated by interpolation of values in a constructed malondialdehyde (MDA) standard curve. Results were originally calculated as nmol of MDA per mg protein, and finally expressed as percent of lipid peroxidation versus control. The content of protein in samples was determined by Bradford's method [15].

2.6. Statistical analysis

Results were expressed as mean values ±SEM. Behavioral data were analyzed by nonparametric ANOVA (Kruskal-Wallis) followed by comparison with Mann-Whitney's test. Biochemical data were analyzed with

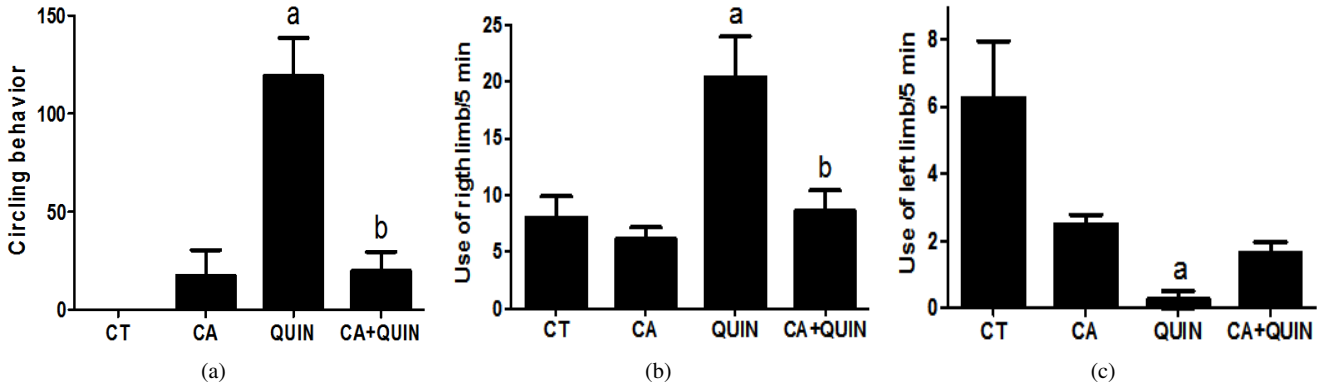

Figure 2: Effect of CA (20 mg/kg, IP) on the behavioral alterations induced by QUIN (240 nmol/μL) in rats. Rats were administered with CA for five consecutive days prior a single infusion of QUIN into the right striatum. Both behavioral markers of motor asymmetry were explored six days after QUIN injection. The preferential use of forelimbs ((b) and (c)) was monitored for 5 min by placing the animals into a plastic cylinder. Immediately thereafter, rats were administered with apomorphine (1 mg/kg, SC) and the number of ipsilateral turns was recorded every 5 min for 60 min. Each bar represents mean values $\pm$ one SEM of seven rats per group. [a]$P < .05$ different of control, [b]$P < .05$ different of QUIN. Data analysis was performed with a nonparametric ANOVA (Kruskal-Wallis) followed by a comparison with Mann-Whitney's test.

a one-way analysis of variance (ANOVA) for repeated measures, followed by post-hoc Tukey's test. The analytical procedures were performed using the scientific statistic software GraphPad Prism 5 (GraphPad Scientific, San Diego, CA, USA). Differences of $P < .05$ were considered as statistically significant.

3. Results

3.1. CA prevented the QUIN-induced behavioral alterations in rats

Figure 2 shows the behavioral results obtained from rats subjected to the rotation behavior test (Figure 2(a)) and the cylinder test (Figures 2(b) and 2(c)).

In Figure 2(a), the control animals—receiving apomorphine six days after intrastriatally infused with vehicle—displayed no rotations, although they exhibited excitement and hyperactivity during the evaluation time (data not shown). The group receiving CA displayed a moderate number of ipsilateral rotations (18 ± 9). The QUIN-lesioned rats displayed a considerable number of rotations in 60 min (121 ± 16; $P < .05$ vs. control) in response to apomorphine, whereas the group pretreated with CA and further infused with QUIN displayed a moderate number of rotations, similar to the CA group (22 ± 6; 82% below QUIN; $P < .05$ vs. QUIN).

Figures 2(b) and 2(c) depict the preferential use of forelimbs in the cylinder test for 5 min. Control rats showed, more or less, and equal use of the right (7.5 ± 2.3 times) versus the left forelimb (6.2 ± 1.7 times). This tendency was not significantly changed in animals receiving CA (6.1 ± 1.2 for the right forelimb vs. 2.3 ± 0.2 for the left forelimb). In contrast, the group receiving QUIN exhibited a remarkable increased preference of the right forelimb (21.0 ± 3.3 times; 180% above the control; $P < .05$) versus the left forelimb (0.4 ± 0.2; 83% below the control; $P < .05$). These tendencies were prevented in QUIN-lesioned rats by the pretreatment with CA (7.8 ± 2.1 for the right forelimb vs. 1.7 ± 0.3 for the left forelimb; 63% below and 325% above QUIN, resp.; $P < .05$ against QUIN for both cases).

3.2. CA reduced the QUIN-induced oxidative damage to lipids in rat striatal slices in a dose-dependent manner

Figure 3 depicts the curve-response effects (as percent values vs. the control) of CA on QUIN-induced striatal lipid peroxidation. QUIN per se produced a significant increase in oxidative damage to lipids when compared to control ($49.5 \pm 7.6\%$ above the control; $P < .05$). CA per se, administered at different concentrations (30, 100, and 300 μM), reduced the levels of lipid peroxidation below the control levels (39, 10, and 19% below the control, resp.), although none of these changes were statistically significant. When preadministered to QUIN-treated slices, CA reduced the oxidative damage to lipids by 18, 40 ($P < .05$) and 87% ($P < .05$) versus QUIN, respectively, in a concentration-dependent manner.

In the upper right panel, 3-NP per se increased the levels of lipid peroxidation by 735% above the control ($P < .05$). Once again, CA per se did not affect the oxidative damage when compared to the control (19% below the control), whereas when preadministered to 3-NP-treated slices, it reduced the 3-NP-induced lipid peroxidation by 32% ($P < .05$).

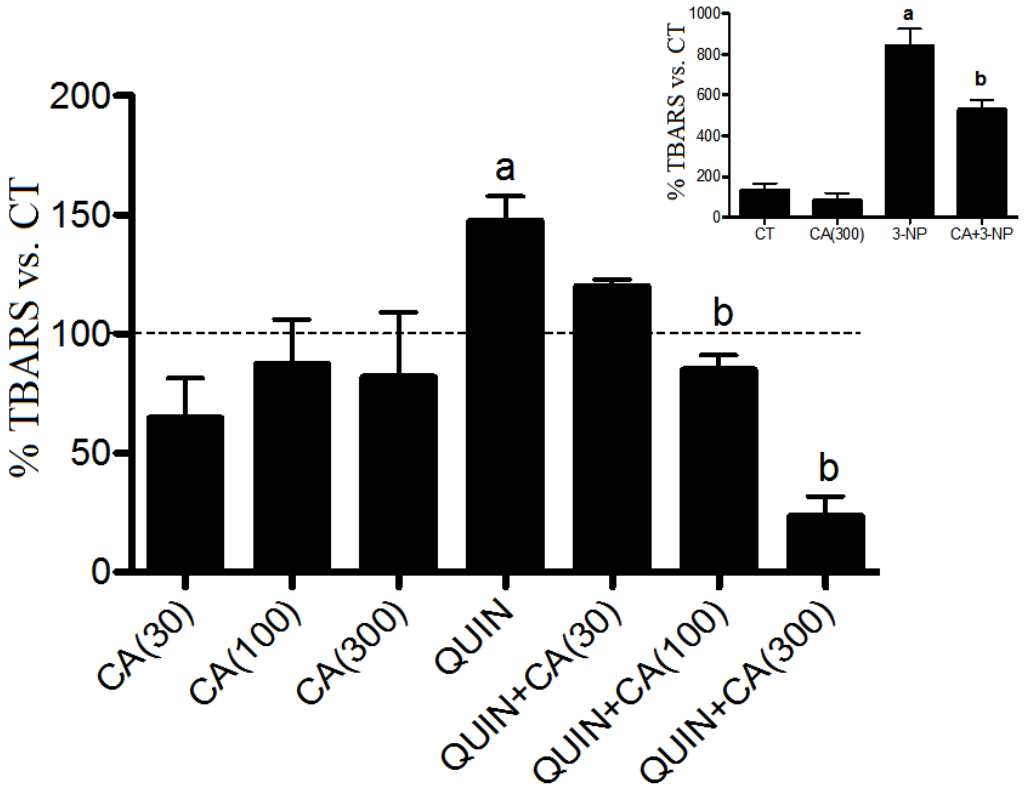

Figure 3: Curve-response effect of CA (30–300 μM) on the striatal oxidative damage to lipids (TBARS formation) induced by QUIN (100 μM) in tissue slices. For comparative purposes, the effect of CA (100 μM) was also tested on the lipoperoxidative effect induced by 3-nitropropionic acid (3-NP, 1 mM) in additional groups (small graph in the right corner). Bars represent mean values ±SEM of seven experiments per group. $^{a}P < .05$ statistically different of control, $^{b}P < .05$ statistically different of QUIN or 3-NP. Data analysis was performed with a one-way ANOVA followed by Tukey's test.

4. Discussion

In this simple study, we found evidence that the natural phenolic compound CA exerted neuroprotective properties against the excitotoxic damage induced by QUIN in the rat striatum. We achieved this conclusion through the positive effects of CA observed on both behavioral and biochemical endpoints of striatal toxicity induced by QUIN. CA was able to significantly attenuate the QUIN-induced circling behavior and the use of the right forelimb, thus confirming that enough degree of neuroprotection is achieved through this agent to prevent the motor asymmetry evoked by the neurotoxin. In addition, it can also be assumed that the preventive effect exerted by CA on QUIN- and 3-NP-induced striatal lipid peroxidation—a major indicator of oxidative damage—is due to the antioxidant properties previously reported for this phenolic compound [7, 8, 9, 10], and this effect might strongly account for its neuroprotective action in the brain.

Our results are complementary to previous findings reported by Kalonia et al. [9], who demonstrated that CA administered per orally (5 mg/kg and 10 mg/kg, PO) was able to prevent alterations in locomotor activity in animals intrastriatally lesioned with QUIN. Despite the obvious experimental differences between Kalonia's report and ours (PO vs. IP administration of CA, range of dosage for CA, and the dose of QUIN employed [300 nmol vs. 240 nmol], all in regard to the in vivo approach), CA demonstrates to be a protective agent that can reduce behavioral and biochemical alterations in the toxic model evoked by QUIN.

Few reports have previously deal with the concept that CA and QUIN are opposite in their effects. Hirai and coworkers [16] described the effects of some natural iron chelators and derivatives on in vitro oxygen consumption rates and superoxide radical formation. Among the agents tested, QUIN and CA were compared. These authors found that QUIN repressed oxygen consumption, whereas CA accelerated it. In turn, these opposite effects establish relevant chemical basis to understand the mechanistic nature of these two different molecules in regard to the induction of superoxide formation (for the case of QUIN) or its repression (for the case of CA). More recently, Minakata et al. [17] tested the capacity of QUIN and CA to modulate the formation of radicals in the reaction mixtures of rat liver microsomes in the presence of ADP,

Fe^{3+}, and NADPH in order to provide information on the protective actions of CA and the neurotoxic actions of QUIN through a redox approach. They demonstrated that while CA inhibited the radical formation, QUIN enhanced their production. The conclusion achieved from these observations is that oxidative stress is part of the toxic pattern elicited by QUIN in the progression of several pathological conditions, whereas antioxidant activity is part of the protective profile of CA and other natural polyphenols. In addition, other groups have provided direct evidence on the in vivo protective effect of CA in other toxic paradigms. For example, Kumar et al. [10] demonstrated that CA is able to reduce different endpoints of oxidative damage and mitochondrial dysfunction in a murine model of chronic fatigue, once again emphasizing the relevance of the antioxidant profile of CA to exert its protective actions. Like this, other studies have provided relevant evidence that CA can prevent different deleterious events linked to oxidative stress and inflammation in chronic pathological conditions.

Of final consideration, phenolic acids, such as CA, may present or combine with other acids in its natural form. Through its combination with other acids, CA yields the formation of another important antioxidant compound, chlorogenic acid (CGA), and such biotransformation occurs by esterification of CA with a cyclic alcohol-acid, quinic acid [18]. Noteworthy, similar to CA, CGA has been also described to exert protective actions in a number of inflammatory and oxidative events [19]. CGA is the most abundant polyphenol found in food and plants [18, 19], thus it can be hypothesized that the presence of both of these agents might induce a simultaneous action, thereby enhancing the protective effect of CA. Of course, this speculation deserves further and more detailed investigation.

In summary, CA is a promising antioxidant tool to investigate the role of oxidative stress in toxic models of neurodegenerative disorders coursing with excitotoxic events. However, more detailed studies are needed to characterize the precise mechanisms underlying the antioxidant and neuroprotective properties of this naturally occurring agent.

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Conflict of interest The authors declare that they have no conflict of interest.

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