Antioxidant propolis attenuates kainate-induced neurotoxicity via adenosine A1 receptor modulation in the rat

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Abstract

We examined the effects of the antioxidant propolis on seizures induced by kainic acid (KA). Sprague–Dawley rats received propolis (75 and 150 mg/kg, p.o.) five times at 12 h intervals. KA (10 mg/kg, i.p.) was injected 1 h after the last propolis treatment. Pretreatment with propolis significantly attenuated KA-induced seizures and KA-induced increases in hippocampal AP-1 DNA binding activity in a dose-dependent manner. KA induced increases in the levels of malondialdehyde and protein carbonyl, and a decrease in the ratio of GSH/GSSG. These oxidative stresses and neuronal degenerations were significantly attenuated by pretreatment with propolis. The neuroprotective effects of propolis appeared to be counteracted by adenosine receptor antagonists [A\textsubscript{1} antagonist, 8-cyclopentyl-1,3-dimethylxanthine (25 or 50 \textmu g/kg); A\textsubscript{2A} antagonist, 1,3,7-trimethyl-8-(3-chlorostyryl)xanthine (0.5 or 1 mg/kg); and A\textsubscript{2B} antagonist, alloxazine (1.5 or 3.0 mg/kg)]. However, this counteraction was most pronounced in the presence of the A\textsubscript{1} antagonist. Our results suggest that the protective effect of propolis against KA-induced neurotoxic oxidative damage is, at least in part, via adenosine A\textsubscript{1} receptor modulation.

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Accumulating evidence indicates that oxidative stress may contribute to the development of the seizures induced by kainic acid (KA) [1,4,6,8–10]. KA has been used as a model for both temporal lobe epilepsy and neurodegenerative disorders [19].

Honeybee propolis has been widely used as a folk medicine. It has been shown to have broad biological activities, which are principally attributed to the presence of flavonoids (major component; rutin, quercetin, galangin, etc.) [7] and caffeic acid phenethyl ester (CAPE) [13]. The prevailing opinion is that the broad biological activities of flavonoids and CAPE are related, in part, to their antioxidant actions [7]. A careful review of the literature indicates that the effects of propolis on neurodegenerative disorders have not yet been demonstrated.

The purine nucleotide adenosine might act as a neuromodulator of the CNS and an endogenous neuroprotectant [2,18]. Adenosine exerts its effects on the neuronal activity via four G protein-coupled receptors, A\textsubscript{1}, A\textsubscript{2A}, A\textsubscript{2B} and A\textsubscript{3} [2]. While adenosine A\textsubscript{1} and A\textsubscript{2B} receptors are widely distributed in the brain, the A\textsubscript{2A} receptor distribution is restricted to the dopamine-nerve terminal regions [2]. A\textsubscript{3} receptors are expressed at low levels in the brain [2]. It has been demonstrated that adenosine A\textsubscript{1} receptors are reduced in the temporal lobe of epileptic [5] and experimental epileptic brains [3]. Furthermore, the adenosine release evoked by KA may be involved in the production of free radicals [1]. In the present study, we examined the role of adenosine receptors in the propolis-mediated pharmacological action in response to KA insult.
All animals were treated in strict accordance with the NIH Guide for the Humane Care and Use of Laboratory Animals. Male Sprague–Dawley rats (Bio Genomics, Charles River Technology, Gapyung-Gun, Gyeonggi-Do, South Korea) weighing about 250 g were maintained on a 12:12 light/dark cycle and fed ad libitum. They were adapted to these conditions for 2 weeks before KA (10 mg/kg, i.p.) administration. All rats were drug and seizure naive before the experiment. Ethanol-extracted propolis (75 or 150 mg/kg; Sigma, St. Louis, MO) suspended in vehicle (2% Tween 80 plus 2% sodium lauryl sulfate in the saline solution) was administered orally five times at 12 h intervals. Control rats received the same volume of vehicle. One hour after the last administration of propolis, KA (10 mg/kg) was administered intraperitoneally.

Thirty minutes before KA, some rats received one of the following adenosine receptor antagonists: the A1 antagonist, 8-cyclopentyl-1,3-dimethylxanthine (CPT, 25 or 50 μg/kg, i.p.; RBI, Natick, MA); the A2A antagonist, 1,3,7-trimethyl-8-(3-chlorostyryl)xanthine (CSC, 0.5 or 1.0 mg/kg, i.p.; Sigma, St. Louis, MO); or the A2B antagonist, alloxazine 8-(3-chlorostyryl)xanthine (CSC, 0.5 or 1.0 mg/kg, i.p.; Sigma, St. Louis, MA). Animals were sacrificed 4 and 48 h after KA administration. Brains were rapidly removed, and hippocampi were dissected and then stored at −80 °C.

Using an automated video tracking system (Noldus Information Technology, Wageningen, The Netherlands) [10], seizure activity was rated according to the scale devised by Racine [16] during a 4 h period following KA challenge. We examined hippocampal AP-1 DNA binding activity following KA to confirm possible convulsant or anticonvulsant effects [15]. The AP-1 oligomer was purchased from Stratagene (La Jolla, CA). Double-stranded oligomer (25 ng) was labeled with [γ-32P]ATP using 10 units of T4 polynucleotide kinase (U.S. Biochemicals, Cleveland, OH). The AP-1 oligomer (22-mer; 5'-CTAGTGATGACCGGTCAGTG-3') contained the consensus sequence (5'-TGAGTCA-3'). To characterize AP-1 DNA binding activity, nuclear extracts were preincubated with a 100-fold excess of unlabeled AP-1 oligomer. A 100-fold excess of an unrelated oligomer (NFI; 5'-TAGGGGTG-GAGTCTCCATG-3'; Research Genetics, Huntsville, AL), normal rabbit serum (NRS; Dako Corporation, Carpinteria, CA) or bovine serum albumin (BSA; Sigma, St. Louis, MO) were used as controls. The addition of cold AP-1 oligomer showed that the AP-1 DNA binding activity was specific. The addition of NRS or NFI did not affect DNA binding. BSA also did not bind to the DNA probe.

The amount of lipid peroxidation was determined [10] by measuring the accumulation of thiobarbituric acid-reactive substances in homogenates of hippocampus, and was expressed as the malondialdehyde (MDA) content. The extent of protein oxidation in the hippocampus was assessed by determining the quantity of protein carbonyl groups, which was measured spectrophotometrically using the 2,4-dinitrophenylhydrazine (DNPH) labeling procedure as described by Oliver et al. [14]. Protein was measured using a BCA protein assay reagent (Pierce, Rockford, IL). GSH and GSSG from dissected hippocampal tissues were measured immediately using a minor modification [10] of the method described by Reed et al. [17]. Cresyl violet stain was used to examine the degeneration of neurons [10]. The significance of the differences in the ratio of convulsing was assessed by chi-square test. Significant differences in seizures were analyzed using the nonparametric Wilcoxon signed rank test. The differences in the levels of MDA, protein carbonyl, GSH, GSSG and neuronal density were compared by Student's t-test for paired data.

Animals pretreated with saline (vehicle) and then given 10 mg/kg of KA showed robust behavioral seizures (3.4 ± 0.6) that lasted 4–5 h. Thirty-one of 36 animals (86.1%) exhibited seizures. Pretreatment with propolis significantly protected against the KA-induced convulsive behaviors in a dose-related manner.

Although the A2A receptor antagonist CSC and the A2B receptor antagonist ALL both appeared to counteract the anticonvulsant effects of propolis slightly, the effect of the A1 receptor antagonist CPT was the most significant (Fig. 1A). There was a low level of AP-1 DNA binding activity in the absence of KA. A significant increase (P < 0.01) in AP-1 DNA binding activity occurred 4 h after KA administration, and was still evident after 48 h. In contrast, the pretreatment with propolis caused significant reductions in AP-1 DNA binding activity (for propolis at 75 mg/kg, P < 0.05; for propolis at 150 mg/kg, P < 0.01) in a dose-related fashion. Consistently at each time-point, CPT most significantly reversed the propolis-induced reduction in AP-1 DNA binding activity, although CSC and ALL both altered propolis-modulated AP-1 DNA binding activity (Fig. 1B, C).

KA produced significant increases in the levels of MDA [unit = nmol/g wet tissue; vehicle: 121.2 ± 13.3 (n = 6) vs. KA: 172.6 ± 12.1 (n = 6), P < 0.01] and carbonyl [unit = nmol/mg protein; vehicle: 4.80 ± 0.52 (n = 6) vs. KA: 7.88 ± 0.38 (n = 6), P < 0.01] which were evident at 4 h after KA administration. The increases in lipid peroxidation and protein oxidation were significantly decreased in a dose-related fashion by pretreatment with propolis [MDA; KA: 172.6 ± 12.1 (n = 6) vs. propolis 75 or 150 mg/kg plus KA: 151.9 ± 9.8 (n = 6) or 133.1 ± 6.8 (n = 6), P < 0.05 or P < 0.01; carbonyl; KA: 7.88 ± 0.38 (n = 6) vs. propolis 75 or 150 mg/kg plus KA: 6.95 ± 0.62 (n = 6) or 5.33 ± 0.28 (n = 6), P < 0.05 or P < 0.01]. CPT dose-dependently reversed the significant propolis-mediated reductions in the levels of MDA [CPT 25 μg + propolis 150 mg/kg plus KA: 146.3 ± 14.1 (n = 6); propolis 150 mg/kg plus KA: 133.1 ± 6.8 (n = 6) vs. CPT 50 μg + propolis 150 mg/kg plus KA: 174.9 ± 12.6 (n = 6), P < 0.05] and carbonyl [CPT 25 μg + propolis 150 mg/kg plus KA: 6.25 ± 0.32 (n = 6); propolis 150 mg/kg plus KA: 5.33 ± 0.28 (n = 6) vs. CPT 50 μg + propolis 150 mg/kg plus KA: 7.80 ± 0.43 (n = 6),
metric Wilcoxon signed rank test or Student’s t-test for convulsing). a

Veh+Veh: mean value is the mean animals in each group for AP-1 DNA binding activity were used. Each group for seizure score and convulsing are in parentheses. Four animals in each group for AP-1 DNA binding activity were used. Each value is the mean ± SEM. *P < 0.002 vs. Veh + KA, **P < 0.001 vs. Veh + KA, ¢P < 0.02 vs. P150 + KA, ¥P < 0.001 vs. P150 + KA (χ² test for convulsing). P < 0.01 vs. Veh + Veh, ¢P < 0.05 vs. Veh + P150, ¥P < 0.01 vs. Veh + KA, ¥P < 0.01 vs. Veh + P150, ¥P < 0.05 vs. Veh + KA, ¥P < 0.01 vs. P150 + KA, ¥P < 0.01 vs. P50 + KA (seizure scores or AP-1 DNA binding activity were analyzed using the non-parametric Wilcoxon signed rank test or Student’s t-test for paired data).

$P < 0.05$, However, CSC [MDA; propolis 150 mg/kg plus KA: 133.1 ± 6.8 (n = 6) vs. CSC 0.5 or 1.0 mg/kg + propolis 150 mg/kg plus KA: 147.2 ± 10.4 (n = 6) or 150.8 ± 10.6 (n = 6); carbonyl: 150 mg/kg plus KA: 5.33 ± 0.28 (n = 6) vs. CSC 0.5 or 1.0 mg/kg + propolis 150 mg/kg plus KA: 6.42 ± 0.62 (n = 6) or 6.85 ± 0.57 (n = 6)], but not ALL, appeared to affect the significant decreases in these oxidative indexes caused by propolis. The KA-induced increase in the MDA and carbonyl value returned to near control (vehicle) levels by 48 h after KA treatment.

No significant differences were observed in the ratio of GSH [unit = nmol/g wet tissue; vehicle: 1.48 ± 0.12 (4 h point) or 1.55 ± 0.11 (2 day point)]/GSSG [unit = nmol/g wet tissue; vehicle: 0.030 ± 0.003 (4 h) or 0.029 ± 0.004 (2 days)] in the absence of KA. After KA treatment, the GSH/GSSG ratio in the hippocampus time-dependently decreased ($P < 0.01$) [4 h after KA, GSH: 1.05 ± 0.08 (n = 6), GSSG: 0.044 ± 0.003 (n = 6); 2 days after KA, GSH: 0.81 ± 0.14 (n = 6), GSSG: 0.057 ± 0.003 (n = 6)]. However, propolis dose-dependently prevented in the GSH/GSSG ratio in the hippocampus (4 h point, propolis 150 mg/kg plus KA vs. KA, $P < 0.05$; 2 day point, propolis 75 or 150 mg/kg plus KA vs. KA, $P < 0.05$ or $P < 0.01$) after KA administration [4 h after KA, GSH: 1.25 ± 0.10 (plus propolis 75 mg/kg) or 1.41 ± 0.10 (plus propolis 150 mg/kg); 2 days after KA, GSH: 1.11 ± 0.11 (plus propolis 75 mg/kg) or 1.24 ± 0.13 (plus propolis 150 mg/kg), GSSG: 0.048 ± 0.004 (plus propolis 75 mg/kg) or 0.042 ± 0.003 (plus propolis 150 mg/kg)]. CPT was the most efficacious in counteracting the significant reductions in the GSH/GSSG ratios mediated by propolis, although both CSC and ALL also appeared to alter the propolis-mediated GSH status (Fig. 2).

The neuronal layers of the hippocampus in the animal without KA were clearly visible with cresyl violet staining. At 2 days after KA injection, significant neuronal losses were observed in the CA1 and CA3 regions; these losses were significantly blocked by pretreatment with propolis at

![Fig. 1.](image1.png)

![Fig. 2.](image2.png)
activity after KA-induced seizures, we cannot rule out the possibility that propolis inhibits the induction of AP-1 transcription factors that respond within hours to seizure-evoked oxidative stress.

KA-induced losses in GSH homeostasis may represent an important index of cellular damage [4,10] because there is a correlation between neuronal loss induced by seizures and the impairment of GSH status [10]. Interestingly, we also observed that impairment of the GSH system occurred in the hippocampus before histological signs of neuronal death. Recently, we demonstrated that cytosolic Cu/Zn-superoxide dismutase (SOD-1) and mitochondrial Mn-superoxide dismutase (SOD-2) protein expressions disappeared within 12 h after KA injection, before significant pyramidal cell losses in the hippocampus [8,9]. Thus, it is possible that the impairment of GSH status in the early stage of a KA insult could be a sensitive index of oxidative stress [4,10]. Recently, it was recognized that a prolonged depletion of endogenous GSH may result in an accumulation of reactive oxygen radicals generated through activation of the KA receptor, which could facilitate the expression of the AP-1 transcription factor in murine hippocampus [11].

Thus, we postulate that propolis significantly blocks seizure-induced neuronal loss by attenuating the impairment of GSH metabolism via, in part, adenosine A1 receptor modulation. The novel antioxidant/anticonvulsant effect could be an important contribution to extending the neuroprotective potential of propolis.

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**References**


