Pharmacological Reports 66 (2014) 1127–1133

Contents lists available at ScienceDirect

Pharmacological Reports

journal homepage: www.elsevier.com/locate/pharep

Original research article

On the toxicity of kynurenic acid *in vivo* and *in vitro*

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ARTICLE INFO

Article history:
Received 16 May 2014
Received in revised form 20 July 2014
Accepted 23 July 2014
Available online 8 August 2014

Keywords:
Kynurenic acid
Toxicity
In vitro
Mice
Rats

ABSTRACT

Background: Kynurenic acid (KYNA), a tryptophan metabolite is an antagonist of ionotropic glutamate receptors and alpha-7 nicotinic receptor. Moreover, it is an agonist of G-protein receptor GPR35. Its neuroprotective, anticonvulsant, anti-inflammatory and antioxidant activity was documented. KYNA is present in food and herbal medicines. However, the data on effects induced by a long-lasting treatment with KYNA is lacking. The aim of the study was the assessment of toxicity of a prolonged administration of KYNA in rodents. The cytotoxicity of KYNA *in vitro* was also examined.
Methods: Adult mice and rats were used. KYNA was administered in the drinking water in concentrations of 25 or 250 mg/L for 3–21 days. The following cells were cultured in an *in vitro* study: mouse fibroblast (NIH/3T3), green monkey kidney cells and primary chick embryo cells (CECC). Cell viability was determined with *methyl* thiazol tetrazolium reduction assay, neutral red uptake assay and lactate dehydrogenase leakage assay.
Results: KYNA affected neither body gain nor body composition. Blood counts were also unaffected. The viability of cells in the culture was lowered at high millimolar concentrations of KYNA. An elevated viability of GMK and CECC cells was detected in the presence of KYNA in micromolar concentrations.
Conclusions: The obtained results showed that a long-term application of KYNA in the drinking water is well-tolerated by rodents. No evidence of a toxic response was recorded. Achieved results indicate that diets containing a high amount of KYNA or enriched with KYNA should not cause any risk to the human health.

Introduction

It is well documented that kynurenic acid (KYNA) is an endogenous substance formed from tryptophan along kynurenine metabolic pathway (see for review [1]). Scientific evidence indicates that KYNA might come from exogenous sources and therefore might be delivered to the human organism with food [2,3] or as a product delivered by intestinal microflora [4]. It is accepted that KYNA is not metabolized in the human body and is excreted mainly with urine (see for review [5]).

KYNA possesses intriguing pharmacological properties. It acts as an antagonist on ionotropic glutamate receptors [6,7] and alpha-7 nicotinic receptor [8]. Moreover, it is an agonist of G-protein receptor GPR35 [9]. Its action on aryl hydrocarbon receptor (AhR) is unclear due to conflicting reports [10,11].

KYNA exerts neuroprotective, anticonvulsant, anti-inflammatory and antioxidant activity (see for review [1,12]. On the other hand elevated KYNA is putatively implicated in the pathogenesis of psychiatric disorders [1].

Most of the available reports describe acute effects of KYNA observed after its single administration. The data on effects induced by a long-lasting treatment with KYNA is lacking. Therefore, the aim of the study was the assessment of toxicity

Abbreviations: AhR, aryl hydrocarbon receptor; CECC, chick embryo cell culture; GPR35, G-protein receptor; GMK, green monkey kidney; KYNA, kynurenic acid; LDH, lactate dehydrogenase; MTT, methyl thiazol tetrazolium; NIH/3T3, mouse fibroblast; NRU, neutral red uptake.

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http://dx.doi.org/10.1016/j.pharep.2014.07.013
1734-1140/

of a prolonged administration of KYNA in rodents. The cytotoxicity of KYNA *in vitro* was also examined.

Materials and methods

Animals

The study was performed on 10–12 weeks old female Balb/c mice, weighing 25–28 g at the beginning of the study, and male adult Wistar rats weighing 220–260 g at the beginning of the study. Animals were kept under standard laboratory conditions (a 12-h light-dark cycle, temperature of 21 ± 1 °C, humidity 55 ± 5%) in colony cages with free access to food and tap water *ad libitum*. The animals were randomly assigned to experimental groups consisting of 6–8 subjects.

Experimental design

All experimental procedures were carried out in accordance with the guidelines of the European Communities Council Directive of 24 November 1986 (86/609/EEC) and approved by the Local Ethics Committee for Animal Experimentation in Lublin and in Olsztyn, Poland.

KYNA was administered in drinking water in concentrations of 25 or 250 mg/L (kynurenic acid, Sigma–Aldrich Inc., St. Louis, MO, USA). Fresh tap water with or without KYNA was refilled every 2–3 days, at which times body weight was recorded.

Measurement by dual energy x-ray absorptiometry (DEXA)

Animals were scanned using Hologic Discovery W QDR Series DEXA system (Hologic Inc. Bedford, MA, USA). The decapitated rats were ventrally positioned and scanned to determine the parameters of body surface [cm^2], bone mineral density (g/cm^2), bone mineral content (g), lean mass (g) fat tissue mass [g] and fat [%]. Analysis was performed using the small-animal mode of the APEX 3.0.1 Software for Windows XP Service Pack 3. The instrument was calibrated at each start.

Blood analysis

Whole blood was obtained from mice by transcutaneous cardiac puncture and placed into heparinized tubes. Complete blood counts were analyzed using an automated *hematology analyzer* Mythic 18 (Orphee S.A., Geneva, Switzerland). White blood cell count, red blood cell count, hemoglobin, hematocrit and platelet count were determined.

Cell cultures

The following cells were cultured: NIH/3T3 (mouse fibroblast, ATCC CRL-1658, purchased from LGC Standards, Poland), GMK (green monkey kidney, purchased from BIOMED Lublin, Poland) and CECC (primary chick embryo cell culture, prepared from 10-day-old chicken embryos according to routine procedure). NIH/3T3 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) and the two other cell types in Minimum Essential Medium Eagle (MEM). All media were supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin and 50 μg/ml streptomycin. All cell culture reagents were purchased from Sigma-Aldrich (Sigma–Aldrich Inc., St. Louis, MO, USA). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2. For the determination of KYNA cytotoxicity the cells were seeded onto 96-well plates (Nunc, Denmark) at a density of 1×10^5 (NIH/3T3) or 2×10^5 (GMK and CECC) cells/ml. After 24 h of incubation the cells were treated with serum free media supplemented with KYNA within a range of concentrations from 0 (control) to 10 mM for 1, 2, 4, 6 and 24 h (LDH assay) and 1, 2, 4, 6, 24, 48 and 72 h (MTT and NRU assay).

MTT assay

The *methyl* thiazol tetrazolium (MTT) reduction colorimetric assay was performed according to protocol described by Mossmann [13] with some modifications. After the incubation time, KYNA supplemented media were replaced with fresh, serum free media containing 0.7 mg/ml of MTT (3-[4,5 dimethylthiazoly-2-yl]-2,5-diphenyltetrazolium bromide, Sigma–Aldrich Inc., St. Louis, MO, USA) and the cells were incubated for the next 3 h at 37 °C. Next, the cells were washed with PBS and 100 μl of DMSO (dimethylsulfoxide, POCh, Gliwice, Poland) was added to each well. After 10 min of gentle shaking, when complete dissolution was achieved, the optical density was measured at a wavelength of 570 nm with 640 nm as a reference wavelength using the Sunrise absorbance reader (Tecan, Austria). The viability of treated cells was expressed as the percent of the control.

NRU assay

The neutral red uptake (NRU) colorimetric assay was performed using a commercially available kit from Sigma-Aldrich (TOX-4; Sigma–Aldrich Inc., St. Louis, MO, USA), according to the manufacturer's instructions. Following exposure to KYNA, the cells were incubated for 3 h with neutral red dye dissolved in serum free media (0.033%). At the end of the incubation period, the medium was carefully removed and the cells were washed with PBS. Afterwards, 100 μl of solubilization solution (1% acetic acid in 50% ethanol) was added to each well and the cells were incubated for 10 min at room temperature. After gentle shaking, the absorbance was measured at a wavelength of 540 nm with 690 nm as a reference wavelength using the Sunrise absorbance reader (Tecan, Austria). The viability of treated cells was expressed as the percent of the control.

LDH leakage assay

The lactate dehydrogenase (LDH) leakage colorimetric assay was performed using a commercially available kit from Sigma–Aldrich (TOX-7; Sigma–Aldrich Inc., St. Louis, MO, USA), according to the manufacturer's instructions. Following exposure to KYNA, the total LDH content (LDH content in media after cell lysing) as well as LDH release (LDH content in cell-free culture media) were measured in the enzymatic assay. Aliquots of media and LDH assay mixture were mixed in a 96-well plate, protected from light and incubated at room temperature for 20–30 min. Afterwards, the enzymatic reaction was stopped by the addition of 1 N HCl and the absorbance was measured at a wavelength of 490 nm with 690 nm as a reference wavelength using the Sunrise absorbance reader (Tecan, Austria). Data were expressed as percentage of control.

Statistical analysis

Data are presented as a mean value and a standard error of the mean (SEM). Data were analyzed statistically by Student's *t*-test or one-way ANOVA with Bonferroni's post test to determine differences between groups. A *p*-value of less than 0.05 was considered significant.

Results

Effect of KYNA on body weight and body composition

KYNA administered in the drinking water at the dose of 250 mg/L for 21 days did not affect body weight gain in rats (Fig. 1) and mice (data not shown).