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Original article

The *in vitro* effect of kynurenic acid on the rainbow trout (*Oncorhynchus mykiss*) leukocyte and splenocyte activity

J. Małaczewska¹, A.K. Siwicki¹, R. Wójcik¹, W.A. Turski², E. Kaczorek¹

¹ Department of Microbiology and Clinical Immunology, Faculty of Veterinary Medicine, University of Warmia and Mazury, Oczapowskiego 13, 10-718 Olsztyn, Poland

² Chair and Department of Experimental and Clinical Pharmacology, II Faculty of Medicine with English Language Division, Medical University, Ceramiczna 1, 20-150 Lublin, Poland

Abstract

Kynurenic acid (KYNA), an endogenous neuroprotectant formed along the kynurenine pathway of tryptophan degradation, is a selective ligand of the GPR35 receptor, which can be found on the surface of various populations of human immune cells. In infections and inflammations, KYNA produces an anti-inflammatory effect through this receptor, by depressing the synthesis of reactive oxygen species and pro-inflammatory cytokines. However, it is still unrecognized whether receptors for kynurenic acid are also localized on immune cells of poikilothermic animals, or whether KYNA is able to affect these cells. The objective of this study has been to determine the effect of different concentrations of kynurenic acid (12.5 μ M to 10 mM) on the viability and mitogenic response of lymphocytes and on the activity of phagocytic cells isolated from blood and the spleen of rainbow trout. The results imply low toxicity of kynurenic acid towards fish immune cells, and the proliferative effect observed at the two lowest concentrations of KYNA (12.5 μ M and 25 μ M) seems indicative of endogenous kynurenic acid being capable of activating fish lymphocytes. Non-toxic, micromole concentrations of KYNA, however, had no influence on the mitogenic response of lymphocytes nor on the activity of phagocytes in rainbow trout under *in vitro* conditions. There is some likelihood that such an effect could be observed at lower, nanomole concentrations of KYNA.

Key words: kynurenic acid, lymphocyte and phagocyte activity, rainbow trout

Introduction

Kynurenic acid (KYNA), a metabolite of tryptophan formed along the kynurenine pathway, an antagonist of ionotropic glutamate receptors (NMDA)

and α -7 cholinergic nicotinic acetylcholine receptors (nACh), is mostly described as a substance having neuroprotective properties. It has been known for some time now that KYNA is also a selective ligand of the G protein-coupled receptor (GPR35), present on

human cells associated with the immune system. Strong expression of this receptor has been detected on monocytes, neutrophils, dendritic cells and T lymphocytes; it was less strongly expressed on B lymphocytes, natural killer T (NKT) cells, basophiles and eosinophiles (Wang et al. 2006, Fallarini et al. 2010). Concentrations of kynurenic acid in blood and tissues of healthy subjects are low, measurable in nanomoles, but they increase to micromole concentrations during infections and inflammations. The reason is the activation of the kynurenine pathway of tryptophan metabolism by inflammation mediators such as bacterial lipopolysaccharides (LPS), free radicals and pro-inflammatory cytokines (IFN γ , TNF α , IL-1 β). Tryptophan metabolites formed along the kynurenine pathway (kynurenines) produce an anti-inflammatory effect through mutual negative feedback, and the main mechanism of action is thought to be the anti-proliferative and pro-apoptotic effect on T lymphocytes (Kudo et al. 2001, Terness et al. 2002, Wang et al. 2006, Moroni et al. 2007, Chen and Guillemin 2009, de Souza et al. 2011, Mándi and Vecsei 2012). Kynurenic acid belongs to these immunotropic kynurenines. Its anti-inflammatory effect stems from the effect on the GPR35 receptor. It is known that this receptor is not activated until KYNA reaches higher than physiological, micromole concentrations (Fallarini et al. 2010, Mándi and Vecsei 2012), and the major effect of its activation is the depressed synthesis of pro-inflammatory cytokines, nitrogen oxide and reactive oxygen species in immune cells (Maes et al. 2007, Moroni et al. 2007, Kaszaki et al. 2008, Lugo-Huitron et al. 2011, Tiszlavicz et al. 2011, Malaczewska et al. 2014). The above findings stimulate an interest in kynurenic acid as a potential immunomodulator, which could find applications in correcting the immune response of an organism, especially in the course of systemic disorders. Immunomodulators are also used in veterinary medicine, especially in aquaculture because of the specificity of the immune response of fish (Magnadóttir 2006). However, no studies have been completed until this day to determine the effect of kynurenic acid on the immune cells of fish.

The purpose of this research has been to determine the effect of various concentrations of kynurenic acid (12.5 μ M – 10 mM) on the viability and activity of immune cells of rainbow trout, isolated from peripheral blood and the spleen, under *in vitro* conditions.

Materials and Methods

Kynurenic acid

Kynurenic acid (KYNA) of the highest available purity was purchased from Sigma-Aldrich. Just before

the use, KYNA was dissolved in the cell growth medium to reach the final concentrations of: 0, 12.5, 25, 62.5, 125, 250, 625 μ M, 1.25 mM, 2.5, 5 and 10 mM.

Fish

The experiment was performed on 24 individuals of the rainbow trout (*Oncorhynchus mykiss*), weighing 350–400 g, supplied by the Inland Fisheries Institute, Olsztyn, Poland. The animals were randomly divided into 6 groups. The immune cells isolated from the individuals within each group were pooled before performing the assays, and tested in triplicate. Circulating blood was collected from fish anaesthetized with 2% Propiscin (Żabieniec, Poland) diluted in water (1 ml l⁻¹) by caudal vein puncture, and the spleen was sampled after bleeding. The experiment has been approved by the Local Ethics Committee.

Isolation of leukocytes

Leukocytes for the tests were isolated from the fish blood and spleen. The spleens were removed aseptically, and pressed through a 60- μ m nylon mesh in RPMI-1640 medium with L-glutamin and sodium bicarbonate (Sigma-Aldrich). The splenocyte cell suspension and the whole heparinized blood diluted 1:2 in RPMI-1640 medium were placed on density gradients in 1:1 ratio (3 ml of gradient and 3ml of cell suspension or diluted blood sample). Gradisol G (Aqua-Medica, Łódź, Poland) was used in order to isolate phagocytic cells and Gradisol L (Aqua-Medica, Łódź, Poland) in order to isolate lymphocytes. After the centrifugation at 400 x g for 40 min at 4°C, the interface cells were collected and washed three times with the RPMI-1640 medium at 400 x g for 5 min. Viability of isolated cells was evaluated by trypan blue exclusion using the EVE automatic cell counter (NanoEnTek, Korea), according to the producer instructions, and was determined to be greater than 95% in each case. Cells were suspended in RPMI-1640 medium containing 10% fetal calf serum (FCS, Sigma-Aldrich), 1% antibiotic-antimycotic solution (Sigma-Aldrich) and different concentrations of kynurenic acid, then dispensed into 96-well plates at a concentration of 5x10⁶ cells ml⁻¹, cultured/incubated at 22°C and used for the following assays.

The viability and mitogenic response of lymphocytes – MTT assay

The mitogenic response of lymphocytes was determined using the MTT colorimetric assay (Mosmann

1983). Cells were suspended in RPMI-1640 growth medium containing mitogens – concanavalin A (ConA, Sigma-Aldrich) at a concentration of 50 $\mu\text{g ml}^{-1}$ as a T-cell mitogen or lipopolysaccharide from *Serratia marcescens* (LPS, Sigma-Aldrich) at a concentration of 25 $\mu\text{g ml}^{-1}$ as a B-cell mitogen and 100 μl of the suspension was added to each well of microtiter plates. The mixture was incubated for 96 h in the presence of different concentrations of kynurenic acid at 22°C. After incubation, 25 μl of solution containing 7 mg ml^{-1} of MTT (3-[4, 5 dimethylthiazolyl-2-yl]-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich) in PBS were added and the plate was incubated for the next 4 h. The supernatant was removed and 100 μl DMSO (dimethylsulfoxide, POCh, Gliwice, Poland) was added to each well. The optical density was measured in a Sunrise absorbance reader (Tecan, Austria) at a wavelength of 570 nm with 640 nm as a reference wavelength. Samples obtained from each group were tested in triplicate. The results of the proliferation assay were expressed as a stimulation index (SI), which was calculated by dividing the mean optical density (OD) of stimulated cultures by the OD of the non-stimulated (control) cultures.

MTT assay was also used to determine the viability of nonadherent cells incubated for 48 h in the presence of kynurenic acid, without mitogens. In this case the results were expressed as the percent of the control cells viability.

Respiratory Burst Activity (RBA) test

The metabolic activity of phagocytes was determined by the measurement of the intracellular respiratory burst activity after stimulation with PMA (phorbol myristate acetate, Sigma-Aldrich), as described by Chung and Secombes (1988) with some modifications described by Chettri et al. (2010). A 100 μl of cell suspension was added to each well of 96-well microtiter plates (Nunc, Denmark). After incubation for 2 h at 22°C, the cells were washed in RPMI 1640 medium to remove nonadherent cells and incubated for the next 24 h with kynurenic acid. Then, 100 μl of PMA (1 $\mu\text{g ml}^{-1}$) in 0.1% NBT (nitroblue tetrazolium, Sigma-Aldrich) solution in RPMI 1640 medium were added to each well. The mixture was incubated for 60 min at 22°C. After the removal of the medium from the cells, the reaction was stopped by the addition of absolute ethanol and then washed twice with 70% ethanol. The formazan produced in the cells was dissolved in 120 μl of 2M KOH and 140 μl of DMSO and the optical density was measured colorimetrically in a Sunrise absorbance reader (Tecan, Austria) at 620 nm. The results

of the RBA assay were expressed as a mean OD of PMA stimulated cells.

Potential Killing Activity (PKA) test

The technique presented by Rook et al. (1985) was used to measure the potential killing activity of phagocytes. A 100 μl of cell suspension was added to each well of 96-well microtiter plates and incubated for 2 h at 22°C. Then, after removing of nonadherent cells and the following 24 h incubation of cells with kynurenic acid, 100 μl of 0.1% NBT solution in PBS (phosphate-buffered saline, Biomed Lublin, Poland) containing *Aeromonas hydrophila* (1×10^8 cells ml^{-1}) were added to each well and incubated for 60 min at 22°C. After incubation the supernatant was removed from each well and adherent cells were fixed with absolute ethanol. Then, 120 μl of 2M KOH and 140 μl of DMSO were added to each well and the plates were mixed. The amount of extracted reduced NBT was measured colorimetrically in a Sunrise absorbance reader (Tecan, Austria) at 620 nm. The results of the PKA assay were expressed as a mean OD of bacteria stimulated cells.

Statistical analysis

Data were analyzed statistically by one-way analysis of variance (ANOVA). Bonferroni's post test was used to determine differences between groups. Statistical evaluation of results was performed using GraphPadPrism software package.

Results

KYNA effect on the lymphocyte viability in the rainbow trout

High, millimole concentrations of KYNA showed the cytotoxic effect towards lymphocytes of rainbow trout, with cells isolated from the spleen being more sensitive than blood cells. A significant decrease in the viability of splenocytes was observed at the KYNA concentrations of 1.25-10 mM (1.25 mM at $p < 0.05$, remaining concentrations at $p < 0.001$), while the viability of peripheral blood lymphocytes began to decrease significantly at the concentration of 2.5 mM (2.5 mM at $p < 0.05$, remaining concentrations at $p < 0.001$). At the same time, the two lowest, micromole concentrations of KYNA had some proliferative effect on the spleen lymphocytes (12.5 μM at $p < 0.05$, 25 μM at $p < 0.01$), as the viability of tested cells was

Table 1. The in vitro effect of KYNA on the viability of rainbow trout lymphocytes (%).

Cells origin	KYNA concentration										
	0 (C)	12.5 μ M	25 μ M	62.5 μ M	125 μ M	250 μ M	625 μ M	1.25 mM	2.5 mM	5 mM	10 mM
Spleen	100	123.729*	127.118**	120.338	111.864	106.779	94.915	76.271*	59.322***	49.153***	33.898***
	\pm 11.058	\pm 14.005	\pm 15.647	\pm 14.336	\pm 11.98	\pm 10.085	\pm 9.328	\pm 9.524	\pm 6.875	\pm 6.199	\pm 4.586
Blood	100	108.196	114.754	107.862	101.639	98.361	95.081	81.967	78.688*	72.131***	65.573***
	\pm 9.956	\pm 8.445	\pm 13.033	\pm 9.751	\pm 10.23	\pm 9.532	\pm 10.221	\pm 8.972	\pm 8.406	\pm 6.594	\pm 7.338

Table 2. The in vitro effect of KYNA on the mitogenic response of rainbow trout lymphocytes (SI).

Cells origin	Mitogen	KYNA concentration										
		0 (C)	12.5 μ M	25 μ M	62.5 μ M	125 μ M	250 μ M	625 μ M	1.25 mM	2.5 mM	5 mM	10 mM
Spleen	ConA	3.875	3.586	3.567	3.321	3.895	3.638	3.121	3.163	3.192	2.593***	2.494***
		\pm 0.543	\pm 0.478	\pm 0.251	\pm 0.361	\pm 0.227	\pm 0.258	\pm 0.409	\pm 0.462	\pm 0.401	\pm 0.317	\pm 0.324
Spleen	LPS	1.672	1.516	1.448	1.389	1.583	1.8	2.031	1.448	1.314	1.303	1.227*
		\pm 0.156	\pm 0.157	\pm 0.214	\pm 0.227	\pm 0.272	\pm 0.184	\pm 0.311	\pm 0.229	\pm 0.196	\pm 0.212	\pm 0.163
Blood	ConA	2.133	1.757	1.861	1.777	2.047	2.053	2.151	1.725	1.526**	1.58*	1.382***
		\pm 0.292	\pm 0.221	\pm 0.192	\pm 0.269	\pm 0.34	\pm 0.308	\pm 0.229	\pm 0.238	\pm 0.162	\pm 0.174	\pm 0.199
Blood	LPS	1.273	1.189	1.205	1.193	1.169	1.251	1.547	1.185	1.156	1.121	1.091
		\pm 0.167	\pm 0.215	\pm 0.218	\pm 0.155	\pm 0.177	\pm 0.146	\pm 0.183	\pm 0.129	\pm 0.109	\pm 0.132	\pm 0.143

Table 3. The in vitro effect of KYNA on the activity of rainbow trout phagocytes (OD).

Cells origin	Test	KYNA concentration										
		0 (C)	12.5 μ M	25 μ M	62.5 μ M	125 μ M	250 μ M	625 μ M	1.25 mM	2.5 mM	5 mM	10 mM
Spleen	RBA	0.632	0.578	0.641	0.754	0.702	0.607	0.612	0.597	0.568	0.495	0.453*
		\pm 0.101	\pm 0.096	\pm 0.082	\pm 0.093	\pm 0.066	\pm 0.072	\pm 0.052	\pm 0.076	\pm 0.081	\pm 0.077	\pm 0.058
Spleen	PKA	0.527	0.514	0.593	0.647	0.603	0.553	0.563	0.527	0.513	0.498	0.462
		\pm 0.076	\pm 0.068	\pm 0.059	\pm 0.081	\pm 0.095	\pm 0.062	\pm 0.059	\pm 0.098	\pm 0.062	\pm 0.077	\pm 0.071
Blood	RBA	0.467	0.478	0.504	0.484	0.492	0.533	0.567	0.523	0.465	0.469	0.417
		\pm 0.059	\pm 0.071	\pm 0.066	\pm 0.087	\pm 0.067	\pm 0.058	\pm 0.079	\pm 0.071	\pm 0.043	\pm 0.047	\pm 0.056
Blood	PKA	0.517	0.553	0.523	0.654	0.573	0.524	0.529	0.516	0.508	0.497	0.483
		\pm 0.069	\pm 0.068	\pm 0.077	\pm 0.066	\pm 0.072	\pm 0.079	\pm 0.095	\pm 0.088	\pm 0.076	\pm 0.048	\pm 0.071

Explanations: All results are expressed as means \pm SD (standard deviation); the p-values (* p <0.05, ** p <0.01, *** p <0.001) refer to the significant differences between control cells (0 mM KYNA) and cells incubated with different concentrations of KYNA.

by 23-27% higher than the control cells viability. No such effect was observed on lymphocytes isolated from blood of the examined fish (Table 1).

KYNA effect on the mitogenic response of the rainbow trout lymphocytes

Toxic concentrations of KYNA decreased the proliferative response of ConA stimulated T lymphocytes, isolated from blood and the spleen of rainbow trout. A significant decrease in the stimulation index of T lymphocytes isolated from the spleen was

observed at the concentrations from 5 to 10 mM (at p <0.001), while for the T cells isolated from blood a significant decrease in the stimulation index appeared at the concentrations of 2.5-10 mM (2.5 mM at p <0.01, 5 mM at p <0.05, 10 mM at p <0.001). Additionally, the highest KYNA concentration depressed the proliferative response of the spleen LPS stimulated B lymphocytes (p <0.05). No such effect was noticed with respect to B lymphocytes isolated from peripheral blood. None of the non-toxic concentrations of KYNA produced a significant effect on the mitogenic response of the rainbow trout lymphocytes (Table 2).

KYNA effect on the phagocyte activity in the rainbow trout

It was only the highest KYNA concentration that significantly decreased the respiratory burst activity of phagocytes isolated from the fish's spleen ($p < 0.05$). No significant effect of KYNA on the potential killing activity of splenocytes was observed. None of the analyzed concentrations affected the activity of peripheral blood phagocytes in rainbow trout (neither respiratory burst nor potential killing activity) (Table 3).

Discussion

All available papers on the effect of KYNA on immunocompetent cells examined *in vitro* deal with the human peripheral blood leukocytes and focus on the influence of KYNA on the synthesis of pro-inflammatory cytokines following mitogenic stimulation of cells (Maes et al. 2007, Fallarini et al. 2010, Tizlavicz et al. 2011). To the authors' best knowledge, this is the first report describing the influence of kynurenic acid on the activity of fish immune cells *in vitro*.

Relatively few papers mention the effect of kynurenic acid on the viability of cells, and most often these are other types of cells than the immune cells analyzed in the present study. The toxicity of KYNA reported by other authors differed, depending on the used cell model, although the level of toxic concentrations was always higher by a few hundred- or even a few thousand-fold than values of KYNA detected in the human organism during the course of an inflammatory process. The adverse influence of KYNA on functions of the rat's myocardial mitochondria was observed at the concentrations of 125 μM – 10 mM/l (Baran et al. 2001), whereas the proliferation of endothelial cells in the bovine aorta was decreased (by 25%) at the concentration of 1 mM (Wejksza et al. 2009). The mixed culture of rainbow trout lymphocytes employed in our study was characterized by a yet lower sensitivity to the adverse effect of high KYNA concentrations. Namely, a decrease in the viability of cells was not observed until the KYNA concentrations reached 1.25 mM and 2.5 mM, and then affected 23.7% and 21.3% of lymphocytes isolated from the spleen and peripheral blood, respectively. Such low sensitivity can be justified by the specific functions performed in an organism by immunocompetent cells. What might be somehow surprising is the proliferative effect of the two lowest concentrations of kynurenic acid (12.5 and 25 μM) because, as mentioned in the introduction to this article, KYNA, as a metabolite of the kynurenic pathway, is regarded as

a compound producing anti-proliferative action, especially towards T lymphocytes. In research on other types of cells than immune ones, very low concentrations of kynurenic acid either failed to produce any effect on this parameter or, analogously to our study, stimulated cells' proliferation. For example, Wejksza et al. (2009) reported that the concentrations of 0.01 and 0.1 mM KYNA did not stimulate the migration and proliferation of endothelial cells, although they neutralized the negative effect of homocysteine on these parameters. Elevated proliferation of cells at the concentrations of 1 and 10 μM of kynurenic acid was observed, however, in respect of the glia cell lines N11 and U-343MG (Di Serio et al. 2005). The proliferative effect of KYNA on rainbow trout lymphocytes may have been caused by the acid-induced activation of receptors on the surface of lymphocytes; although the question whether the GPR35 receptor occurs in fish at all remains unanswered.

Only very high concentrations of kynurenic acid, 2.5 mM and above, significantly affected the mitogenic response of rainbow trout peripheral blood lymphocytes; for splenocytes, the lowest effective concentration was even higher, i.e. 5 mM. The decrease in the proliferation of ConA-stimulated T lymphocytes observed in that case was most certainly caused by the cytotoxic effect of high concentrations of KYNA rather than its anti-proliferative effect, because the same, high concentrations of this acid coincided with a decreased viability of lymphocytes. A decrease in the LPS-stimulated B cells was observed only in the case of splenocytes and only at the highest KYNA concentration applied, therefore it may be suspected that the cytotoxicity of high KYNA concentrations affected mainly T cells. It is possible that in fish, like in man, the expression of receptors for kynurenic acid on T cells is much stronger than on B cells (Wang et al. 2006, Fallarini et al. 2010). However, we lack relevant data to verify this hypothesis. On the other hand, the fact that low KYNA concentrations had no effect on the mitogenic response of fish lymphocytes *in vitro* is unsurprising. Kudo et al. (2001) also found out that kynurenic acid in the concentrations of 10 and 100 μM did not affect the proliferation of human peripheral blood mononuclear cells stimulated by PHA.

Regarding the phagocytic cells of rainbow trout, there was only one example of a significant change, namely a decrease in the respiratory burst activity of splenocytes induced by the highest tested KYNA concentration, and the said effect was most probably caused by the toxicity of the acid. However, the literature contains reports verifying the antioxidant activity of KYNA, both in *in vitro* experiments and in animal organisms, leading to a reduced synthesis of reactive

oxygen species (ROS) and even to a considerable decrease in the intensity of respiratory burst of the peripheral blood phagocytes (Moroni et al. 2007, Kaszaki et al. 2008, Lugo-Huitron et al. 2011, Malaczewska et al. 2014). However, if the ROS scavenging properties of KYNA were to be responsible for the observed effect, then similar changes in the oxidative burst should be observable in a broader range of concentrations of this acid.

Recapitulating, kynurenic acid is characterized by low toxicity towards the immune cells of rainbow trout. At the same time, the proliferative effect of the two lowest KYNA concentrations (alone, without addition of mitogens) seems to suggest that endogenous kynurenic acid is able to activate fish lymphocytes. Non-toxic, micromole concentrations of KYNA, however, had no influence on the mitogenic response of lymphocytes nor on the activity of phagocytes in rainbow trout under *in vitro* conditions. It cannot be excluded that such an effect could occur at the lower, nanomole concentrations of KYNA, only slightly exceeding its physiological levels observed in humans, as reported by Maes et al. (2007), where the most significant effects of kynurenic acid on the synthesis of pro-inflammatory cytokines by the human peripheral blood leukocytes *in vitro* were found at the concentration of 280 nM. However, this hypothesis needs verification.

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