Short communication

Kynurenic acid enhances expression of p21 Waf1/Cip1 in colon cancer HT-29 cells

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Abstract:

Background: Kynurenic acid (KYNA), a tryptophan metabolite, was found in the mucus of rat small intestine. However, its role in the gastrointestinal tract is still not fully elucidated.

Methods: To verify whether KYNA affects cell cycle regulators, the protein expression of cyclin-dependent kinase inhibitor p21 Waf1/Cip1 was investigated in colon adenocarcinoma HT-29 cells exposed to KYNA. MTT, BrdU assay and siRNA technology were used to evaluate the effect of KYNA on cancer cell proliferation.

Results: KYNA significantly enhanced the expression of p21 Waf1/Cip1. Importantly, the overexpression of this protein was involved in inhibition of proliferation and DNA synthesis in HT-29 cells.

Conclusions: KYNA may be considered as a potential chemoprevention agent against colon cancer.

Key words:

kynurenic acid, colon cancer, p21 Waf1/Cip1, proliferation

Abbreviations: BrdU – 5'-bromo-2'-deoxy-uridine, CDK – cyclin-dependent kinases, DMEM – Dulbecco's modified Eagle's medium, DTT – dithiothreitol, EDTA – ethylenediaminetetraacetic acid, EGTA – ethylene glycol tetraacetic acid, FBS – fetal bovine serum, GPR – G-protein-coupled receptor, KYNA – kynurenic acid, MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, PMSF – phenylmethanesulfonyl fluoride, PVDF – polyvinylidene fluoride, SDS – sodium dodecyl sulfate, SEM – standard error of the mean

Introduction

Kynurenic acid (KYNA), an endogenous metabolite of the kynurenine pathway of tryptophan degradation, is an agonist of orphan G-protein-coupled receptor (GPR35) which is predominantly expressed in immune and gastrointestinal tissues [24], a non-competi-

tive antagonist of $\alpha 7$ nicotinic receptor and a broad spectrum antagonist of all types of ionotropic glutamate receptors [8, 20]. Recently, it was also reported that KYNA is an agonist of aryl hydrocarbon receptor [2]. KYNA was found in human saliva [10], gastric juice, bile, pancreatic juice [15], mucus of rat small intestine [11] and mucus of pig colon [15]. Importantly, KYNA was also detected in several products of daily human diet and medicinal herbs [21, 22]. The highest concentration was reported in honeybee products and some fresh vegetables including broccoli and potato [22]. Although the role of KYNA in the gastrointestinal tract is still not fully elucidated, previous studies revealed its involvement in a complex duodenal mucosa protection system [5–7], modulation of intestinal motility, and inflammatory response in experimental colonic obstruction in dogs [9] and experimental colitis in rats [23]. Moreover, taking into consideration that KYNA supported the growth of probiotic bacteria *in vitro*, its potential role in the regulation of bacterial growth in the digestive system is suggested [3].

In this study, we report that KYNA significantly enhanced the expression of cell cycle regulator cyclin-dependent kinase (CDK) inhibitor p21 Waf1/Cip1 in colon adenocarcinoma HT-29 cells. Moreover, KYNA-induced overexpression of p21 Waf1/Cip1 was involved in inhibition of proliferation and DNA synthesis in HT-29 cells.

Materials and Methods

Cell culture

Colon adenocarcinoma HT-29 cells were obtained from ECACC (European Collection of Cell Cultures, Centre for Applied Microbiology and Research, Salisbury, UK). Cells were grown in 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and nutrient mixture Ham F-12 supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 μ g/ml) and were maintained in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. All reagents were obtained from Sigma (Sigma Chemicals, St. Louis, MO, USA).

MTT assay

HT-29 cells were plated on 96-well microplates (Nunc, Roskilde, Denmark) at a density of 3×10^4 cells/ml. Next day, the culture medium was removed and the cells were exposed to serial dilutions of KYNA (0.00001–10 mM; Sigma) in a fresh medium and incubated for 48 h. Cell proliferation was assessed by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method previously described by Rzeski et al. [19].

BrdU assay

HT-29 cells were plated on 96-well microplates (Nunc) at a density of 3×10^4 cells/ml. Next day, the culture medium was removed and the cells were exposed to serial dilutions of KYNA (0.00001–10 mM) in a fresh medium supplemented with 10% FBS for 48 h. The incorporation of 5'-bromo-2'-deoxyuridine (BrdU) into newly synthesised DNA of actively proliferating cells was measured by means of the BrdU method, previously described by Rzeski et al. [19].

Gene silencing (siRNA)

HT-29 cells were plated on 96-well microplates at a density of 3×10^4 cells/ml the day before transfection with p21 siRNA (100 nM; Cell Signaling Technology Inc., Danvers, MA, USA). Transfection was carried out by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The following day, the cells were exposed to KYNA (0.01, 0.1 and 1 mM) in a fresh medium for 48 h. MTT assay was performed to evaluate cell proliferation. The p21 silencing was confirmed by western blot.

Western blot analysis

HT-29 cells were lysed in RIPA buffer (1% NP40 (Tergitol), 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM EGTA, 1 mM Na₃VO₄, 20 mM NaF, 0.5 mM DTT, 1 mM PMSF, protease inhibitor mixture in PBS, pH 7.4) and centrifuged at $14,000 \times g$ for 10 min. Protein content in supernatants was determined by BCA Protein Assay Kit (Pierce Biotechnology, Rockford, USA). Supernatants were solubilized in sample buffer (30% glycerol, 10% SDS, 0.5 M Tris-HCl, pH 6.8, 0.012% bromophenol blue, 5% β -mercaptoethanol), and boiled for 5 min. For west-

ern blotting, equal amounts of proteins were electrophoresed on 10% SDS-PAGE and transferred to PVDF membrane. After blocking for 1 h at room temperature with 5% non-fat dry milk in TBS-0.1% Tween 20 (TBS-T), membranes were probed at 4°C overnight with primary antibodies (p21 Waf1/Cip1 1:1000, β -actin 1:2000; Cell Signaling Technology). The membranes were then washed in TBS-T buffer and incubated with secondary antibody coupled to horseradish peroxidase (1:2000 in 5% non-fat milk in TBS-T) for 1 h at room temperature and visualized by using enhanced chemiluminescence (Pierce Biotechnology). Serial exposures were made on Kodak BioMax Light film (Eastman Kodak Company, Rochester, New York, USA).

Data analysis

Data were presented as the mean value and standard error of the mean (SEM). Statistical analysis was performed using one-way ANOVA with Tukey *post-hoc* test; $p < 0.05$ was considered statistically significant. The IC_{50} value (the concentration of drug necessary to induce 50% inhibition), together with confidence limits, was calculated using computerized linear regression analysis of quantal log dose-probit functions, according to the method of Litchfield and Wilcoxon [12].

Results and Discussion

We report that KYNA significantly enhanced the expression of cell cycle regulator CDK inhibitor p21 Waf1/Cip1 in colon adenocarcinoma cells. HT-29 cells were exposed to 2.5 mM KYNA for 5 min – 48 h. Western blot analysis revealed that KYNA significantly increased protein levels of p21 Waf1/Cip1 starting at 4 h of exposition to tested compound (Fig. 1a). Interestingly, at 24 h of incubation with KYNA a considerable decrease of p21 Waf1/Cip1 expression was observed. Although the reason of this phenomenon is unknown, the significant differences in basal expression of p21 Waf1/Cip1 in control cells (without KYNA) after 4 and 24 h of incubation may suggest that expression of this protein is dynamic through long-term experiment. Importantly, incubation with serial dilutions of KYNA (0.01–5 mM) for 4, 24 or 48 h revealed that the expression of p21 Waf1/Cip1

Fig. 1. Regulation of p21 Waf1/Cip1 by KYNA. Western blot analysis of p21 Waf1/Cip1 in HT-29 cells after treatment with (a) KYNA 2.5 mM for 5 min – 48 h, or (b) treatment with serial dilutions of KYNA (0.01–5 mM) for 4, 24 or 48 h (C – control; not treated)

was increased also in a dose-dependent manner (Fig. 1b). p21 Waf1/Cip1 is a cyclin-dependent kinase inhibitor involved in the regulation of cell cycle, apoptosis and cell differentiation [27]. In the intestinal tract, p21 Waf1/Cip1 is expressed in cells which exit the proliferative compartment, and disruption of this process may lead to carcinogenesis [17].

Although the preliminary character of this study do not allow to clearly specify the biological consequences of KYNA-induced overexpression of p21 Waf1/Cip1, our results suggested that enhanced expression of this protein may be involved in growth inhibition of colon adenocarcinoma HT-29 cells. We report that KYNA not only significantly inhibited proliferation of HT-29 cells (MTT assay; $IC_{50} = 2$ mM) but also decreased DNA synthesis (BrdU assay; $IC_{50} = 4.4$ mM) after 48 h of incubation (Fig. 2a, 2b). A similar effect was previously reported in synovio-cytes [16]. In contrast, Di Serio et al. [1] reported the stimulatory effect of KYNA on the proliferation rate of mouse microglia and human glioblastoma cells *in vitro*. Although we investigated the effect of KYNA in a broad range of concentrations from 0.00001 to 10 mM, we did not observe the stimulatory activity of KYNA in HT-29 cells. Importantly, the involvement of p21 Waf1/Cip1 in KYNA-induced growth inhibition of HT-29 cells was confirmed by means of gene silencing technology. We did not observe the antiprolifera-

Fig. 2. The antiproliferative effect of KYNA on colon adenocarcinoma cells HT-29. Cells were exposed to fresh medium (control) or KYNA (0.00001–10 mM) for 48 h. Proliferation was assessed by means of MTT assay (**a**) or BrdU assay (**b**). Data represent a mean value (% of control) $\pm$ SEM of six independent experiments. Values significant in comparison to control (100%) with * $p < 0.05$ (one-way ANOVA with Tukey *post-hoc* test). (**c**) Proliferation of HT-29 and HT-29 siRNA p21 Waf1/Cip1 cells after treatment with KYNA (0.01–1 mM) for 48 h (MTT assay; data represent a mean value (% of control) $\pm$ SEM of five independent experiments). (**d**) Confirmation of silencing p21 Waf1/Cip1 gene in HT-29 (western blot analysis); C – control; p21 siRNA – cells with silenced p21 Waf1/Cip1 gen

tive effect of KYNA in cells with silenced p21 Waf1/Cip1 gene (Fig. 2c, 2d). Our results are consistent with previous studies where Yamamoto et al. [26] revealed that overexpression of p21 Waf1/Cip1 in HT-29 cells is strongly associated with increased doubling time as well as decreased saturation density and plating efficiency. Moreover, p21 Waf1/Cip1, as an inhibitor of cell proliferation, plays an important role in drug-induced tumor suppression [13].

Since p21 Waf1/Cip1 is also involved in differentiation and apoptosis [4, 14, 18, 25], at this stage of our studies its role in those cellular processes in KYNA-treated HT-29 cells cannot be excluded. Several *in vivo* and *in vitro* experiments revealed that p21 Waf1/Cip1 expression is induced during terminal differentiation [14], however, this protein was also shown to decrease sodium butyrate-induced differen-

tiation in HT-29 cells [26]. The role of p21 Waf1/Cip1 in apoptosis has not been unequivocally established. Generally, p21 Waf1/Cip1 protects cells from undergoing p53-mediated apoptosis [4]. However, previous studies indicated also that p21 Waf1/Cip1 in cells expressing mutated p53 may play a crucial role in promotion of apoptosis through a distinct apoptotic pathway [18, 25]. Although it needs further investigations, KYNA-induced overexpression of p21 Waf1/Cip1 in HT-29 cells harboring mutated p53 may suggest its involvement in apoptosis in colon adenocarcinoma cells.

Nowadays, since colon cancer was emerged as a growing global health problem, the scientific effort is focused on designing the molecular targeted cancer therapies, significantly increasing the effectiveness of treatment. Taking into consideration antiproliferative activity, KYNA-induced overexpression of p21 Waf1/

Cip1 and long-term, multistep process of colon carcinogenesis, KYNA may be suggested as a novel chemopreventive agent against colon cancer. Interestingly, previous studies indicated that the loss of the p21 gene and Western-style diet were additive on colon tumor formation in the murine model [27]. It should be emphasized that KYNA content found in mucus of rat ileum (0.003–0.016 mM) [11] and in mucus of pig colon (~0.0015 mM) [15] is high enough to influence expression of p21 Waf1/Cip1 and proliferation of HT-29 cells *in vitro*. Importantly, KYNA concentration in gastrointestinal tract may be easily increased. Since KYNA is present in food [22] and medicinal herbs [21], it can be administered *per os* as a constituent of diet.

Although the specific biological consequences of enhanced expression of p21 Waf1/Cip1 in KYNA-treated HT-29 cells need further studies, its involvement in growth inhibition of colon adenocarcinoma cells suggested that KYNA may be considered as a potential chemoprevention agent against colon cancer.

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