Saffron Extract Ameliorates Oxidative Damage and Mitochondrial Dysfunction in the Rat Brain

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Abstract

Given its widely reported antioxidant properties, the effects of saffron extract (SE) were tested in striatal synaptosomes isolated from the brain of rats exposed to the mitochondrial toxin 3-nitropropionic acid (3-NPA). The assays of thiobarbituric acid-reactive substances (TBARS) formation and 3-(3,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) reduction were used as current markers of lipid peroxidation (LP) and synaptosomal viability, respectively. Our results show that 3-NPA alone (20 mg/kg/day, i.p.), given to rats for 3 days, produced a significant increase in LP and decreased the mitochondrial function in synaptosomal fractions when compared with control. In contrast, striatal synaptosomes from those animals receiving SE (1 mg/kg/day, i.p.) for 5 days (administration schedule starting 1 day before and finishing 1 day after 3-NPA), exhibited levels of LP similar to control, while preserved their mitochondrial function. Another index of LP, the formation of lipid fluorescent products, was tested in brain homogenates incubated with 3-NPA and/or SE. 3-NPA significantly increased LP by 90 %, while SE decreased this marker. Altogether, these data suggest that protective actions of SE produced in the 3-NPA model may be related with its antioxidant properties, as saffron is rich in antioxidant carotenoids. Our data also open a research field on the potential neuroprotective properties of saffron compounds against neurodegenerative disorders with energy impairment and excitotoxic components.

INTRODUCTION

Cellular depletion of ATP in the CNS represents a physiopathologic event leading to a series of morphologic, biochemical and physiologic derangements involving energy deficit, reactive oxygen species (ROS) formation and oxidative stress. Indeed, ROS generation is an important mechanism accounting for cellular injury in many neurodegenerative disorders (Valko et al., 2006), affecting the structure and function of macromolecules such as membrane lipids, proteins and nucleic acids (Bulkley, 1994). Although cellular defense against ROS-mediated injury
is provided by enzymatic (catalase, superoxide dismutase, glutathione peroxidase, etc.) and nonenzymatic (GSH, α-tocopherol, vitamin C, urate, etc.) radical scavenging systems (Abdollahi et al., 2004), these systems are commonly susceptible of being affected and diminished under pathologic conditions. The use of antioxidants such as α-tocopherol, ascorbic acid, and β-carotene in different paradigms is considered a promising approach for neuroprotection. However, clinical evidence that antioxidants can combat the deleterious actions of ROS is still relatively scarce. Recent overwhelming attention to plant products and alternative medicine has encouraged plant chemists, pharmacologists, biochemists, and molecular biologists to combine their efforts in search for natural agents that can limit ROS-mediated injuries.

In traditional Chinese medicine, Crocus sativus extract (saffron) has been largely used for anodyne and emmenagogue. Saffron is employed in folk medicine as an effective antispasmodic, eupetic, gingival sedative, anticatarrhal, nerve sedative, carminative, diaphoretic, expectorant, stimulant, stomachic and aphrodisiac (Rios et al., 1996). Furthermore, recent pharmacological studies have demonstrated that saffron extract (SE), or its active constituents, present anticonvulsant (Hosseinzadeh and Khosravan, 2002), antidepressant (Hosseinzadeh et al., 2004), anti-inflammatory (Hosseinzadeh and Younesi, 2002), antitumoral and radical scavenging properties (Abdullaev, 1993; Zhang et al., 1994; Escribano et al., 1996; Rios et al., 1996; Abe et al., 1999), while promote diffusion of oxygen in different tissues (Rios et al., 1996).

SE also has chemopreventive and genoprotective effects against genotoxin-induced oxidative stress in mice (Nair et al., 1995; Premkumar et al., 2001, 2003; Abdullaev et al., 2002).

The fungal toxin 3-nitropropionic acid (3-NPA) is known to affect the CNS by disrupting mitochondrial electron transport through the inhibition of succinate dehydrogenase, thus leading to deficit in cellular energy and neurodegenerative events in mammals (Alexi et al., 1998). 3-NPA produces selective striatal lesions through the secondary activation of glutamate receptors (Greene et al., 1998), also involving acute necrotic and delayed apoptotic cell death pathways (Pang and Geddes, 1997), as well as oxidative stress as major contributors to neurotoxicity (La Fontaine et al., 2000a). In this regard, the toxin is able to produce depleted levels of reduced glutathione (GSH), altered profiles in antioxidant enzyme activities, and increased levels of reactive oxygen/nitrogen species (ROS/RNS) (Binienda et al., 1998, Nam et al., 2005). All these toxic features serve to propose 3-NPA as a suitable model for investigating oxidative stress in the brain, such as lipid peroxidation and mitochondrial dysfunction. In spite of the many antioxidant properties described for SE, in this study we evaluated whether SE may reduce, or even avoid, some oxidative markers of 3-NPA-induced toxicity in rat brain preparations.

MATERIALS AND METHODS

Reagents

Malondialdehyde (MDA), 3-NPA and 3-(3,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromite (MTT) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Pure SE (“Baby brand”) was obtained from a street market in Delhi, India (Khari baoli). All other reagents were obtained from known commercial sources. Deionized water was obtained from a Milli-RQ system (Millipore, MA, USA) and used for preparation of all solutions.

Animals
Sixty male Wistar bred-in-house rats (270-300) obtained from the vivariums of the Instituto Nacional de Neurología y Neurocirugía (México) and the Instituto Nacional de Pediatría (México), were used throughout the study. Animals were housed five per cage in acrylic box cages and received food and water *ad libitum*; they were always maintained under standard conditions of temperature and humidity. The number of independent experiments per condition was always maintained to a minimum of six, independently of the total number of animals employed. All procedures with animals were carried out according to the “National Institutes of Health Guide for the Care and Use of Laboratory Animals”. During the experiments, all efforts were made to minimize animal suffering. For all experimental purposes, animals were housed five per cage under standard conditions.

**Experimental Protocol**

For the first part of the experimental protocol, animals were divided into four groups (6 rats per group) and treated as follows: Group I (Control) received a total of eight injections of saline i.p. (five of them mimicking SE administration and the other three mimicking 3-NPA); Group II received SE (1 mg/kg/day, i.p.) for five days; Group III received 3-NPA (20 mg/kg/day, i.p.) for three days, starting just one day after the first injection of SE; Group IV received SE plus 3-NPA under the same conditions described above. Two hours after the last SE administration, animals were killed by decapitation, brains were dissected out and their striata (caudate nuclei from both sides) properly collected and preserved at -75 ºC until used for isolation of synaptosomal fractions.

The second part of the protocol consisted of preparation of crude brain homogenates. Briefly, a total number of twenty rats were killed by decapitation and their brains removed. Each brain was homogenized in 20 ml of PBS. A pool of brain homogenates was then formed by mixing them. From this pool, the following conditions were prepared: 1) Control (970 µl of tissue homogenate plus 30 µl of PBS); 2) SE (970 µl of tissue homogenate plus 30 µl of SE 20 µg/ml); 3) 3-NPA (970 µl of tissue homogenate plus 30 µl of 3-NPA 100 µM); and 4) SE + 3-NPA (940 µl of tissue homogenate plus 30 µl of SE 5, 10 or 20 µg/ml plus 30 µl of 3-NPA 100 µM). Once prepared, samples were incubated at 37 ºC for 60 min in a shaking water bath and used for determination of LP by the formation of lipid fluorescent products.

**Preparation of Synaptosomal P2 Fractions**

From the first part of the experimental protocol, synaptosomes were obtained from the striata by a method previously described by us (Pérez-De La Cruz et al., 2006). Tissue samples were homogenized in sucrose (0.32 M). Homogenates were centrifuged at 1,073 x g for 10 min, and supernatants re-centrifuged at 17,172 x g for 15 min. The resulting pellets were re-suspended in HEPES buffer (NaCl 0.1 M, NaH_{2}PO_{4} 0.001 M, NaHCO_{3} 0.005 M, CaCl_{2} 0.001 M, glucose 0.006 M, and HEPES 0.01 M, pH 7.4). Synaptosomal fractions were then immediately used for estimation of LP and MTT reduction.

**Estimation of LP in Synaptosomes by the Assay of TBA-reactive Substances (TBARS)**

LP was evaluated by thiobarbituric acid (TBA) reactive assay, according to a previous report (Pérez-De La Cruz et al., 2006). Briefly, aliquots of 250 µl containing synaptosomal fractions were added with 500 µl of the TBA reagent (0.75 g of TBA + 15 g of trichloroacetic acid + 2.54 ml HCl) and mixtures were heated at 94 ºC for 30
min. Samples were then kept on ice for 5 min and centrifuged at 3,000 x g for 15 min. The optical density of supernatants was measured in a ThermoSpectronic Genesis 8 spectrophotometer at a wavelength of 532 nm. Final concentrations of TBARS were obtained by interpolation in a standard curve of malondialdehyde and corrected by the total content of protein per sample, measured by the reaction with the Folin-phenol reagent (Lowry et al., 1951). Results were expressed as nanomoles of TBARS per mg of protein.

**MTT Reduction Assay in Synaptosomal Fractions**

Mitochondrial function was estimated by the MTT reduction assay, following general descriptions of original reports (Mosmann, 1983; Berridge and Tan, 1993). This method is currently employed as an index of the functional status of the respiratory chain as the formation of formazan salts occurs through the action of mitochondrial dehydrogenases in viable cells or cell fractions. Four hundred µl-aliquots of the synaptosomal fractions were added with 8 µl of the MTT reagent (10 mg/ml) and incubated at 37 ºC for 90 min. Quantification of formazan was assessed by estimation of optical density in a ThermoSpectronic Genesis 8 spectrometer at 570 nm. Results were expressed as the percentage of MTT reduction with respect of control values.

**Estimation of LP in Brain Homogenates by Fluorescent Products**

From the second part of the experimental protocol, two aliquots (1 ml) from each sample (each corresponding to brain homogenates incubated in the presence of SE and/or 3-NPA) were added with 4 ml of a mixture of chloroform-methanol (2:1) and vortexed for 5 seconds. Samples were then preserved on ice and protected from light for 30 min. At the end of phase separation, the upper phase was removed with a vacuum pump and aliquots of 900 µl were obtained from the remaining phases and added with 100 µl of methanol. Fluorescent signals were measured in samples in a Perkin-Elmer LS-55 luminescence spectrometer at 370 nm of emission and 430 nm of excitation wavelengths. Final signals were corrected by the content of protein in each sample (Lowry et al., 1951). Results were expresses as the Fluorescence Units produced per mg of protein.

**Statistical Analysis**

All data were expressed as the mean ± one S.E.M. and analyzed by one-way ANOVA followed by Tukey’s test for multiple comparisons. Values of P<0.05 were considered of statistical significance.

**RESULTS**

The effects of SE and 3-NPA on lipid peroxidation (LP) in isolated synaptosomes from rat striatum are presented in Fig. 1. 3-NPA significantly increased TBA-RS formation when compared with control (49 % above), while treatment with SE to 3-NPA-injected rats resulted in a complete prevention of TBA-RS formation, up to basal levels (0.01 % below the control, 33 % of prevention when compared with 3-NPA alone). SE alone produced no significant changes in TBA-RS formation when compared with control animals (7 % above).

Synaptosomal fractions of 3-NPA-treated rats exhibited a significant decrease in MTT reduction with respect to control values (84 % below). In contrast, SE partially prevented the decreased mitochondrial capability of MTT reduction induced
by 3-NPA by 57%, while SE alone given to rats produced no significant effect when compared with control values (Fig. 2).

The concentration-response effect of SE on 3-NPA-induced LP in brain homogenates is shown in Fig. 3. The incubation of brain homogenates in the presence of 3-NPA alone produced a significant increase in lipid fluorescence when compared with control (97% above). The co-incubation of homogenates with 3-NPA plus increasing concentrations of SE (5, 10 or 20 µg/ml) produced a concentration-dependent decrease in LP, where the lower concentration tested exhibited no differences with respect of 3-NPA alone (10% below), the middle concentration produced significant protection (30% below 3-NPA alone and 36% above control), and the higher concentration prevented lipid fluorescence formation almost completely (37% below 3-NPA alone and 23% above control).

DISCUSSION

Results of this work demonstrate that SE is able to prevent LP and mitochondrial dysfunction induced by the neurotoxin 3-NPA in two biological preparations—striatal synaptosomes and brain homogenates—through a mechanism likely involving antioxidant properties. The antioxidant effect of SE against 3-NPA resulted in a concentration-dependent response when tested in brain homogenates. Oxidative stress induced by 3-NPA includes the following characteristics: ROS formation, changes in endogenous antioxidants, increased levels of 3-NT (a biomarker of ONOO- formation), and the oxidative disruption of the mitochondrial respiratory chain and further excitotoxicity (Binienda et al., 1998; Klivenyi et al., 2000). Therefore, given these major features of 3-NPA toxicity, it is likely that the protective actions produced by SE in this study could be mostly related to its documented ability to scavenge ROS (Assimopoulou et al., 2005). These considerations are further supported by previous reports demonstrating that 3-NPA-induced neurotoxicity can be attenuated and/or blocked by the use of different antioxidants, such as melatonin (Túnez et al., 2004b), nicotine (Túnez et al., 2004a), vitamin E (La Fontaine et al., 2000b), and S-allylcysteine (Pérez-De La Cruz et al., 2006). Moreover, as a first approach to characterize protection by SE in the 3-NPA toxic model, the use of striatal synaptosomal fractions and brain homogenates suggest wide antioxidant actions of saffron under different conditions.

As mentioned above, SE is known to scavenge ROS (Assimopoulou et al., 2005), and has been shown to exert protective actions at central level in different toxic models, including well-characterized anticonvulsant (Hosseinzadeh and Khosravan, 2002), antinociceptive, anti-inflammatory (Hosseinzadeh and Younesi, 2002), and antidepressant (Hosseinzadeh et al., 2004) effects in mice. In this regard, although the mechanisms by which SE might be exerting protection in the oxidative model evoked by 3-NPA are not completely clear, based on our observations we suggest that such effects may be due to its content in several antioxidant compounds, such as the different carotenoids present in SE i.e., safranal, crocetin, crocin and others (Rios et al., 1996), which in turn may be able to preserve the cell redox status and energy metabolism. In particular, crocin and crocetin, both carotenoids and active components of saffron, are acting at various levels on biological systems, and their positive actions are consistently linked to low risk of cancer (Van Popper, 1993). Increased activities of GST, GPx and CAT in lung and liver tissues from mice treated with crocetin have been reported, thereby suggesting that this carotenoid could be influencing host detoxification processes. Crocetin also enhances glutathione activity, thus emphasizing a potential protective role against oxidative damage in systemic
models. Furthermore, crocetin has been shown to exert neuroprotective actions in a hemi-parkinsonian rat model (Ahmad et al., 2005), whereas safranal has exhibited anticonvulsant activity against pentylenetetrazole-induced convulsions in mice (Hosseinzadeh and Talebzadeh, 2005). It is therefore evident, from these observations and our own results, that all these SE constituents may be important candidates to be considered responsible for inhibition of lipid peroxidation and inducers of antioxidant status and metabolic preservation. Nevertheless, these suggestions have to be demonstrated in this and other neurotoxic models in further studies through their direct application. In addition, although the saffron compounds are likely to be able to effectively cross the blood-brain barrier (Hosseinzadeh and Khosravan, 2002; Hosseinzadeh et al., 2004), more detailed pharmacokinetic studies are needed to support their use in neurotoxic models under in vivo conditions.

In summary, the results of this work describe for the first time the antioxidant properties of SE against specific neurotoxic insults involving impairment of energy metabolism and oxidative stress, and thus, open an interesting field of research devoted to the potential use of saffron components against neurodegenerative events in animal models and humans.

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Literature Cited


Fig. 1. Effect of saffron extract (SE) on 3-nitropropionic acid (3-NPA)-induced lipid peroxidation (formation of TBA-reactive substances) in rat striatal synaptosomes. Synaptosomal fractions were obtained from control, 3-NPA- (20 mg/kg/day for 3 days, i.p.), SE- (1 mg/kg/day for 5 days, i.p.), or SE + 3-NPA-treated animals. Mean values ± S.E.M. of 6 rats per group. *P<0.01, differences against control values; one-way ANOVA followed by Tukey’s test.

Fig. 2. Effect of saffron extract (SE) on 3-nitropropionic acid (3-NPA)-induced mitochondrial dysfunction (decreased reduction of MTT) in rat striatal synaptosomes. Synaptosomal fractions were obtained from control, 3-NPA- (20 mg/kg/day for 3 days, i.p.), SE- (1 mg/kg/day for 5 days, i.p.), or SE + 3-NPA-treated animals. Mean values ± S.E.M. of 6 rats per group. *P<0.05 and **P<0.01, differences against control values; one-way ANOVA followed by Tukey’s test.
Fig. 3. Effect of saffron extract (SE) on 3-nitropropionic acid (3-NPA)-induced lipid peroxidation (formation of lipid fluorescent products) in rat brain homogenates. Crude homogenates were incubated at 37°C for 60 minutes in the presence of 3-NPA (100 µM) and/or SE (5, 10 or 20 µg/ml). Mean values ± S.E.M. of 6 experiments per group. *P<0.05 and **P<0.01, differences against control values; one-way ANOVA followed by Tukey’s test.