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Short communication

Clenbuterol enhances the production of kynurenic acid in brain cortical slices and glial cultures

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Abstract:
The effect of a β_2-adrenergic agonist, clenbuterol on the production of a glutamate receptor antagonist, kynurenic acid was studied *in vitro*. Clenbuterol enhanced the production of kynurenic acid in brain cortical slices (0.1–1.0 mM) and in glial cultures (1–50 μM). Timolol, a non-selective β-adrenergic antagonist prevented this effect. The presented data indicate a novel mechanism of action of β_2-adrenoceptor agonists and suggest that an increased formation of the endogenous glutamate receptor antagonist, kynurenic acid could partially contribute to their neuroprotective activity.

Key words:
β-adrenergic agonist, kynurenic acid, glial culture, *in vitro*

Abbreviations: GFAP – glial fibrillary acidic protein, HPLC – high performance liquid chromatography, KAT – kynurenine aminotransferase, KRB – Krebs-Ringer buffer, NGF – nerve growth factor, NMDA – N-methyl-D-aspartate

Introduction

Kynurenic acid is the only known endogenous compound able to block the glycine site of N-methyl-D-aspartate (NMDA) receptors and to antagonize α7 subtype of cholinergic nicotinic receptors [15]. It is formed in the brain and in the periphery [10, 15, 18] from a direct bioprecursor, L-kynurenine, *via* enzymatic reaction mediated by kynurenine aminotransferases I and II (KAT I and II). Kynurenic acid exerts anticonvulsant and neuroprotective activity in experimental models, and its disturbed metabolism was suggested to be one of the causative factors in human neuropathology [15, 17]. Kynurenic acid may also inhibit glutamate release, and this effect is not shared by other NMDA or α7 cholinergic antagonists [2]. It blocks presynaptic NMDA autoreceptors already at low, submicromolar concentrations, thereby regulating glutamate release [12]. Kynurenic acid formation is controlled by several distinct intra- and intercellular mechanisms, such as e.g. endogenous excitatory amino acid agonists or D,L-homocysteine [3, 8, 13, 17]. Here, we demonstrate that β_2-adrenergic agonist clenbuterol enhances the production of kynurenic acid *ex vivo*, in rat brain cortical slices and in mixed glial cultures.

Materials and Methods

Animals

Cortical slices were obtained from male Wistar rats, weighing 220–250 g. Newborn Wistar rats (postnatal day 1–2) were used for the preparation of glial cultures. Animals were housed under standard laboratory conditions, at ambient temperature of 20°C, with food and water available *ad libitum*. All experimental procedures were approved by the Local Ethics Committee in Lublin and are in agreement with European Communities Council Directive on the use of animals in experimental studies.

Substances

L-kynurenine (sulfate salt), kynurenic acid, L-pyruvate, pyridoxal-5'-phosphate, 2-mercaptoethanol, clenbuterol and timolol and cellulose membrane dialysis tubing were obtained from Sigma-Aldrich (St. Louis, USA), whereas all high pressure liquid chromatography (HPLC) reagents were obtained from J.T. Baker Laboratory Chemicals. All other chemicals were purchased from Sigma-Aldrich (St. Louis, USA).

In vitro experiments – cortical slices

Production of kynurenic acid in freshly prepared cortical slices was performed as previously described [8]. Each well contained 6 slices, and each concentration of the drug was studied in 6 wells (N = 6). Briefly, slices were incubated (2 h, 37°C) in Krebs-Ringer buffer (KRB), pH 7.4 (118.5 mM NaCl, 4.75 mM KCl, 1.18 mM $MgSO_4$, 1.77 mM $CaCl_2$, 12.9 mM NaH_2PO_4, 3 mM Na_2HPO_4 and 5 mM D-glucose) saturated with 95% O_2/5% CO_2, in the presence of L-kynurenine (10 μM) and various concentrations of the studied substances. Kynurenic acid was separated from the incubation medium and quantified fluorimetrically using a high performance liquid chromatography (HPLC) system (Varian, USA) and ESA catecholamine HR-80, 3 μm, C18 reverse-phase column. The mean control production of kynurenic acid in the presence of 10 μM L-kynurenine was 2.98 ± 0.61 pmol/h/well.

In vitro experiments – glial cultures

Mixed glial cultures were obtained from cerebral hemispheres of rat pups (postnatal day 1–2) as described by others [5, 19], with our modification. Briefly, brain tissue of newborn rats was seeded at a density of one brain/75 cm^2 culture flasks (Falcon, Switzerland) containing 10 ml of Basal Medium Eagle with Earle's salts (BME, Biochrom AG, Berlin), supplemented with 2 mM L-glutamine, penicillin-streptomycin (500 IU/ml – 500 UG/ml; Gibco-BRL) and containing 10% heat-inactivated fetal bovine serum (Gibco, InVitrogen). The medium was replaced twice a week. Cultures were maintained at 37°C in a 5% CO_2/95% air atmosphere. After 14–15 days, the cells were replated on poly-L-lysine-coated 24-microwell plates (Nunc), and the medium was replaced every 3 days. After 1 week, a confluent astrocytic monolayer developed with scattered oligodendrocytes and microglia on top. Approx. 75–80% of the cells were glial fibrillary acidic protein (GFAP) – positive (as revealed by immunostaining). The production of kynurenic acid was assessed in tissue cultures after 20–22 days *in vitro*. On the day of experiment, the culture medium was changed and the studied substances were added to cultures. After 22 h, the medium was changed into the freshly prepared KRB of the above composition. Cells were further incubated in the presence of L-kynurenine (final concentration 10 μM; 2 h) and the studied substances (total incubation time 24 h). Each concentration of the drug was studied in 6 wells (N = 6). The mean control production of kynurenic acid by glial cells in the presence of 10 μM L-kynurenine was 11.8 ± 1.25 pmol/h/well.

Activities of kynurenine aminotransferases (KATs)

The activities of KAT I and KAT II were assayed in the dialyzed homogenate of cortical brain tissue, as described before [8]. Briefly, freshly obtained cortical brain tissue was homogenized (1:9; w/v) in 5 mM Tris-acetate buffer, pH 8.0, containing 50 μM pyridoxal-5'-phosphate and 10 mM 2-mercaptoethanol. The resulting homogenate was centrifuged (12,000 rpm for 10 min), and the supernatant was dialyzed overnight at 8°C, using cellulose membrane dialysis tubing, against 4 l of the buffer composed as above. The enzyme preparation was incubated in the reaction mixture containing 2 μM L-kynurenine, 1 mM pyruvate, 70 μM pyridoxal-5'-phosphate, 150 mM Tris-acetate buffer, at pH of 7.0 or 9.5, for KAT II or KAT I, respectively (all given concentrations are the final ones) and solutions of the tested drugs at varying con-

centrations. When KAT II activity was assayed, glutamine (final concentration 2 mM), the KAT I inhibitor, was added to the samples. Further procedures were performed as described above. The mean control activity of KAT I and KAT II in the presence of 2 μM kynurenine was 1.72 ± 0.09 and 0.78 ± 0.07 pmol of kynurenic acid/mg of tissue/h, respectively.

Each experiment was repeated at least twice. Statistical comparisons were preformed using one-way analysis of variance (ANOVA) with Bonferroni *post-hoc* test.

Results

Clenbuterol enhanced the kynurenic acid synthesis in cortical slices (0.25–1.0 mM) and in glial cultures (1–50 μM) (Fig. 1). The non-selective β-adrenergic antagonist timolol, used at the 50 μM concentration in cortical slices and at 5 μM concentration in glial cultures, has reversed its action in both experiments (Fig. 1). KAT I and KAT II activities were not altered by clenbuterol at up to 0.5 mM concentration (Tab. 1).

Discussion

Central noradrenergic system may modulate neuronal survival, neuroplasticity and neurogenesis [14]. Neurons and glial cells express several subtypes of functional α- and β-adrenergic receptors, moreover, β_2-adrenergic receptors are located predominantly on the glial cells [14]. Agonists of β_2-adrenoceptors display neuroprotective effects *in vitro* and *in vivo* [7, 14]. Clenbuterol protected mouse cerebral cortex and rat hippocampus from ischemic damage and attenuated hippocampal neuronal loss induced by L-glutamate in culture [16]. These effects seemed to result from the induction of nerve growth factor (NGF) synthesis [4, 16]. Moreover, the compound was shown to increase brain levels of tryptophan [11]. Clinically, drugs activating β_2-adrenergic receptors are commonly used to treat and to prevent the attacks of bronchial asthma due to their potent peripheral effects, including bronchodilation and enhancement of mucociliary clearance [1].

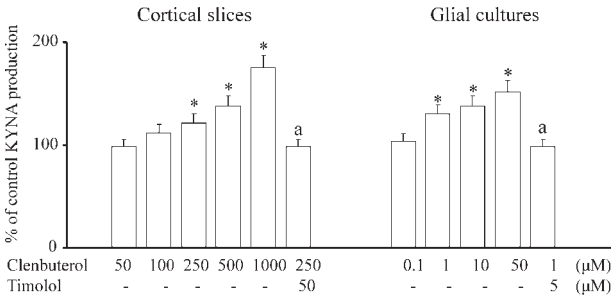

Fig. 1. The effect of clenbuterol on the kynurenic acid production in rat brain cortical slices and in mixed glial cultures. Cortical slices were incubated for 2 h in KRB, pH 7.4, in the presence of 10 μM L-kynurenine and 50–1000 μM clenbuterol. Timolol (50 μM) was added to the incubation media 10 min prior to clenbuterol. Glial cells were incubated in the presence 0.1–50 μM clenbuterol for 24 h. L-kynurenine (final concentration 10 μM) was present in the incubation media during the last 2 h of incubation. Timolol (5 μM) was added to the incubation media 10 min prior to clenbuterol.* $p < 0.05$ *vs.* control (ANOVA); [a] $p < 0.05$ *vs.* clenbuterol (ANOVA)

Tab. 1. The effect of clenbuterol on the activity of kynurenine aminotransferases (KATs) I and II

Clenbuterol (μM)	KAT I (% of control)	KAT II (% of control)
10	101.8 ± 4.0	98.5 ± 3.7
50	97.8 ± 5.3	102.7 ± 4.8
100	98.1 ± 5.5	95.2 ± 8.5
500	104.7 ± 4.5	95.7 ± 6.2

Data are presented as the percentage of control values ± SD

The data presented herein suggest a novel central mechanism of action of β_2-adrenergic agonists involving an increased production of glutamate receptor antagonist, kynurenic acid. Timolol, a non-selective β-adrenergic antagonist reversed the action of clenbuterol *in vitro*; KATs activities were not changed by clenbuterol. Thus, the observed increase in the kynurenic acid production seems to result from a direct stimulation of the β_2-adrenergic receptors and not from the activation of kynurenic acid biosynthetic enzymes. The regulation of kynurenic acid synthesis is governed by various extracellular and intracellular mechanisms. Notably, only few drugs, e.g. some antiepileptics, are able to stimulate kynurenic acid production, but when used at higher, close to millimolar concentrations [9]. The concentrations of clenbuterol effective in glial cultures (1–50 μM) are comparable with the neuroprotective concentrations of clenbuterol (1 μM) re-

ported by others [7]. The underlying cellular mechanism most probably is associated with an increased formation of intracellular cAMP, the second messenger mediating the effects of β_2-adrenergic stimulation [6, 7]. Further experiments will clarify this issue.

An enhanced synthesis of kynurenic acid may be one of the mechanisms contributing to the neuroprotective effects of β-adrenoceptor agonists. Development of drugs modifying noradrenergic receptor activity and/or downstream signaling, especially in astrocytes, was suggested for treatment of several neurological and psychiatric disorders and as a novel therapy in neurodegeneration [7]. Our current research focuses on the assessment of the effects exerted by a broad range of β-adrenergic agonists on the brain synthesis of kynurenic acid *in vitro* and identification of cellular mechanisms underlying this action.

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