Pharmacological Reports
2006, 58, 393–398
ISSN 1734-1140

Kynurenic acid in human saliva – does it influence oral microflora?

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Abstract:
Kynurenic acid (KYNA) is an endogenous antagonist of α7 nicotinic receptors and all ionotropic glutamate receptors. Its neuroprotective activity has been suggested. In this study, the presence of KYNA in human saliva and its potential bactericidal role was investigated. KYNA was found in all samples of human saliva with mean concentration of 3.4 nM. The concentration of KYNA in saliva obtained from patients with odontogenic abscesses was 3.5 times higher than in healthy subjects. We have shown that the human gingival fibroblasts produce KYNA and an inflammatory stimulant, lipopolysaccharide, enhanced its synthesis *in vitro*. The bactericidal effect of KYNA was also presented. We hypothesize that KYNA may contribute to the control of oral microflora.

Key words:
kynurenic acid, saliva, human, fibroblast, bacteria

Abbreviations: HGF – human gingival fibroblasts, IDO – indoleamino-2,3-dioxygenase, KYNA – kynurenic acid, LPS – lipopolysaccharide, NMDA – N-methyl-D-aspartate, SEM – standard error of the mean

Introduction

Kynurenic acid (KYNA) is the only known endogenous antagonist of all subtypes of ionotropic glutamate receptors [18]. It is preferentially active at the glycine allosteric site of the N-methyl-D-aspartate (NMDA) receptor [3]. It has been also demonstrated that KYNA is a noncompetitive antagonist of α7 nicotinic receptor [9]. Its anticonvulsant and neuroprotective properties were experimentally proven. However, the question of the physiological role of KYNA in the regulation of brain neurotransmission is still a matter of controversy. Although it was concluded from electrophysiological studies performed *in vitro* that endogenous brain levels of KYNA are below those nec-

essary to antagonize glutamate receptors [17], some recent studies indicate that physiological levels of brain KYNA are sufficient to antagonize NMDA receptors [21] and *in vivo* electrophysiological experiments demonstrate that a moderate (3–5-fold) increase in whole brain KYNA is associated with a marked activation of rat dopamine neurons [4]. Moreover, it was found that at physiologically relevant concentrations [15, 26] KYNA inhibited α7 nicotinic receptor [9]. These effects indicate its physiological role and suggest that KYNA is involved in neurotransmission.

In recent years, significant interest has been shown in identifying KYNA and its potential role outside the brain. The presence of KYNA has been documented in urine, serum, amniotic fluid, and synovial fluid [11, 13, 16]. KYNA is a metabolite of tryptophan formed along the kynurenine pathway. The conversion from tryptophan to kynurenine is achieved by tryptophan-2,3-dioxygenase and indoleamino-2,3-dioxygenase (IDO), and KYNA is produced irreversibly from kynurenine by kynurenine aminotransferases [22]. It is well established that IDO activity is elevated during infectious diseases. It was shown that IDO expression could be induced in many cell types by interferon-γ and other products including that of bacterial and viral origin [7, 8, 14]. The elevated KYNA level was found in cerebrospinal fluid of rhesus macaques inoculated with poliovirus directly into the spinal cord, which is a model of focal inflammatory neurological disease [8] in both lumbar cerebrospinal fluid and post-mortem brain tissue of patients with inflammatory diseases (bacterial, viral, fungal and parasitic infections, meningitis, autoimmune diseases and septicemia) [7] and in HIV-1-infected patients [2, 6].

In this study, the presence of KYNA and its potential bactericidal role in human saliva was investigated.

Materials and Methods

Subjects

Male patients admitted for therapy to the dentist's surgery were invited to participate in the study. Informed written consent was obtained. A total of 32 patients comprising 16 control subjects and 16 patients with odontogenic abscesses (8 patients with subperiosteal abscess, 6 patients with submandibular abscess and 2 patients with buccal abscess) were enrolled. The study was approved by the Ethics Committee of the Medical University in Lublin, Poland.

Saliva

The unstimulated saliva was collected between 9 and 12 a.m. Patients refrained from eating, drinking, smoking and oral hygiene for 2 h before the collection. Saliva was sampled by spitting it directly into a plastic tube. Samples were immediately frozen and stored at –70°C until the time of analysis.

KYNA determination

KYNA was investigated according to the method of Turski et al. [26]. Specimens were immersed in a boiling water bath for 1 min. The denaturated proteins were removed by centrifugation (10 min, 20 000 rpm), and the supernatant was applied to the columns containing cation-exchange resin (Dowex 50 W^+: 200–400 mesh) prewashed with 0.1 M HCl. Subsequently, the columns were washed with 1ml of 0.1 M HCl and 1 ml of water, and the fraction containing KYNA was eluted with 2 ml of water. Eluate was subjected to HPLC, and KYNA was detected fluorometrically (Hewlett Packard 1050 HPLC system: ESA catecholamine HR-80, 3 μm, C_{18} reverse-phase column, flow rate of 1.0 ml/min; Hewlett Packard 1046A fluorescence detector: excitation 344 nm, emission 398 nm). KYNA was obtained from Sigma. All HPLC reagents used in the study were obtained from Baker (Griesheim, Germany) and were of the highest available purity.

Cell culture

Human gingival fibroblasts (HGF) were obtained by outgrowth technique from tissue explants taken from a healthy child. Tissue was cut into small fragments (1 mm^3) then washed in phosphate-buffered saline (PBS), suspended in culture medium and placed into 25 cm^2 culture flasks (Nunc, Rossilde, Denmark). The culture medium consisted of Dulbecco's Modified Eagel's Medium (DMEM, Sigma, USA) supplemented with 10% fetal bovine serum (FBS) (Life Technologies, Karlsruhe, Germany), penicillin (100 μ/ml) and streptomycin (100 μg/ml) (Sigma, USA). Cells were grown at 37°C in an humidified atmosphere of 95% air and 5% CO_2. After reaching confluence, the cells

were subcultured every 4 days using 0.25% trypsin-EDTA (Sigma, USA) solution. For experiments, cells from subcultures 2–5 were used.

KYNA production

Cells plated on 24-well plates at a density of 1×10^5/ml were allowed to grow for 24 h. The next day, cells were washed 3 times with Hank's balanced salt solution (HBSS, Sigma, USA) and subsequently incubated with L-kynurenine dissolved in HBSS at 37°C in a 95% air and 5% CO_2 atmosphere. At the end of incubation time supernatants were collected and treated with 50% trichloroacetic acid. Precipitated proteins were removed by centrifugation. Supernatants were analyzed for KYNA content as previously described. Aminooxyacetic acid (Sigma, USA) and lipopolysaccharide (LPS, Sigma, USA) were added to the incubation medium 15 min before L-kynurenine.

Antibacterial activity determination

Staphylococcus aureus ATCC 25923 and *Streptococcus mutans strains*; CAPM 6067, DSM 20381 were grown in brain heart infusion broth, (BHI, bioMerieux SA, Marcy I'Etoile, France) and *Escherichia coli* ATCC 25922 in Luria-Bertani broth, (LB, Lab. Conda, Madrid, Spain) at 37°C to the early exponential phase (OD_{600} 0.1–0.2). KYNA was added to the suspension of the cells of the final density of 2×10^2 colony forming units (CFU) per ml. The final concentration of KYNA was 0.1, 0.5 and 2.5 mM. Samples were incubated in the presence of KYNA at 37°C for 2 or 24 h. Viable cells were determined by plating a dilution series in triplicate onto appropriate (BHI or LB) agar plates without KYNA and incubated at 37°C for 18 h (*Escherichia coli*, *Staphylococcus aureus*) or 36 h (*Streptococcus mutans strains*) prior to counting.

Statistics

Data were presented as the mean value and standard error of the mean (SEM). Statistical analysis was performed using the Student's *t*-test. Significance was accepted at $p < 0.05$.

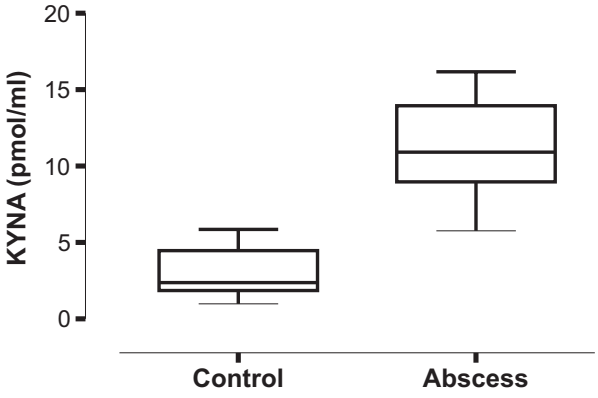

Fig. 1. Kynurenic acid (KYNA) concentration in saline collected from 16 healthy subjects (control) and 16 patients with odontogenic abscesses. Results are expressed as pmol/ml. The box shows median, upper and lower quartiles of the data; the whiskers indicate the minimum and maximum values

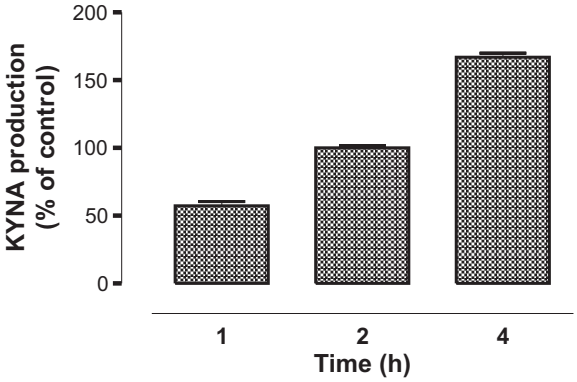

Fig. 2. Time-dependence of kynurenic acid (KYNA) synthesis in human gingival fibroblasts (HGF) *in vitro*. Results are expressed as percentage of control (time 2 h); mean ± SEM; n = 6 independent experiments

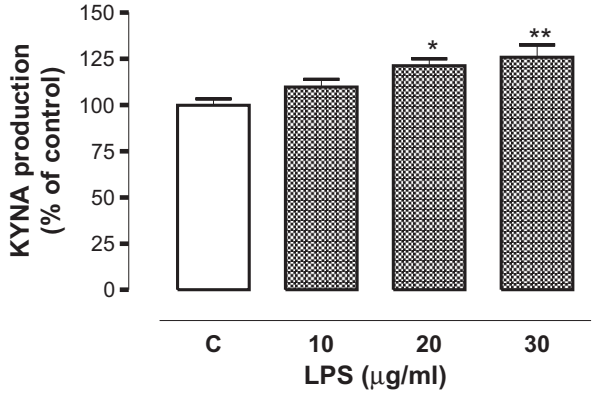

Fig. 3. Effect of lipopolysaccharide (LPS) on kynurenic acid (KYNA) synthesis in human gingival fibroblasts (HGF). Results are expressed as percentage of control (C); mean ± SEM; n = 6 independent experiments; * $p < 0.05$, ** $p < 0.01$ *vs.* control (C, 100%)

Tab. 1. Bactericidal effect of kynurenic acid (KYNA)

Strain	KYNA concentration [mM]		
	0.1	0.5	2.5
Staphylococcus aureus ATCC 25923	83.5 (78.7–88.5)	41.0 (38.3–45.6)	27.3 (25.6–30.5)
Streptococcus mutans CAPM 6067	99.5 (93.6–100)	99.0 (89.5–100)	0.32 (0.28–0.50)
Streptococcus mutans DSM 20381	99.7 (95.0–100)	99.2 (89.7–100)	10.2 (8.9–11.8)
Escherichia coli ATCC 25922	73.5 (68.3–80.7)	26.5 (25.0–35.7)	8.8 (5.3–12.8)

Bacteria were incubated in medium containing KYNA for 2 h and viable cells were determined after subsequent incubation without KYNA lasting for 18 or 36 h. The results are presented as percentage of control (mean and range in parenthesis). The number of viable bacteria in respective controls (without KYNA) was considered 100%

Results

KYNA in saliva

The presence of KYNA was determined in all samples of saliva. In saliva samples to which authentic KYNA was added, only one peak was recorded on the chromatogram (data not shown). The mean concentration of KYNA in saliva obtained from healthy subjects (mean age = 31.2 ± 3.6 years; n = 16) was 3.41 ± 0.66 pmol/ml. The mean concentration of KYNA in saliva obtained from patients with odontogenic abscesses (mean age = 35.6 ± 2.8 years; n = 16) was 12.06 ± 1.61 pmol/ml, which is considerably higher than in the control group ($p < 0.001$) (Fig. 1).

KYNA production in HGF

In the presence of L-kynurenine, HGF produced KYNA in a concentration- (2.5–10 μM, 2 h; data not shown) and time-dependent manner (Fig. 2). The production of KYNA under control conditions (L-kynurenine 5 μM, incubation 2 h) was 4.27 ± 0.66 pmol/1 × 10^5 cells/2 h. In the presence of aminooxyacetic acid (25 μM), KYNA production was inhibited by 48.9% (data not shown). LPS at concentrations of 20 and 30 μg/ml enhanced KYNA synthesis. LPS at 10 μg/ml was ineffective (Fig. 3).

KYNA antibacterial activity

The susceptibility to bactericidal action of KYNA was assayed on *Streptococcus mutans, Staphylococcus aureus* and *Escherichia coli* strain. In all examined strains, the bactericidal action was observed at low cell density. Exposure to KYNA (0.1–2.5 mM) for 2 h resulted in the bactericidal effect in *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922. In *Streptococcus mutans* strains CAPM 6067 and DSM 20381 the bactericidal effect of KYNA was found only at high concentration of 2.5 mM (Tab. 1). Exposure to KYNA (2.5 mM) for 24 h resulted in the bactericidal effect in *Streptococcus mutans* CAPM 6067 only (data not shown).

Discussion

This study documented the presence of KYNA in unstimulated whole saliva obtained from healthy male adults. KYNA was determined in all 16 studied samples of saliva, with a mean concentration of 3.4 nM. The presence of KYNA in nanomolar concentration range has been documented also in urine, amniotic fluid [11, 13], synovial fluid [16], cerebrospinal fluid [24, 26], brain tissue [24, 26] and retina [19].

The origin of KYNA in saliva is unknown. Here, we have shown that HGF produces KYNA in a concentration- and time dependent manner from its precursor, L-kynurenine *in vitro*. The synthesis of KYNA was strongly inhibited by aminooxyacetic acid, the non-specific inhibitor of the kynurenine aminotransferases. KYNA production was also found to occur in cultured neurons [20], glia cells [12, 29], oligodendrocytes [27] and bovine aortic endothelial cells [28]. It was reported to be synthesized in endothelial [23], brain [25], liver and kidney [5] slices. It can be as-

sumed that kynurenine aminotransferases, the enzymes responsible for KYNA synthesis are widely distributed. Since, the predominant cell type of the soft connective tissues of the periodontium, fibroblasts, synthesize KYNA, it can be suggested that KYNA present in saliva is produced locally in the oral cavity. The synthesis of KYNA by cells of salivary glands was not investigated in our study, however, such possibility should also be considered.

Here, we found 3.5 times higher mean concentration of KYNA in the whole saliva obtained from patients with odontogenic abscesses. Moreover, LPS an inflammatory stimulant derived from cell walls of Gram-negative bacteria increased KYNA production in HGF *in vitro*. Finally, the bactericidal effect of KYNA was presented. We found that KYNA exerted the short-lasting bactericidal effect on *Escherichia coli*, *Streptococcus mutans* DSM 20381 and *Staphylococcus aureus*, bacteria detected in oral cavity. The concentration of KYNA effective *in vitro* (~0.5 mM) was higher than that found in saliva in patients with odontogenic abscesses (12 nM). These findings, however, do not necessarily exclude the possibility that KYNA may contribute to the control of bacterial growth *in vivo*. In our study, the concentration of KYNA was measured in the whole saliva and not in the immediate vicinity of bacteria, e.g. inside the abscesses. Due to the low concentration of KYNA in the saliva, it can be hypothesized, that it operates in concert with defense mechanisms known to be present in saliva. It was shown that the antibacterial activity of saliva depends on many immunologic and nonimmunologic components. These are secretory immunoglobulins, IgA, IgG and IgM and various proteins, mucins, peptides and enzymes, which maintain the physiological flora in the oral cavity and protect teeth against microbial assault [10]. It should also be mentioned that in patients with hyposalivation caused by radiation therapy, primary Sjögren's syndrome and unknown factors the marked changes in the oral microflora were found [1].

In conclusion, we provide evidence that KYNA is an endogenous constituent of human saliva, and hypothesize its contribution to the control of oral microflora.

Acknowledgment:
This study was supported by a grant from the Medical University, Lublin, Poland.

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Received:
April 6, 2006; in revised form: May 15, 2006.