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Paradoxical effects of adenosine receptor ligands on hydroxyl radical generation by L-DOPA in the rat striatum

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Abstract:
Parkinson's disease (PD) is a progressive neurodegenerative disorder associated with selective loss of dopaminergic neurons in substantia nigra pars compacta. Among the proposed mechanisms of dopaminergic degeneration, oxidative stress is believed to play an important role. On the other hand, L-DOPA used as the main medication in PD and overproduction of dopamine (DA) in striatal neurons could elicit toxic effects due to formation of free radicals (FRs). Adenosine, an endogenous neuromodulator was shown in various experimental models to have neuroprotective properties. In our study, we investigated the role of adenosine A_1 and A_{2A} receptor ligands in hydroxyl radical generation by L-DOPA in the rat striatum. The hydroxyl radical was assayed by HPLC-ED as a product of its reaction with p-hydroxybenzoic acid (PBA). Intrastriatal infusion of L-DOPA (50 μM) markedly increased dialysate level of DA and 3,4-dihydroxybenzoic acid (3,4-DHBA). An adenosine A_1 receptor agonist N^6-cyclopentyladenosine (CPA, 25–50 μM), non-selective A_1/A_{2A} receptor agonist 2-chloroadenosine (2-CADO, 50–100 μM), and selective A_{2A} receptor agonist CGS 21680 (25–50 μM) decreased the level of 3,4-DHBA. A non-selective A_1/A_{2A} adenosine receptor antagonist caffeine (100 μM) produced similar effect on 3,4-DHBA level. At the same time, CPA and 2-CADO, but not CGS 21680 or caffeine, decreased L-DOPA-induced DA release. The adenosine receptor ligands alone only weakly changed extracellular DA level and did not influence hydroxyl radical production. However, they showed scavenging activity in Fenton reaction *in vitro*. The primary caffeine metabolite in rodents, 1,3,7-trimethyl uric acid (1,3,7-mUA) decreased both, DA synthesis and 3,4-DHBA level. Thus, paradoxically, both agonists of A_1 receptor and agonist of A_{2A} receptor as well as antagonist of A_1 and A_{2A} receptors (caffeine), all decreased generation of FRs. Our study suggests that a decrease in hydroxyl radical generation caused by adenosine receptor ligands results from attenuation of L-DOPA-induced DA release or from their scavenging activity.

Key words:
hydroxyl radical, L-DOPA, adenosine, *in vivo* microdialysis

Abbreviations: aCSF – artificial cerebrospinal fluid, 2-CADO – 2-chloroadenosine, CGS 21680 – 2-*p*-(2-carboxyethyl) phenethylamino-5'-N-ethylcarboxamidoadenosine, CPA – N^6-cyclopentyladenosine, DA – dopamine, 3,4-DHBA – 3,4-dihydroxybenzoic acid, L-DOPA – 3,4-dihydroxyphenylalanine, DOPAC – 3,4-dihydroxyphenylacetic acid, DMSO – dimethylsulfoxide, FRs – free radicals, GPx – glutathione peroxidase, GSH – glutathione, HPLC – high performance liquid chromatography, HVA – homovanillic acid, MAO – monoamine oxidase, 1,3,7-mUA – 1,3,7-trimethyl uric acid, NO – nitric oxide, $ONOO^-$ – peroxynitrite, ONOOH – peroxynitrous acid, PD – Parkinson's disease, PBA – p-hydroxybenzoic acid

Introduction

Free radicals (FRs) are generated under normal and pathological conditions. Due to the presence of an un-

paired electron, FRs are highly unstable and tend to react with cellular elements. Particularly, the most reactive hydroxyl radical, when generated in excess, causes cellular damage leading to cell death [26]. Hydroxyl radical is generated *via* the Fenton reaction from hydrogen peroxide in the presence of ferrous ions or *via* the Heber-Weiss reaction from hydrogen peroxide and superoxide radical [27].

$Fe^{2+} + H_2O_2 = Fe^{3+} + {}^{\bullet}OH + OH^{-}$ (Fenton's reaction)

$H_2O_2 + {}^{\bullet}O_2^{-} = O_2 + OH^{-} + {}^{\bullet}OH$ (Haber-Weiss reaction)

Brain antioxidant defense system includes the antioxidant enzymes: superoxide dismutase (SOD) that removes hydroxyl radical by catalyzing its dismutation [26] and glutathione peroxidase (GPx) which catalyzes reaction of hydrogen peroxide with a thiol-containing tripeptide, glutathione (GSH) [8]. The brain is also rich in non-enzymatic antioxidants, such as ascorbic acid [46], α-tocopherol [28], coenzyme Q [5], which respond, together with antioxidant enzymes, to hydrogen radicals, and constitute the antioxidant brain defense system. An impairment of the brain defense system and overproduction of various reactive species causes oxidative stress which plays an important role in pathogenesis of several neurodegenerative disorders such as Parkinson's disease (PD), Alzheimer's disease, amyotrophic lateral sclerosis, Huntington's disease [26]. In PD, oxidative stress induced by FRs damages neuronal membrane lipids, proteins and other components of brain tissue. There are several potential sources of the increased FR production in PD, including mitochondrial dysfunction, increased free iron levels and increased dopamine (DA) metabolism [39]. Mitochondrial dysfunction and particularly defect of mitochondrial complex-I of the respiratory chain contributes to oxidative stress and neuronal damage [39].

A number of data have shown that catecholamines and particularly dopamine (DA) are an important source of FRs in the brain [12]. As long as DA is stored in synaptic vesicles, it is stable. However, when it is in excess in cytosol, then it is easily metabolized by monoamine oxidase (MAO) to produce hydrogen peroxide or by autooxidation to form quinones [3]. Autooxidation of DA or L-DOPA *via* quinone formation generates FRs such as superoxide radical and hydrogen peroxide. Moreover, DA and L-DOPA quinones are easily oxidized to aminochromes and finally polymerize to form melanin [3]. Thus, L-DOPA therapy leading to high brain concentration of DA may potentially contribute to progression of oxidative damage of DAergic cells in patients with PD.

Prevention of oxidative stress with low molecular weight antioxidants, such as ascorbate, α-tocopherol, β-carotene, coenzyme Q, glutathione or drugs displaying scavenging properties such as DA agonists (pergolide, ropinirole, apomorphine), selegiline, tolcapone has been proposed as neuroprotective therapy in various neurodegenerative disorders [9, 15, 23, 26, 31]. Recently, it has been suggested that a neuromodulator adenosine may be considered to be an endogenous neuroprotectant as shown by a number of evidences [17, 19, 40, 51]. Adenosine acts through four types of G-protein-coupled receptors A_1, A_{2A}, A_{2B}, A_3 which are expressed on neurons and non-neuronal cells [22, 44]. A_1 receptors have widespread brain distribution, while A_{2A} receptors are mainly located in regions with dopaminergic innervation, such as the striatum, nucleus accumbens, tuberculum olfactorium. A density of A_{2B} and A_3 receptors in the brain is relatively low [17, 20]. By activating A_1 receptors, adenosine depresses neuronal activity and release of neurotransmitters, whereas by activating presynaptic A_{2A} receptors, it stimulates neurotransmitter release or increases neuronal activity through postsynaptic A_{2A} receptors [17]. Thus, adenosine can alter synaptic level of neurotransmitters such as DA or glutamate [25] and can influence the secretion of cytokines, growth factors, glutamate, nitric oxide (NO) from non-neuronal cells such as astrocytes or microglia [14, 37, 47]. A contribution of these factors to the generation of oxidative stress is well established [26, 29, 50]. Moreover, adenosine level and A_{2A} receptor-density are altered in the striatum of DA-lesioned animals or in brains of parkinsonian patients [30, 42]. Thus, activity of adenosine receptors in controlling cellular redox state may be crucial for initiation of oxidative stress.

Epidemiological data as well as animal models of PD suggest that caffeine, a weak and non-selective A_1/A_{2A} adenosine receptor antagonist is neuroprotective [4, 48]. The mechanism of this neuroprotection is not fully understood, but the inhibition of MAOB activity and scavenging of FRs by caffeine have been proposed as causative factors of this neuroprotection [13, 36]. Moreover, both the adenosine receptor agonists as well as antagonists have shown to be active neuroprotectants in various animal models of neurotoxicity [7, 19, 40, 48]. Our study was undertaken to determine whether activation of adenosine A_1 and A_{2A} receptors by agonists or their blockade by caf-

feine may influence production of FRs induced by the administration of exogenous L-DOPA. A_1 adenosine receptor agonists N^6-cyclopentyladenosine (CPA) and 2-chloroadenosine (2-CADO), an A_{2A} receptor agonist 2-*p*-(2-carboxyethyl)phenethylamino-5'-N-ethylcarboxamidoadenosine (CGS 21680) and caffeine were applied into the striatum through microdialysis probe and their effect on FR generation induced by infusion of L-DOPA was studied. The primary metabolite of caffeine in rodents is 1,3,7-trimethyl uric acid (1,3,7-mUA), as demonstrated in rat liver microsomes [6, 18]. It is also known that xanthines are substrates of xanthine oxidase, and this reaction predominantly generates the superoxide radical [34]. Therefore, we also investigated whether both caffeine and its biologically active metabolite1,3,7-mUA are involved in the process of FR generation in the rat striatum. Additionally, we also studied the effect of adenosine receptor ligands on FR generation in Fenton reaction *in vitro* to establish the possible scavenging properties of adenosine agonists and caffeine.

Materials and Methods

Animals

The microdialysis studies were conducted using male Wistar rats (250–300 g) bred at the Institute of Pharmacology, the Polish Academy of Sciences, Kraków, Poland. The rats were housed in temperature- and humidity-controlled rooms with a 12-h light/dark cycle, with *ad libitum* access to filtered tap water and standard pelleted laboratory chow throughout the study. The experimental procedures and housing conditions used were in strict accordance with the Polish governmental regulations concerning experiments on animals (Dz. U. 05.33.289). All the experimental protocols were approved by the Local Ethics Commission for Experimentation on Animals.

Drugs

L-3,4-Dihydroxyphenylalanine (L-DOPA), N^6-cyclopentyladenosine (CPA), 2-chloroadenosine (2-CADO), 2-*p*-(2-carboxyethyl)phenethylamino-5'-N-ethylcarboxamidoadenosine (CGS 21680) and caffeine were obtained from RBI (Poznań, Poland), while 1,3,7-trimethyl uric acid (1,3,7-mUA), benserazide and p-hydroxybenzoic acid (PBA) were obtained from Sigma-Aldrich (Poznań, Poland). All the chemicals used for high performance liquid chromatography (HPLC) were purchased from Merck (Warszawa, Poland). L-DOPA, caffeine as well as CPA, 2-CADO, CGS 21680 and 1,3,7-mUA were dissolved in an artificial cerebrospinal fluid (aCSF) or in a small amount of dimethylsulfoxide (DMSO) and then diluted to an appropriate volume with aCSF. The final concentration of DMSO in the perfusion fluid did not exceed 0.02–0.05%.

Microdialysis

Rats were anesthetized with ketamine (75 mg/kg *im*) and xylazine (10 mg/kg *im*) and placed into a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA). The skulls were exposed and small holes were drilled for insertion of the microdialysis probes in the striatum using the following coordinates: 1.8 mm anterior from the bregma; 2.8 mm lateral from the sagittal suture; –7.0 mm ventral from the dura [41]. Vertical microdialysis probes were constructed as described in detail elsewhere [24]. All probes were connected to a syringe pump (BAS, IN, USA) which delivered an aCSF composed of [mM]: NaCl 145, KCl 2.7, $MgCl_2$ 1.0, $CaCl_2$ 1.2; pH = 7.4 at a flow rate of 1.5 μl/min. Baseline samples were collected every 20 min after the washout period to obtain a stable extracellular neurotransmitter level. Appropriate drugs were then administered and dialysate fractions were collected for 240 min. PBA given in order to bind hydroxyl radical was infused together with the tested drugs. At the end of the experiment, the rats were sacrificed and their brains were histologically examined to validate probe placement.

Analytical procedure

DA, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were analyzed by HPLC with electrochemical detection. Chromatography was performed using an LC-10 AD pump (Shimadzu Europa GmbH, Warszawa, Poland), LC-4B amperometric detector with a cross-flow detector cell (BAS, IN, USA) and BDS-Hypersil C18 analytical column (3 × 100 mm, a 3 μm, Thermo Electron Corp., UK). The mobile phase was composed of 0.1 M monochloroacetic acid adjusted to pH = 3.7 with 3 M sodium hydroxide, 0.5 mM EDTA, 13 mg/l 1-octanesulfonic acid sodium salt, a 5.7% methanol and a 0.5% aceto-

nitrile. The flow rate was 0.5 ml/min, and the applied potential of a 3 mm glassy carbon electrode was +600 mV with a sensitivity of 2 nA/V. The level of hydroxyl radical was estimated as its reaction product with PBA, 3,4-dihydroxybenzoic acid (3,4-DHBA), and measured in striatal dialysates together with DA and its metabolites. The chromatographic data were processed by Chromax 2001 (Pol-Lab, Warszawa, Poland) software run on a personal computer. The values were not corrected for *in vitro* probe recovery, which was approximately 10–15%.

Fenton reaction

The hydroxyl radical was generated from hydrogen peroxide in the presence of ferrous iron. Typically, 50 µl of 0.6% hydrogen peroxide, 50 µl of 50 µM $FeCl_2$/EDTA and 400 µl of PBA were mixed with an appropriate concentrations of adenosine ligands (all dissolved in 100 mM phosphate buffer, pH = 7.0, 37°C) and incubated for 10 min. The reaction was terminated by injection of 1 µl of incubation mixture to HPLC. Hydroxyl radical was detected as its reaction product with PBA as described above.

Data analysis

An average concentration of three stable samples prior to drug administration was regarded as a control value and was considered to be 100%. The statistical significance was calculated using repeated-measures ANOVA, followed by Tukey's *post-hoc* test. The results were considered statistically significant when $p < 0.05$.

Results

Effect of L-DOPA on DA, DOPAC, HVA and 3,4-DHBA level in dialysates from the rat striatum

L-DOPA given peripherally (100 mg/kg in the presence of benserazide 50 mg/kg) or in local infusion

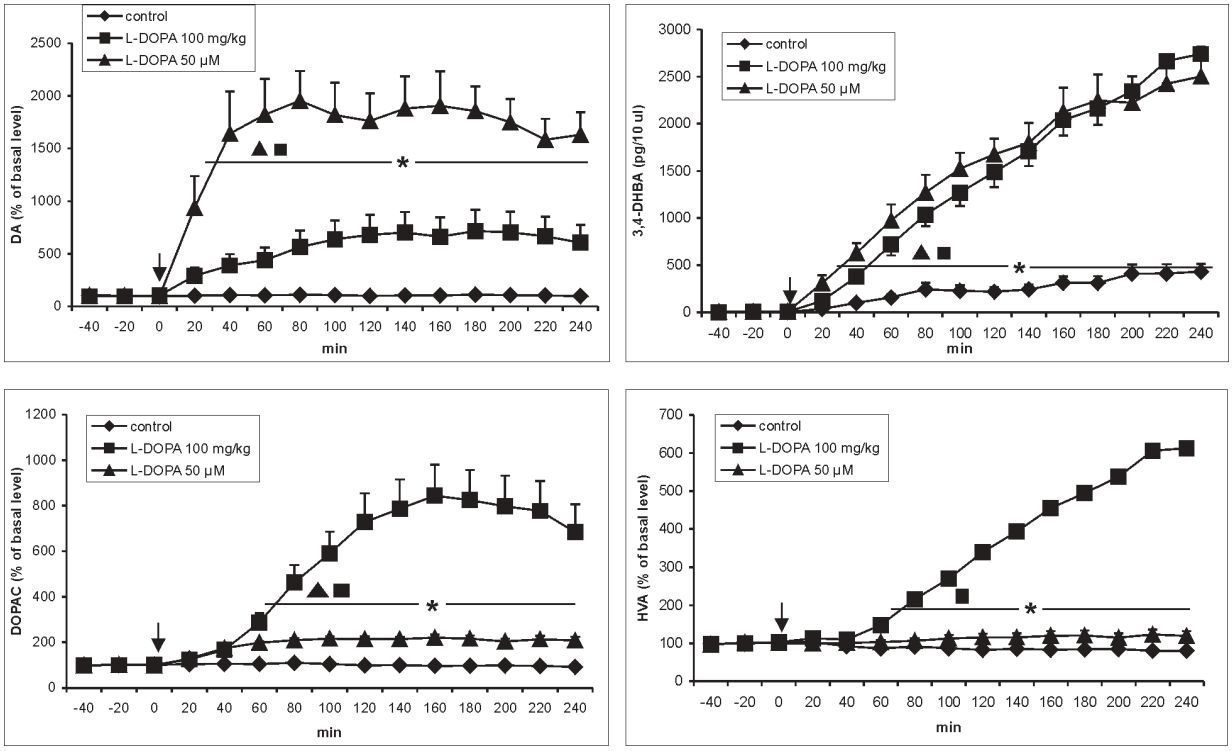

Fig. 1. A comparison of the effect of local and peripheral L-DOPA administration on DA, DOPAC, HVA and 3,4-DHBA level in the rat striatal dialysates. L-DOPA was given locally through the microdialysis probe at a concentration of 50 µM or peripherally at a dose of 100 mg/kg together with benseraside at 50 mg/kg (indicated by an arrow). Time-course of the effect is shown. Each value is the mean ± SEM of 8–13 measurements. The basal concentrations of DA, DOPAC and HVA (pg/10 µl) in a control group were: 13.0 ± 2.09, 2752 ± 220, 2015 ± 128; in the group treated with a 50 µM L-DOPA: 10.0 ± 1.23, 2453 ± 255, 2020 ± 203, respectively; in the group treated with L-DOPA at a dose of 100 mg/kg: 9.0 ± 0.63, 2723 ± 243, 1986 ± 169, respectively. * $p < 0.01$ different from basal level

(50 μM) through microdialysis probe significantly increased DA, DOPAC and HVA over basal level (Fig. 1). L-DOPA infused locally was more potent in raising extracellular DA concentration as maximal increase reached ca. 2,000% of basal level, while L-DOPA given peripherally increased the extracellular DA concentration to ca. 500% of basal level. On the other hand, the local infusion of L-DOPA was less effective in increasing extracellular level of DOPAC and HVA (Fig. 1). L-DOPA infused through a microdialysis probe enhanced DOPAC concentration to 200% of basal level and was without influence on HVA concentration, while L-DOPA given peripherally increased level of both DOPAC and HVA to similar extent, to ca. 600–800% of basal level. L-DOPA applied locally or peripherally increased 3,4-DHBA level to a similar extent reaching values of ca. 2,500% at 240 min after administration (Fig. 1).

Effect of adenosine receptor agonists on L-DOPA-induced increase in the striatal level of DA and 3,4-DHBA

A selective agonist of adenosine A_1 receptor CPA (25 and 50 μM) significantly and in a dose-dependent manner decreased L-DOPA-induced DA release (Fig. 2). 2-CADO, a less selective adenosine A_1 receptor agonist, also decreased DA level, but the lower 2-CADO concentration (50 μM) was more effective than the higher one (100 μM). The difference in DA level between L-DOPA treatment and joint application of L-DOPA and 2-CADO (50 μM) was significant from 80 to 240 min of infusion (Fig. 2). A selective A_{2A} adenosine receptor agonist, CGS 21680 (50 μM) slightly increased L-DOPA-induced DA release and the difference between control group and the group receiving CGS 21680 was significant from 180 to 240 min of agonist infusion (Fig. 2). In con-

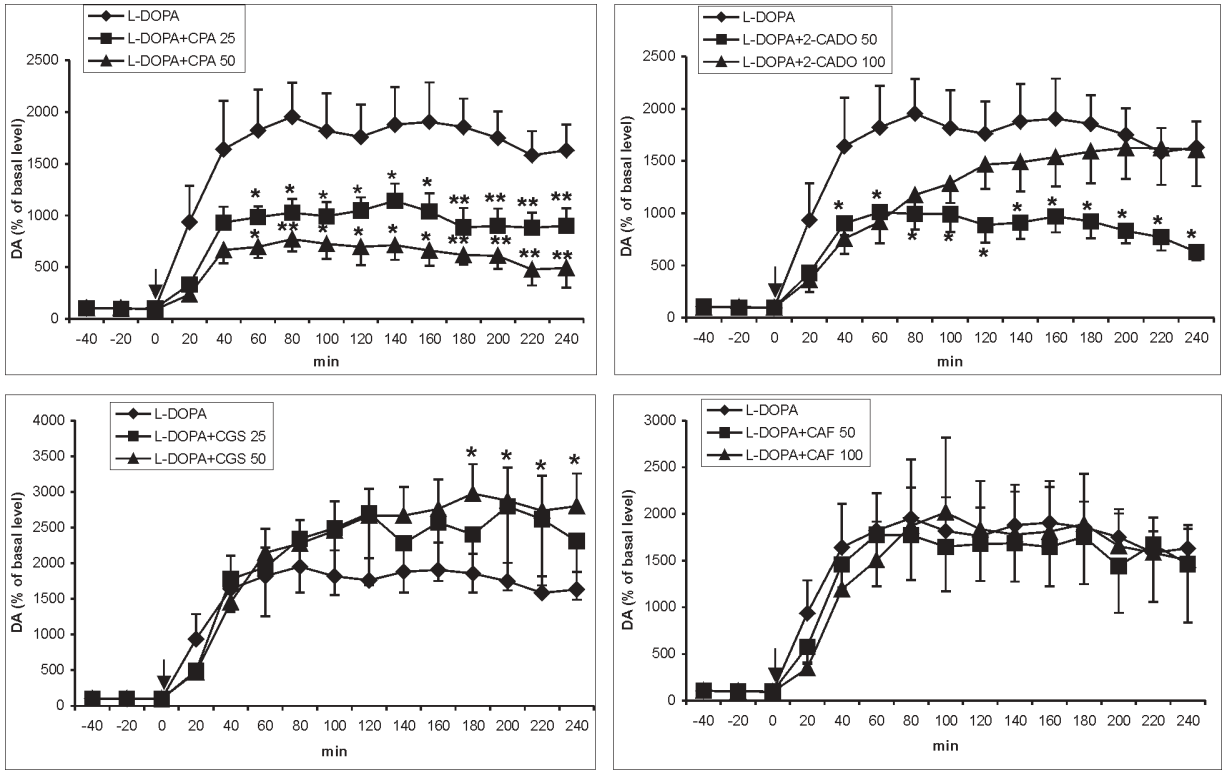

Fig. 2. Effect of adenosine A_1 receptor agonists CPA (25, 50 μM), 2-CADO (50, 100 μM), an A_{2A} adenosine receptor agonist CGS 21680 (25, 50 μM) and A_1/A_{2A} receptor antagonist caffeine (CAF, 50, 100 μM) on L-DOPA (50 μM)-induced increase in DA release in striatal dialysates. Time-course of the effect is shown. Start of drug infusion is indicated by an arrow. Each value is the mean ± SEM of 5–6 measurements. The basal concentration of DA (pg/10 μl) in control group was 13.0 ± 2.09; in the group treated with CPA 25 μM: 11.8 ± 0.86 and in the group treated with CPA 50 μM: 8.3 ± 1.02; in the group treated with CGS 21680 25 μM: 8.9 ± 1.29; in the group treated with CGS 21680 50 μM: 8.3 ± 0.69; in the group treated with CAF 50 μM: 8.0 ± 0.80; in the group treated with CAF 100 μM: 8.2 ± 1.21. * $p < 0.05$, ** $p < 0.01$ different from control group. All points of the curves corresponding to drug treatments were significantly different from the basal level ($p < 0.05$–0.01)

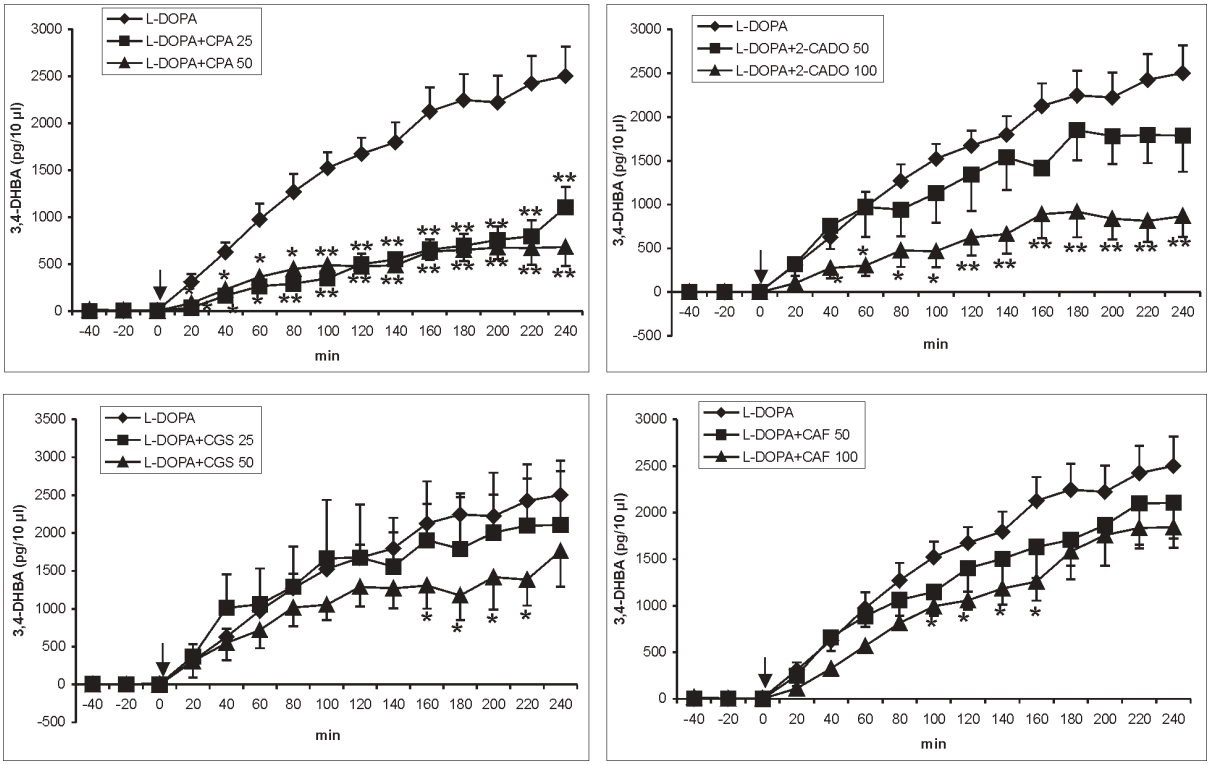

Fig. 3. Effect of adenosine A_1 receptor agonists CPA (25, 50 µM), 2-CADO (50, 100 µM), an A_{2A} adenosine receptor agonist CGS 21680 (25, 50 µM) and A_1/A_{2A} receptor antagonist caffeine (CAF, 50, 100 µM) on L-DOPA (50 µM)-induced increase in the striatal level of 3,4-DHBA. Time-course of the effect is shown. Start of drug infusion is indicated by an arrow. Each value is the mean ± SEM of 5–9 measurements. * $p < 0.05$, ** $p < 0.01$ different from control group. All points of the curves corresponding to drug treatments were significantly different from the basal level ($p < 0.05$–0.01)

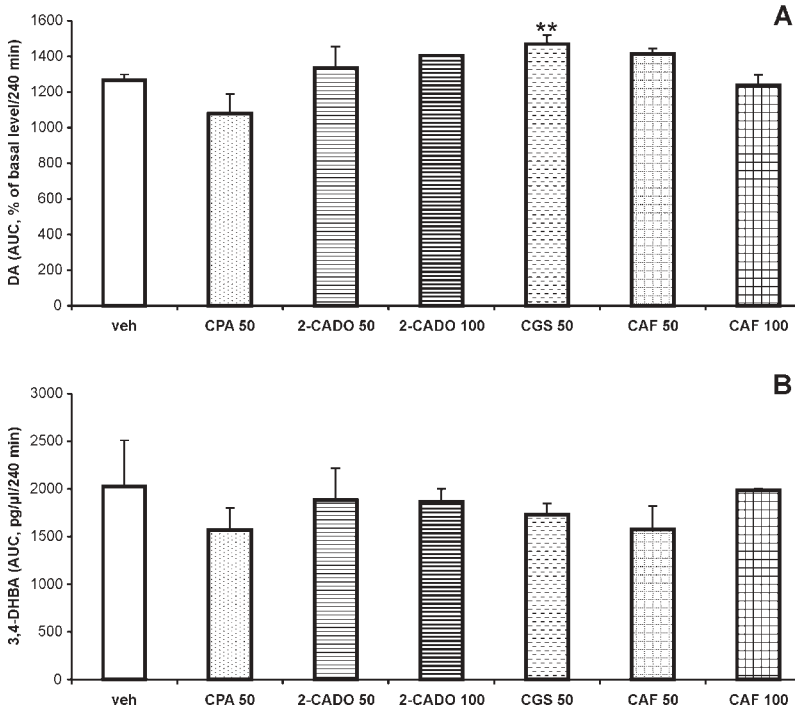

Fig. 4. Effect of adenosine receptor ligands on striatal level of DA **(A)** and 3,4-DHBA **(B)**. Total effect expressed in percent/240 min is shown as an area under the curve (AUC). Each value is the mean ± SEM of 5–14 measurements. The basal concentration of DA (pg/10 µl) in a control group was: 13.0 ± 2.1; in the group treated with CPA (50 µM): 14.7 ± 1.1; in the groups treated with 2-CADO 50 µM or 100 µM: 13.0 ± 0,54 and 10.6 ± 0,71, respectively; in the group treated with CGS 21680 (50 µM): 14.3 ± 0,81; in the groups treated with caffeine (CAF) 50 µM or 100 µM: 12.7 ± 2.3 and 12.4 ± 1.6, respectively. ** $p < 0.01$ different from control group

trast, a non-selective A_1/A_{2A} adenosine receptor antagonist caffeine (50 and 100 μM) did not influence L-DOPA-induced striatal release of DA (Fig. 2).

The striatal levels of DOPAC and HVA, increased by infusion of L-DOPA, were not changed by CPA, 2-CADO, CGS 21680 and caffeine (results not shown).

The formation of 3,4-DHBA by L-DOPA was significantly attenuated by both concentrations of CPA and the higher concentration of 2-CADO (Fig. 3). CGS 21680 (50 μM) significantly decreased 3,4-DHBA formation from 160 to 220 min after administration, whereas caffeine (100 μM) inhibited only slightly production of hydroxyl radical, but the difference between L-DOPA-treated group and the group receiving joint application of L-DOPA and caffeine (100 μM) was significant from 100 to 160 min of infusion (Fig. 3).

Effect of adenosine receptor ligands on striatal level of DA, DOPAC, HVA and 3,4-DHBA

A summary of the effect of adenosine receptor ligands given alone on DA and 3,4-DHBA level is shown in Figure 4A and 4B. 2-CADO and caffeine did not influence striatal extracellular DA level (Fig. 4A), CPA slightly decreased it (by 15%) while CGS 21680 significantly increased (by 16%) extracellular DA level [$F(1,13) = 9.7$; $p < 0.01$] (Fig. 4A). The production of 3,4-DHBA was slightly decreased by CPA and was not affected by 2-CADO, CGS 21680 and caffeine (Fig. 4B).

Effect of 1,3,7-trimethyl uric acid on L-DOPA-induced increase in the striatal level of DA, DOPAC, HVA and 3,4-DHBA

1,3,7-Trimethyl uric acid (1,3,7-mUA) (50 μM) given alone had no influence on DA, DOPAC, HVA level and hydroxyl radical formation (Fig. 5). On the other hand, L-DOPA-induced enhancement in DA, DOPAC and HVA level was attenuated by 1,3,7-mUA, but the effect of 1,3,7-mUA acid on DA and HVA level did not reach statistical significance (Fig. 5). Similarly, L-DOPA-induced production of hydroxyl radical was significantly inhibited by 1,3,7-mUA (Fig. 5). Statisti-

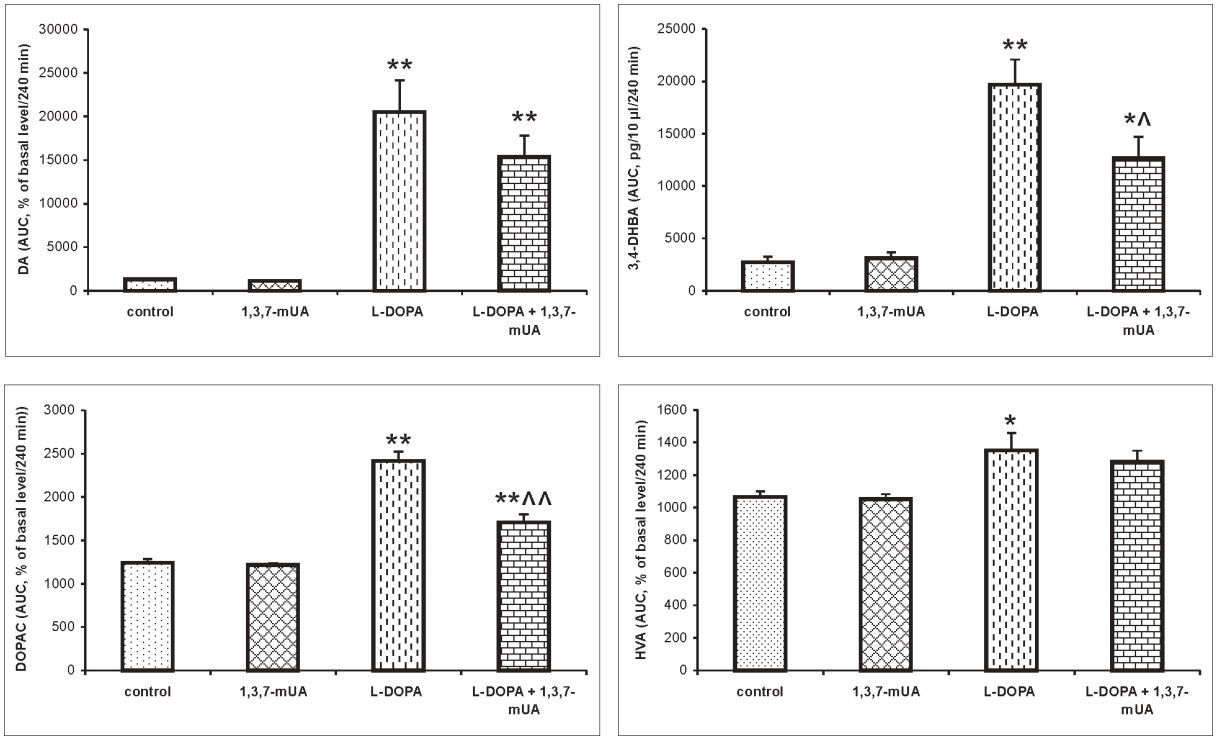

Fig. 5. Effect of 1,3,7-trimethyl uric acid (1,3,7-mUA, 50 μM) on L-DOPA (50 μM)-induced increase in the striatal level of DA, DOPAC, HVA and 3,4-DHBA. Total effect expressed in percent/240 min is shown as an area under the curve (AUC). Each value is the mean ± SEM of 6–9 measurements. The basal concentrations of DA, DOPAC and HVA (pg/10 μl) in a control group were respectively: 10.9 ± 1.21, 2752 ± 220 and 2015 ± 128; in the group treated with 1,3,7-mUA: 8.0 ± 1.0, 2633 ± 189 and 1819 ± 159, respectively; in the group treated with L-DOPA: 9.0 ± 0.9, 2453 ± 255 and 2020 ± 203; in a group treated with 1,3,7-mUA and L-DOPA: 6.9 ± 0.36, 3129 ± 269 and 2269 ± 118, respectively. * $p < 0.05$, ** $p < 0.001$ different from control group; ^ $p < 0.05$, ^^ $p < 0.01$ different from group treated with L-DOPA alone

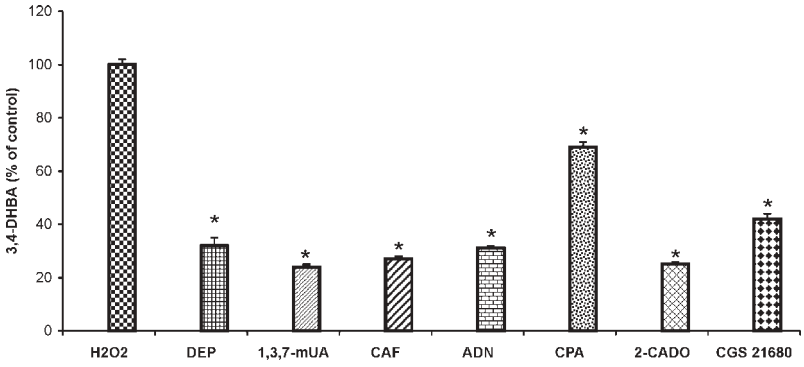

Fig. 6. Effect of adenosine receptor ligands CPA, 2-CADO, CGS 21680, caffeine (CAF), adenosine (ADN), R-(–)-deprenyl (DEP) and 1,3,7-mUA (all at a concentration of 1.67 mM) on *in vitro* formation of hydroxyl radical. Values are the mean ± SEM of 3 measurements and represent a percent of hydroxyl radical amount produced from hydroxyl peroxide (H_2O_2). ** $p < 0.001$ *vs.* hydroxyl peroxide group

cally significant differences in the overall effect between the group treated with L-DOPA and the group treated with L-DOPA and 1,3,7-mUA acid were as follows: for DA $F(1,11) = 1.45$, $p < 0.2$; for DOPAC $F(1,14) = 23.19$, $p < 0.001$; for HVA $F(1,14) = 0.27$, $p < 0.6$ and for 3,4-DHBA $F(1,13) = 4.36$, $p < 0.05$.

Effect of adenosine receptor ligands and 1,3,7-trimethyluric acid on *in vitro* formation of hydroxyl radical

Production of hydroxyl radical in Fenton reaction was diminished by CPA, 2-CADO, CGS 21680, 1,3,7-mUA and caffeine (Fig. 6). Adenosine and deprenyl showed similar free radical trapping activity (Fig. 6). Statistically significant differences between samples with hydroxyl peroxide alone and samples containing drugs were as follows: deprenyl – $F(1,4) = 6734$, $p = 0$; 1,3,7-mUA – $F(1,4) = 8438$, $p = 0$; caffeine – $F(1,4) = 7776$; $p = 0$; adenosine – $F(1,4) = 6936$, $p = 0$; CPA – $F(1,4) = 1350$, $p = 0$; 2-CADO – $F(1,4) = 8214$, $p = 0$; CGS 21680 – $F(1,4) = 4874$, $p = 0$.

Discussion

As reviewed in the introduction, there is some evidence showing a relationship between DA and hydroxyl radical generation. The current study confirms that a massive increase in the DA concentration in striatal dialysates after administration of L-DOPA is a source of hydroxyl radical formation. DA is degraded enzymatically by MAO and is transformed intraneuronally to DOPAC and hydrogen peroxide [16]. Then, in the presence of ferrous iron, hydrogen peroxide is transformed *via* Fenton reaction into hydroxyl radical [27]. In addition, DA is transformed by means of autoxidation into semiquinones and quinones followed by formation of hydrogen peroxide and superoxide radical [3]. In our study, peripherally administered L-DOPA increased synthesis of DA and its utilization through MAO, which was reflected by a marked increase in DOPAC and HVA. On the other hand, L-DOPA infused through a microdialysis probe was very effective in increasing DA release, but was less potent in increasing the level of its metabolites DOPAC and HVA. The differences in DA metabolism between groups treated peripherally and locally with L-DOPA may be related to the fact that L-DOPA given peripherally is incorporated into neurons in a more physiological manner than when it is applied directly into extracellular space in the brain. Most of L-DOPA transported from the periphery to the brain is converted to DA. Consequently, DA is then stored in synaptic vesicles or is released from dopaminergic neuronal terminals in a tonic or phasic manner. DA can be cleared from synaptic and extrasynaptic space by enzymes MAO and catechol-O-methyltransferase (COMT). There is evidence that a major portion of DOPAC converted further to HVA is derived from an intraneuronal pool of newly synthesized DA which has not been released [53]. On the other hand, L-DOPA exogenously applied *via* a microdialysis probe is converted into DA in extracellular space in the brain, constituting a very large portion of DA stored in the brain. However, deamination of extracellular DA is likely to occur outside the DAergic neurons, but this process is not as efficient as the one taking place inside DAergic neurons. For this reason the level of DOPAC and HVA converted from DA after local L-DOPA administration was much lower when compared to the level of those metabolites following peripheral injection of L-DOPA. Nevertheless, L-DOPA given by these two routs was equipotent in formation

of hydroxyl radicals in the rat striatum. Thus, the generation of hydroxyl radicals by means of DA autoxidation or intraneuronally by its enzymatic degradation with concomitant formation of hydrogen peroxide might induce oxidative stress. In our study, we had chosen local administration of all drugs, to avoid differences between adenosine ligands due to their uneven penetration from periphery to the brain. Assuming 10% recovery of the dialysis probe, the concentration of the studied drugs in the brain tissue should not exceed nM range, allowing for stimulation of adenosine receptors.

CPA and 2-CADO are known to be agents suppressing neuronal activity, and diminishing synthesis and release of DA [25, 52]. Thus, the inhibition of L-DOPA-induced DA synthesis by CPA and 2-CADO and the decrease in enzymatic degradation of cytosolic DA by these compounds explains the decrease in hydroxyl radical generation. However, the effect of 2-CADO was not dose-dependent, since the higher concentration of this A_1 agonist was weaker in inhibiting DA release. This discrepancy could be attributed to the fact that 2-CADO is less potent in binding to A_1 receptor than CPA (16-fold) and at high concentration it may activate A_{2A} adenosine receptor thereby stimulating DA release [11]. Nevertheless, both CPA and 2-CADO inhibited L-DOPA-induced formation of hydroxyl radical in a dose-dependent manner, despite that both agonists differed in their influence on DA release. A marked inhibitory effect of the higher concentration of 2-CADO on hydroxyl radical formation indicates that beside suppression of DA synthesis by 2-CADO, other properties of this compound may be involved in its effect on FR generation. For instance, our *in vitro* studies indicate quenching effect of 2-CADO on Fenton reaction. These properties of 2-CADO may, therefore, account for its effectiveness in attenuation of hydroxyl radical formation by L-DOPA. Similarly, A_{2A} agonist CGS 21680, despite increasing L-DOPA-induced DA release, at the same time diminished formation of hydroxyl radical. Thus, the lack of corresponding changes in DA and hydroxyl radical level in the presence of CGS 21680 points to a non-receptor-mediated mechanism, likely connected with scavenging activity of the drug. Our *in vitro* data showing scavenging activity of CGS 21680 corroborate this possibility. Interestingly, adenosine A_1 and A_{2A} agonists, except for a slight stimulatory effect of CGS 21680, did not affect markedly basal DA release and they did not generate FRs when given alone. Thus, CPA, 2-CADO and CGS 21680 were effective antioxidants in the presence of L-DOPA, i.e. in a FR-generating system.

Adenosine receptors are expressed not only on neuronal but also on glial cells where they regulate release of cytokines, growth factors, calcium fluxes and various signaling molecules, like NO or glutamate [1, 21, 38, 45, 47]. NO is a FR which easily reacts with superoxide radical to generate peroxynitrite ($ONOO^-$) [26]. Peroxynitrite is a potent oxidant but it is protonated to peroxynitrous acid, ONOOH and fissioned to hydroxyl radical [26]. It was shown elsewhere that adenosine A_{2A} receptor stimulation potentiated lipopolysaccharide-induced NO release by activated microglia [47], but inhibited NO production by reactive astrocytes [10]. Thus, on the one hand, an A_{2A} agonist may increase oxidative stress, but on the other, it may also depress nitrosative stress. It is, thus, likely that A_{2A} receptor may be involved in this paradoxical effects of NO in various biological systems, particularly in the brain [49]. Thus, depressive effect of A_{2A} agonist on L-DOPA-induced production of FRs in our *in vivo* model may result from the inhibitory effect of CGS 21680 on reactive astrocytes. However, this hypothesis and the role of A_{2A} receptor in oxidative stress needs further studies. Antioxidant activity of adenosine receptor ligands in the presence of FR-radical generating system may be also linked with adenosine itself. It was shown that in hippocampal slices FRs induced adenosine release, which then, by A_1 receptor activation attenuated release of various factors involved in FR formation from neuronal and glial cells [2].

Caffeine is a weak and non-selective adenosine A_1/A_{2A} antagonist. Its affinity for A_1 and A_{2A} adenosine receptors is 29 and 48 μM as estimated by receptor binding studies [33]. In our study, caffeine was used at a 50–100 μM concentration, but taking into account ca. 10% recovery of dialysis probe, tissue caffeine concentration in the vicinity of the probe was in nM range, thus too low to block adenosine receptors. In fact, we did not observe any caffeine effect on basal extracellular DA level or on L-DOPA-elicited DA release. However, we observed a decrease in hydroxyl radical generation by caffeine when it was infused in the presence of L-DOPA. This effect was highly significant when total effect of drugs expressed as an area under the curve (AUC) was compared. The level of 3,4-DHBA in the group treated with L-DOPA was 19,702 ± 2378 pg/240 min, while in the group re-

ceiving L-DOPA plus caffeine (100 μM) it was 13,337 ± 531 pg/240 min [$F(1,11) = 4.88$, $p < 0.049$]. We also found that caffeine showed scavenging activity *in vitro* in the presence of FR generating system. Therefore, it is likely that suppressing caffeine effect on hydroxyl radical level in the presence of free radical generating system may be related to its antioxidant properties resulting from caffeine chemical molecular structure. In fact, some derivatives of xanthic or caffeic acid were effective scavengers of FRs in the brain tissue as shown by Lauderback et al. [35] and Ihan et al. [32]. These observations are in line with the epidemiological evidence showing an inverse relationship between caffeine consumption and risk of developing Parkinson's disease [4]. It has also been suggested that neuroprotective effect of caffeine in various animal neurotoxicity models seems to be linked with A_{2A} receptor blockade as selective antagonists of adenosine A_{2A} receptor decelerate the neurodegeneration of DA cells [51] and are also effective in models of ischemic and excitotoxic damage [43]. However, data shown in our work indicate that the antioxidant effect of caffeine is not related to caffeine influence on DA synthesis *via* A_{2A} receptor blockade.

As 1,3,7-mUA is the primary metabolite of caffeine in rodents [6, 18], we studied its effect on hydroxyl radical generation induced by L-DOPA. This caffeine metabolite was inactive when given alone, but significantly decreased generation of hydroxyl radical by L-DOPA, decreased L-DOPA-derived DOPAC level and also not significantly decreased DA release. Therefore, it may be possible that 1,3,7-mUA can interfere with DA synthesis. However, it is uncertain whether the amount of 1,3,7-mUA produced endogenously from locally applied caffeine may have any impact on hydroxyl radical generation by L-DOPA, as 1,3,7-mUA was not effective when used at the lower concentration (results not shown). Nevertheless, our *in vitro* data show that 1,3,7-mUA, similarly to adenosine, adenosine analogs and deprenyl, may have quenching effect on FRs. Thus, 1,3,7-mUA may provide an additional clue to the mechanism of antioxidant and neuroprotective effects of caffeine.

In conclusion, our findings show involvement of adenosine receptors in the mechanism of neuroprotection. Paradoxically, both agonists of A_1 receptor and agonist of A_{2A} receptor as well as antagonist of A_1 and A_{2A} receptors (caffeine), all decreased generation of FRs. Receptor-mediated effects of A_1 agonists on DA synthesis on the one hand and scavenging activity of the studied agents on the other, indicate the complexity of neuroprotective processes. It is also likely that the regulation of neuronal and glial cell function and resulting stimulation of various antioxidant defense systems, like enzymes (GPx, SOD) and low-molecular-weight antioxidants (GSH), may account for quenching of oxidative stress by adenosine receptor ligands in the brain tissue. Further studies are needed to explain in detail all issues related to adenosine effect on the brain antioxidant defense systems.

References:

1. Alloisio S, Cugnoli C, Ferroni S, Nobile M: Differential modulation of ATP-induced calcium signaling by A_1 and A_2 adenosine receptors in cultured cortical astrocytes. Br J Pharmacol, 2004, 141, 935–942.
2. Almeida CG, de Mendonça A, Cunha RA, Ribeiro JA: Adenosine promotes neuronal recovery from reactive oxygen species induced lesion in rat hippocampal slices. Neurosci Lett, 2003, 339, 127–130.
3. Asanuma M, Miyazaki I, Ogawa N: Dopamine- or L-DOPA-induced neurotoxicity: the role of dopamine quinone formation and tyrosinase in a model of Parkinson's disease. Neurotox Res, 2003, 5, 165–176.
4. Ascherio A, Chen H: Caffeinated clues from epidemiology of Parkinson's disease. Neurology, 2003, 61, S51–S54.
5. Beal MF, Shults CW: Effects of coenzyme Q10 in Huntington's disease and early Parkinson's disease. Biofactors, 2003, 18, 153–161.
6. Berthou F, Guillois B, Riche C, Dreano Y, Jacqz-Aigrain E, Beaune P: Interspecies variations in caffeine metabolism related to cytochrome P4501A enzymes. Xenobiotica, 1992, 22, 671–680.
7. Bishnoi M, Chopra K, Kulkarni SK: Theophylline, adenosine receptor antagonist prevents behavioral, biochemical and neurochemical changes associated with an animal model of tardive dyskinesia. Pharmacol Rep, 2007, 59, 181–191.
8. Brigelius-Flohe R: Tissue-specific functions of individual glutathione peroxidases. Free Radic Biol Med, 1999, 27, 951–965.
9. Bozzi Y, Borelli E: Dopamine in neurotoxicity and neuroprotection: what do D_2 receptors have to do with it? Trends Neurosci, 2006, 29, 167–174.
10. Brodie C, Blumberg PM, Jacobson KA: Activation of the A_{2A} adenosine receptor inhibits nitric oxide production in glial cells. FEBS Lett, 1998, 429, 139–142.
11. Bruns RF, Lu GH, Pugsley TA: Characterization of the A_2 adenosine receptor labeled by [^{3}H]NECA in rat striatal membranes. Mol Pharmacol, 1986, 29, 331–346.
12. Cadet JL, Brannock C: Free radicals and the pathobiology of brain dopamine systems. Neurochem Int, 1998, 32, 117–131.
13. Castagnoli N, Petzer JP, Steyn S, Castagnoli K, Chen J-F, Schwarzschild MA, Van der Schyf CJ: Monoamine oxi-

dase B inhibition and neuroprotection. Neurology, 2003, 61, S62–S68.
14. Ciccarelli R, Di Iorio P, Bruno V, Battaglia G, D'Alimonte I, D'Onofrio M, Nicoletti F, Caciagli F: Activation of A_1 adenosine or mGlu3 metabotropic glutamate receptors enhances the release of nerve growth factor and S-100 β protein from cultured astrocytes. Glia, 1999, 27, 275–281.
15. Chen Y-K, Lin H-C, Liu J-C, Wan F-J: Potent, hydroxyl radical-scavenging effect of apomorphine with iron and dopamine perfusion in rat striatum. Brain Res, 2001, 896, 165–168.
16. Cohen G: Oxy-radical toxicity in catecholamine neurons. Neurotoxicology, 1984, 5, 77–82.
17. Cunha RA: Neuroprotection by adenosine in the brain: from A_1 receptor to A_{2A} receptor blockade. Purinergic Signal, 2005, 1, 111–134.
18. Daniel WA, Kot M, Wójcikowski J: Effects of classic and newer antidepressants on the oxidation pathways of caffeine in rat liver. *In vitro* study. Pol J Pharmacol, 2003, 55, 1045–1053.
19. de Mendonça A, Sebastião AM, Ribeiro JA: Adenosine: does it have a neuroprotective role at all? Brain Res Rev, 2000, 33, 258–274.
20. Dixon AK, Gubitz AK, Sirinathsinghji DJ, Richardson PJ, Freeman TC: Tissue distribution of adenosine receptor mRNAs in the rat. Br J Pharmacol, 1996, 118, 1461–1468.
21. Fiacco TA, McCarthy KD: Intracellular astrocyte calcium waves *in situ* increase the frequency of spontaneous AMPA receptor currents in CA 1 pyramidal neurons. J Neurosci, 2004, 24, 722–732.
22. Fredholm BB, Abbracchio MP, Burnstock G, Daly JW, Harden TK, Jacobson KA, Leff P, Williams A: IV. Nomenclature and classification of purinoceptors. Pharmacol Rev, 1994, 46, 143–156.
23. Gabryel B, Małecki A: Ebselen attenuates oxidative stress in ischemic astrocytes depleted of glutathione. Comparison with glutathione precursors. Pharmacol Rep, 2006, 58, 381–392.
24. Gołembiowska K, Dziubina A: Striatal adenosine A_{2A} receptor blockade increases extracellular dopamine release following L-DOPA administration in intact and dopamine-denervated rats. Neuropharmacology, 2004, 47, 414–426.
25. Gołembiowska K, Żylewska A: Adenosine receptors – the role in modulation of dopamine and glutamate release in the rat striatum. Pol J Pharmacol, 1997, 49, 317–322.
26. Halliwell B: Oxidative stress and neurodegeneration: where are we now? J Neurochem, 2006, 97, 1634–1658.
27. Halliwell B, Gutteridge JMC: Free Radicals in Biology and Medicine, 4th edn., Oxford University Press, Oxford, 2006.
28. Hayton SM, Muller DP: Vitamin E in neural and visual function. Ann NY Acad Sci, 2004, 1031, 263–270.
29. Herrera AJ, Tomás-Camardiel M, Venero JL, Cano J, Machado A: Inflammatory process as a determinant factor for the degeneration of substantia nigra dopaminergic cells. J Neural Transm, 2005, 112, 111–119.
30. Hurley MJ, Mash DC, Jenner P: Adenosine A_{2A} receptor mRNA expression in Parkinson's disease. Neurosci Lett, 2000, 291, 54–58.
31. Iida M, Miyazaki I, Tanaka K, Kabuto H, Iwata-Ichikawa E, Ogawa N: Dopamine D2 receptor-mediated antioxidant and neuroprotective effects of ropinirole, a dopamine agonist. Brain Res, 1999, 838, 51–59.
32. Ihan A, Akyol O, Gurel A, Armutcu F, Iraz M, Oztas E: Protective effects of caffeic acid phenethyl ester against experimental allergic encephalomyelitis-induced oxidative stress in rats. Free Radic Biol Med, 2004, 37, 386–394.
33. Jacobson KA, van Rhee AM: Development of selective purinoceptor agonists and antagonists. In: Purinergic Approaches in Experimental Therapeutics. Ed. Jacobson KA, Jarvis MF, Wiley-Liss, New York, USA, 1997, 101–128.
34. Kellog EWI, Fridovich I: Liposome oxidation and erythrocyte lysis by enzymatically generated superoxide and hydrogen peroxide. J Biol Chem, 1997, 252, 6721–6728.
35. Lauderback CM, Drake J, Zhou D, Hackett JM, Castegna A, Kanski J, Tsoras M et al.: Derivatives of xanthic acid are novel antioxidants: application to synaptosomes. Free Radic Res, 2003, 37, 355–365.
36. Lee C: Antioxidant ability of caffeine and its metabolites based on the study of oxygen radical absorbing capacity and inhibition of LDL peroxidation. Clin Chim Acta, 2000, 295, 141–154.
37. Li XX, Nomura T, Aihara H, Nishizaki T: Adenosine enhances glial glutamate efflux via A_{2A} adenosine receptors. Life Sci, 2001, 68, 1343–1350.
38. Liu QS, Xu Q, Arcuino G, Kang J, Niedergaard M: Astrocyte mediated activation of neuronal kainate receptors. Proc Natl Acad Sci USA, 2004, 1201, 3172–3177.
39. Moore DJ, West AB, Dawson VL, Dawson TM: Molecular pathophysiology of Parkinson's disease. Annu Rev Neurosci, 2005, 28, 57–87.
40. Morelli M, Wardas J: Adenosine A_{2A} receptor antagonists: potential therapeutic and neuroprotective effects in Parkinson's disease. Neurotox Res, 2001, 3, 545–556.
41. Paxinos G, Watson C: The Rat Brain in Stereotaxic Coordinates. Ed. Paxinos G, Watson C, Academic Press, San Diego, USA, 1998.
42. Pinna A, Corsi C, Carta AR, Valentini V, Pedata F, Morelli M: Modification of adenosine extracellular levels and adenosine A_{2A} receptor mRNA by dopamine denervation. Eur J Pharmacol, 2002, 446, 75–82.
43. Popoli P, Blum D, Pintor A, Tebano MT, Frank C, Gianfriddo M, Domenici MR et al.: The controversial role of adenosine A_{2A} receptor antagonists as neuroprotective agents. Curr Med Chem – CNS Agents, 2004, 4, 35–45.
44. Ralevic V, Burnstock G: Receptors for purines and pyrimidines. Pharmacol Rev, 1998, 50, 413–492.
45. Rathbone MP, Middlemiss PJ, Gysbers JW, Andrew C, Herman MAR, Reed JK, Ciccarelli R et al.: Trophic effects of purines in neurons and glial cells. Prog Neurobiol, 1999, 59, 663–690.
46. Rice ME: Ascorbate regulation and its neuroprotective role in the brain. Trends Neurosci, 2000, 23, 209–216.
47. Saura J, Angulo E, Ejarque A, Casadó V, Tusell JM, Moratalla R, Chen J-F et al.: Adenosine A_{2A} receptor

stimulation potentiates nitric oxide release by activated microglia. J Neurochem, 2005, 95, 919–929.
48. Schwarzschild MA, Xu K, Oztas E, Petzer JP, Castagnoli K, Castagnoli N, Chen J-F: Neuroprotection by caffeine and more specific A_{2A} receptor antagonists in animal models of Parkinson's disease. Neurology, 2003, 61, S55–S61.
49. Sharpe MA, Robb SJ, Clark JB: Nitric oxide and Fenton/Haber-Weiss chemistry: nitric oxide is a potent antioxidant at physiological concentrations. J Neurochem, 2003, 87, 386–394.
50. Smythies J: The neurotoxicity of glutamate, dopamine, iron and reactive oxygen species: functional interrelationships in health and disease: a review – discussion. Neurotox Res, 1999, 1, 27–39.
51. Xu K, Bastia E, Schwarzschild M: Therapeutic potential of adenosine A_{2A} receptor antagonists in Parkinson's disease. Pharmacol Ther, 2005, 105, 267–310.
52. Zetterström T, Fillenz M: Adenosine agonists can both inhibit and enhance *in vivo* striatal dopamine release. Eur J Pharmacol, 1990, 180, 137–143.
53. Zetterström T, Sharp T, Collin AK, Ungerstedt U: *In vivo* measurement of extracellular dopamine and DOPAC in rat striatum after various dopamine-releasing drugs; implications for the origin of extracellular DOPAC. Eur J Pharmacol, 1988, 148, 327–334.

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