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TRANSLATIONAL NEUROSCIENCES - ORIGINAL ARTICLE

Kynurenic acid and its derivatives are able to modulate the adhesion and locomotion of brain endothelial cells

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Abstract

The neuroprotective actions of kynurenic acid (KYNA) and its derivatives in several neurodegenerative disorders [characterized by damage to the cerebral endothelium and to the blood–brain barrier (BBB)] are well established. Cell–extracellular matrix (ECM) adhesion is supposedly involved in recovery of impaired cerebral endothelium integrity (endothelial repair). The present work aimed to investigate the effects of KYNA and its synthetic derivatives on cellular behaviour (e.g. adhesion and locomotion) and on morphology of the GP8 rat brain endothelial cell line, modeling the BBB endothelium. The effects of KYNA and its derivatives on cell adhesion were measured using an impedance-based technique, the xCELLigence SP system. Holographic microscopy (Holomonitor™ M4) was used to analyse both chemokinetic responses and morphometry. The GP8 cells proved to be a suitable model cell line for investigating cell adhesion and the locomotion modulator effects of kynurenines. KYNA enhanced cell adhesion and spreading, and also decreased the migration/motility of GP8 cells at physiological concentrations (10^{-9} and 10^{-7} mol/L). The derivatives containing an amide side-chain at the C2 position (KYNA-A1 and A2) had lower adhesion inducer effects compared to KYNA. All synthetic analogues (except KYNA-A5) had a time-dependent inhibitory effect on GP8 cell adhesion at a supraphysiological concentration (10^{-3} mol/L). The immobilization promoting effect of KYNA and the adhesion inducer activity of its derivatives indicate that these compounds could contribute to maintaining or restoring the protective function of brain endothelium; they also suggest that cell–ECM adhesion and related cell responses (e.g. migration/motility) could be potential new targets of KYNA.

Keywords Endothelial repair · Kynurenic acid · Holographic microscopy · Impedimetry · Synthetic kynurenines · Brain endothelial cell · Kynurenic acid (PubChem CID: 3845) · L-Kynurenine (Pubchem CID: 161166)

Introduction

L-Kynurenine (KYN) and kynurenic acid (KYNA) are endogenous metabolites of tryptophan. The kynurenine pathway is the main pathway for breaking down tryptophan

Kálmán Magyar : Deceased.

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in the brain; it is also expressed to a varying degree in astrocytes, neurons, glia, macrophages, endothelial cells and dendritic cells. Approximately 95% of tryptophan is believed to convert to KYN by two catalytic steps: the first is a catalysis by either tryptophan- or indoleamine 2,3-dioxygenases (TDO or IDO-1, IDO-2) and the second is mediated by formamidase (Fig. 1) (Vecsei et al. 2013). KYN serves as a key molecule between neurotoxic and neuroprotective metabolites since it can be metabolized via three distinct pathways to form: (1) 3-hydroxy-L-kynurenine, (2) anthranilic acid and (3) kynurenic acid by the action of kynurenine 3-monooxygenase, kynureninase and kynurenine aminotransferases (KATs), respectively. Four subtypes of KATs (KAT I, II, III and IV) have been identified and all of them showed transamination activity towards KYN, leading to synthesis of KYNA; however, there are differences in their substrate specificity, their pH profiles and their contribution to KYNA formation in the brain (Han et al. 2010). 3-Hydroxy-L-kynurenine can be further transformed, leading to the formation of quinolinic acid via intermediates that include 3-hydroxyanthranilic acid. Quinolinic acid and its intermediates are known neurotoxic compounds; they generate free radicals, oxidative stress, lipid peroxidation and subsequently excitotoxicity due to excitatory amino acid receptors (Moroni et al. 2012; Vecsei et al. 2013). In contrast, KYNA has been widely shown to have a characteristic neuroprotective effect and has been proven to act protectively amidst degenerative processes [Alzheimer's- (Zwilling et al. 2011), Huntington's- (Beal et al. 1990; Zadori et al. 2011) and Parkinson's diseases (Ogawa et al. 1992; Silva-Adaya et al. 2011)]. Furthermore, KYNA's actions are also implicated in migraines (Vecsei et al. 2013), as well as in inflammatory (Varga et al. 2010) and immunological (Mandi and Vecsei 2012; Tiszlavicz et al. 2011) mechanisms.

KYNA has been described as a potential blocker of endogenous excitatory amino acid receptors (e.g. ionotropic glutamate receptors): glycine binding site of *N*-methyl-D-aspartate (NMDA),

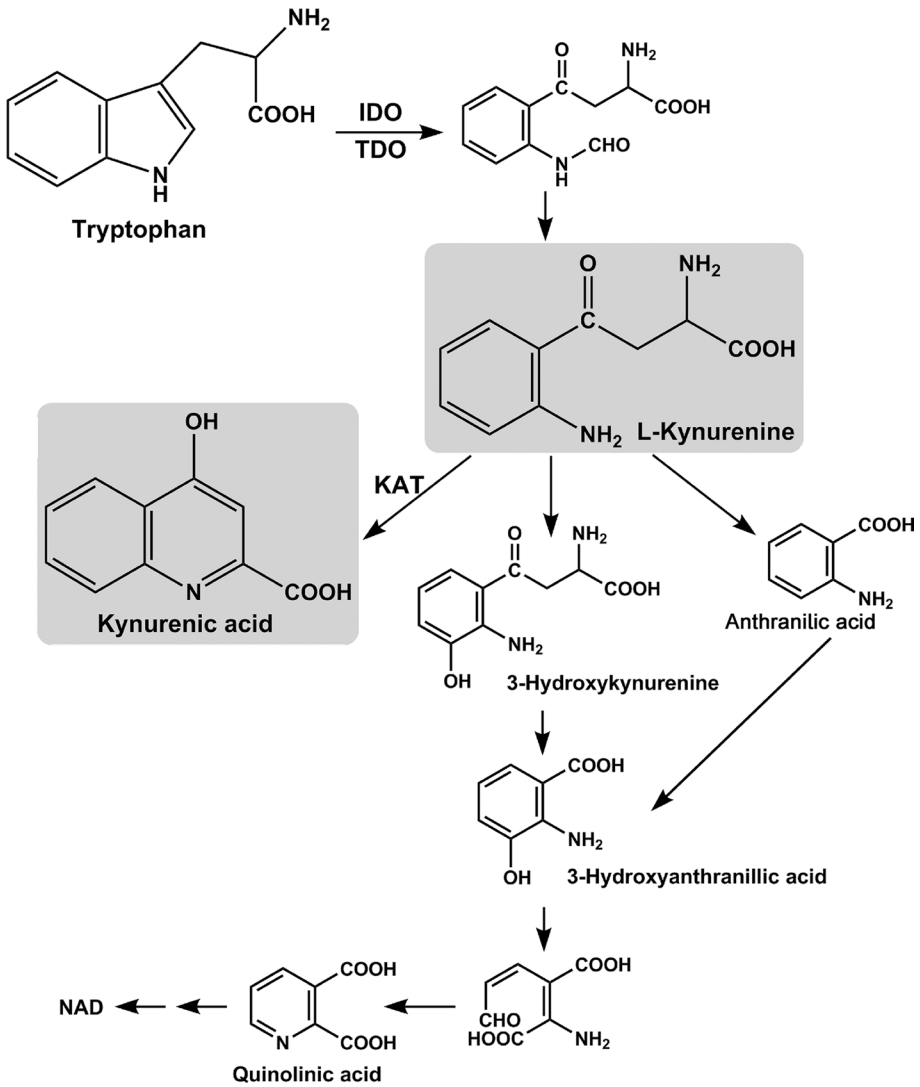

Fig. 1 Tryptophan–kynurenine metabolism. *TDO/IDO* tryptophan- or indoleamine 2,3-dioxygenases-1 and -2, *KAT* kynurenine aminotransferase

α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainite receptors (Perkins and Stone 1982; Prescott et al. 2006; Wu et al. 1995) and the α-7-nicotinic acetylcholine (nACh) receptor (Hilmas et al. 2001; Zwilling et al. 2011). However, interesting dose-dependent effects of KYNA were revealed in in vitro electrophysiological examinations of young rat hippocampi. It was confirmed that KYNA (referred to as Janus-face molecule) exerts inhibitory effects at micromolar concentrations, while at nanomolar concentrations, it facilitates field excitatory postsynaptic potentials (Rozsa et al. 2008). Furthermore, KYNA acts as an agonist of the G-protein-coupled orphan receptor (GPR35), which can contribute to decreased glutamate as well as to pro-inflammatory mediator release from glia and immune cells by means of attenuated cAMP and intracellular Ca^{2+} concentration. The exact role of GPR35 in the nervous system remains unknown, but it has been demonstrated that the KYNA–GPR35 relationship is important in certain neurodevelopmental processes (Cosi et al. 2011).

The above-mentioned pathological conditions, where the alteration of the kynurenine pathway was observed, such as inflammation (e.g. multiple sclerosis), neurodegeneration (e.g. Alzheimer's disease) and cerebral ischemia (e.g. stroke); are characterized by disruption of the functional/structural integrity of brain endothelial cells and impairment in the function of the blood–brain barrier (BBB) (del Zoppo and Milner 2006; Lyros et al. 2014; Persidsky et al. 2006).

From the point of view of barrier function, the brain endothelial cells are considered to be the most important cell type of the neurovascular unit; consisting of astrocytes, pericytes, neurons and extracellular matrix (ECM) of the basement membrane (Persidsky et al. 2006; Wilhelm et al. 2011). The permeability of cerebral endothelial cells is based mainly on two structures: (1) inter-endothelial junctions (tight and adherent junctions); (2) cell–ECM interactions involving integrins, dystroglycans of the endothelial cells and the constituent components of the intact basement membrane (del Zoppo and Milner 2006). Cell–ECM interactions contribute to ensuring the morphology and function of cerebral endothelial cells in quiescent state and to maintaining the BBB by (1) regulating key cellular processes (proliferation, differentiation and migration); (2) modifying VE-cadherin junctions and consequently endothelial permeability, as well as (3) anchoring the endothelial cells in place and regulating their migration (Baeten and Akassoglou 2011; del Zoppo and Milner 2006; Persidsky et al. 2006). The components of the basement membrane (e.g. collagen IV, laminin, perlecan), which together represent the other side of cell–ECM interaction, also regulate endothelial function by (1) interacting with matrix adhesion receptors; (2) modulating the availability of growth factors and (3) increasing transendothelial electrical resistance (barrier properties) (Baeten and Akassoglou 2011).

During neuropathological changes, initial disturbances in cerebral microcirculation, pro-inflammatory and autoimmune conditions lead to endothelial/BBB dysfunction and increased permeability (Lyros et al. 2014; Vecsei et al. 2013). As an early consequence, the endothelium becomes dysfunctional and reactive oxygen species (ROS) are generated; and the expression of adhesion molecules and chemokines induce the recruitment of leukocytes (Baeten and Akassoglou 2011; Kelleher and Soiza 2013). The prolonged presence of oxidative/inflammatory factors can result in changes to or loss of tight junctions and integrins (e.g. β_1, α_v, α_6 integrins), leading to senescence and detachment-mediated cell death (anoikis) (Baeten and Akassoglou 2011; del Zoppo and Milner 2006). These changes together contribute to secondary inflammation, further breakdown of the BBB with leakage of plasma proteins and neurotoxic substances, or to thrombus formation by exposure of subendothelial tissues (Baeten and Akassoglou 2011; Kelleher and Soiza 2013; Lyros et al. 2014).

Endothelial cells have regenerative capacity, helping to restore normal structure and function. There are two main kinds of endogenous endothelial repair process (Fig. 2): (1) proliferation and migration of endothelial cells surrounding the lesion, leading to rapid re-endothelization; the repair capacity in this case is limited; (2) incorporation of circulating endothelial progenitor cells as an alternative mechanism to replacing lost and/or injured endothelial cells (Deanfield et al. 2007). The capacity of endogenous endothelial repair mechanisms is dependent on several factors that can increase proliferation [e.g. vascular endothelial growth factor (VEGF)] and migration [e.g. stromal cell-derived factor 1 (SDF-1) chemokine; nitric oxide] of the endothelial cells, or can upregulate the expression of several cell–ECM adhesion molecules (e.g. α5β1 integrin—fibronectin receptor, α4 and β2 integrin, E-selectin ligand); these were shown to be involved in the homing of endothelial progenitor cells or in local endothelial repair (Fig. 2) (Besler et al. 2008; Deanfield et al. 2007; Zampetaki et al. 2008).

A considerable amount of literature indicates that KYNA can also have remarkable functions in endothelial cells. Wejksza and her co-workers have revealed the presence of KAT I and KAT II as well as de novo synthesis of KYNA from KYN in cultured bovine aortic endothelial cells. Endothelial cells are able to regulate the concentration of kynurenines in the central nervous system (CNS) by taking up KYN and converting it to KYNA (Wejksza et al. 2004). It is assumed that the expression changes of IDO in endothelial cells by inflammatory mediators may regulate vascular tone and subsequently neuronal activity, neurodegeneration and cognition. There is evidence to suggest that the KYN may be secreted from the basolateral pole of endothelial cells, gaining direct access to the brain (Owe-Young et al. 2008); this is especially important

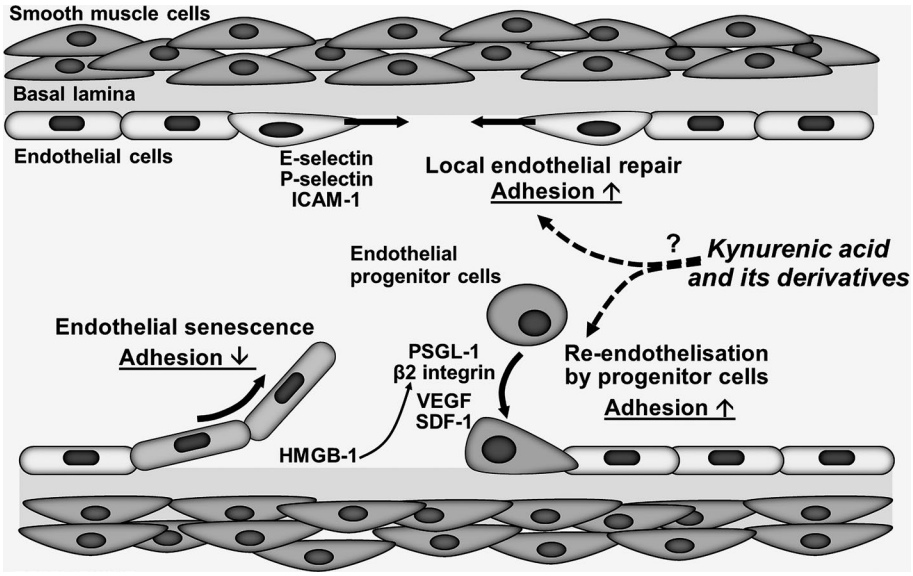

Fig. 2 Role of cell adhesion in maintenance of endothelial integrity. The endothelium can become dysfunctional in certain pathological conditions (e.g. neurodegeneration), which can progress to senescence and detachment of endothelial cells. The endogenous repair processes can restore endothelial integrity. The neighbouring endothelial cells can proliferate and adhere to the injury; the incorporation of circulating endothelial progenitor cells can also ensure re-endothelization. *ICAM-1* intercellular adhesion molecule 1, *HMGB1* high-mobility group protein B1, PSGL-1 P-selectin glycoprotein ligand-1, *VEGF* vascular endothelial growth factor, *SDF-1* stromal cell-derived factor 1

in conditions with a compromised BBB (Kandanearatchi and Brew 2012). It is also suggested that KYNA may have modulatory effects on the function of the BBB. The administration of KYNA could reduce the number of KCl-induced cortical spreading depression (CSD) waves and in parallel decrease CSD-associated permeability of the BBB (Oláh et al. 2013).

To study the function and the molecular and cellular mechanisms of BBB endothelium, several co-culture techniques (including primary brain endothelial cells) have been developed (Dehouck et al. 1990). However, these experimental BBB systems have some drawbacks: (1) the phenotypic instability of primary cerebral endothelial cultures; (2) the difficulty in eliminating all non-endothelial cell contaminants and (3) and insufficiently high throughput required for toxicity studies (Culot et al. 2008; Roux and Couraud 2005). The different immortalized brain endothelial cell lines [e.g. GP8 (Greenwood et al. 1996), GPNT (Regina et al. 1999) or hCMEC/D3 (Weksler et al. 2005)] were shown to be powerful and simplified alternatives of primary cerebral endothelial cells for neurotoxicity and permeability studies (Wilhelm et al. 2011). In our current study, the GP8 rat brain endothelial cell line was used, as it retains both cerebral endothelial morphology (cobblestone shape) and the expression of adhesion molecules (e.g. ICAM-1 or PECAM-1), transporters (e.g. *P*-glycoprotein) (Greenwood et al. 1996; Roux and Couraud 2005; Wilhelm et al. 2011) and vasoactive substances (e.g. arachidonic acid metabolites) (Kis et al. 1999) which are considered to be characteristic for BBB endothelium.

The above-mentioned findings indicate that influential endothelial cell–ECM adhesion and locomotion may be involved in the pharmacological effects of KYNA and its derivatives.

In the present work, our main objectives were:

1. To characterize the cell adhesion/morphology and locomotion of the GP8 brain endothelial cell line exposed to KYNA by label-free and real-time methods: impedimetry and holographic imaging.
2. To characterize and compare the cell–ECM adhesion modulator effects of different synthetic kynurenines in the GP8 cell line.

Materials and methods

Kynurenic acid and its analogues

KYN and KYNA were obtained from Sigma Aldrich (Steinheim, Germany). The KYNA analogues (Table 1) were synthesized and tested at the laboratory of the Institute of Pharmaceutical Chemistry, University of Szeged, according to procedures described earlier (Demeter et al. 2012; Fülöp et al. 2012; Nagy et al. 2011). All KYNA analogues contain a cationic centre in their side-chains.

Table 1 Empirical formula and analytical parameters of investigated synthetic kynurenic acid analogues

Name	Empirical formula	Molecular weight (g/mol)	Melting point (°C) ≈	Dissolves in H_2O
Kynurenic acid (KYNA)	$C_{10}H_7NO_3$	189.17	282–283	+
L-Kynurenine (KYN)	$C_{10}H_{12}N_2O_3$	208.21	302	+
KYNA-A1	$C_{14}H_{18}ClN_3O_2$	295.76	178–180	+
KYNA-A2	$C_{16}H_{20}ClN_3O_2$	321.80	227–230	+
KYNA-A4	$C_{19}H_{26}N_4O_3$	358.20	> 350	+ (< 10^{-3} mol/L)
KYNA-A5	$C_{19}H_{26}N_4O_2$	342.21	> 350	+ (< 10^{-3} mol/L)

Kynurenic acid derivatives—amides of C2-carboxylic function (KYNA-A1 and -A2) and amides of C2-carboxylic function + side-chains on C3 (KYNA-A4 and -A5)

KYNA and KYN analogues were dissolved in a 1:1 mixture of DMEM and F12 media (pH = 7.4) to prepare stock solutions of 10^{-2} mol/L concentration. The stock solutions were further diluted to the final concentrations of 10^{-9}, 10^{-7}, 10^{-5} and 10^{-3} mol/L, in complete medium used for cell culturing (see below). The selection of tested concentrations was based on the Janus-face character of KYNA (Rozsa et al. 2008), as well as on the plasma concentrations of KYNA (e.g. normal conditions, inflammation) (Mandi and Vecsei 2012). The negative control was the adequate volume of complete cell culture medium without test compounds.

Cell culturing

Cell adhesion induced by KYNA and its derivatives was investigated on the GP8 rat brain endothelial cell line (Greenwood et al. 1996).

The cells were maintained in a T25 flask (Orange Scientific, Braine-l'Alleud, Belgium), coated previously with rat tail collagen for 60 min at room temperature. The cells were passaged twice a week in a 1:1 mixture of DMEM and F12 (Sigma Ltd. St. Louis, MO, USA) containing 10% foetal bovine serum (Lonza Group Ltd., Switzerland), L-glutamine (2 mmol/L) (Gibco®/Invitrogen Corporation, New York, NY, USA), 0.1 mg/mL heparin (Teva, Debrecen, Hungary), 1 ng/mL basic fibroblast growth factor (Roche Applied Science, Indianapolis, IN, USA), 100 µg/mL penicillin/streptomycin (Gibco®/Invitrogen Corporation, New York, NY, USA).

Cell adhesion assay

The effects of KYNA and its analogues on adhesion of the GP8 cells were assessed using the xCELLigence SP System (Roche Applied Science, Indianapolis, IN, USA), which measured the change in impedance of gold microelectrodes integrated on the bottom of a tissue culture plate (E-plate) in real-time manner. More cells attached to the electrode, or higher spreading of cells creates a larger increase in impedance. Changes in impedance are represented by the cell index (CI). The CI is a relative and dimensionless value, calculated by the following formula:

$$\mathrm{CI} = \frac{\left(Z_i - Z_0\right)}{F_i},$$

where Z_i is the impedance at an individual point of time during the experiment, Z_0 is the impedance at the start of the experiment, and F_i is a constant depending on the frequency ($F_{10\ \mathrm{kHz}} = 15$).

During the characterization of the adhesion behaviour of GP8 rat brain endothelial cells, two types of ECM substrate (collagen, mixture of gelatin and fibronectin) were applied. In this experiment, the surface of the electrodes was coated either with rat tail collagen for 60 min at room temperature, or with a mixture of 1 µg/cm^2 human fibronectin (Chemicon® International Inc., Temecula, Canada) in 0.1% gelatin (Sigma Ltd. St. Louis, MO, USA) for 20 min incubation at 4 °C. The uncoated surface served as a control. After the coating, a background measurement was performed by adding 100 µL of complete culture medium and then recording the cell index for 1 h to gain constant background impedance curves. The cells were then added to the well at the final concentration of 10^4 cells per well. The attachment and spreading of the model cell were characterized by a time course study of Delta CI values. Delta CI refers to the difference of CI value at the time point of cell inoculation and the CI value at the given point in time. The adhesive capacity of the cells was described by the slope of Delta CI, calculated at a 3-h time interval (from the inoculation of the cells to the maximum of CI increase). The slope values were expressed as a percentage of the control.

In order to measure the adhesion modulator effects of KYNA and its derivatives, the electrodes were coated with 1 µg/cm^2 human fibronectin (Chemicon® International Inc., Temecula, Canada) in 0.1% gelatin (Sigma Ltd. St. Louis, MO, USA). The solutions of KYNA derivatives at the final

concentrations of 10^{-9}, 10^{-7}, 10^{-5}, 10^{-3} mol/L were added to the wells, and GP8 cells were applied at a concentration of 10^4 cells/well. Compound-free wells served as a control. The adhesion of the GP8 cells was monitored every 20 s for 24 h at 10 kHz.

The Delta CI values obtained at the 5, 10 and 15 h for each concentration of kynurenine were normalized to the untreated control and were given as Norm. Delta CI (Normalized Delta Cell Index) as a percentage.

Digital holographic microscopy

The Holomonitor™ M4 microscope (Phase Holographic Imaging AB, Lund, Sweden) was used for imaging and tracking both GP8 cell movement and morphological changes induced by KYNA. This technique provides quantitative and long-term kinetic cellular analysis without any cell labelling. The principle behind this method is the detection of the phase shift of the probing laser light transmitting or reflecting through a cell sample and comparison to the reference light (Persson et al. 2010; Peter et al. 2015).

The GP8 cells were seeded (2.5×10^5 cells) on a Petri dish (diameter: 35 mm), coated with 0.1% gelatin containing human 1 µg/cm^2 human fibronectin and allowed to adhere for 24 h. After automatic calibration of the background and microscope objective (20×), one field of each sample was focused on by the digital autofocus feature. The cells were then treated with KYNA at the final concentrations of 10^{-9}, 10^{-7} mol/L, or with an equivalent volume of culture medium, and captured every 30 s for 2 h.

For the evaluation, at each time-lapse sequence, 50 cells were identified and selected by minimum error histogram-based threshold algorithm of the software (HoloStudio M4 2.6.2). By tracking the cells, the movement and morphological changes were automatically analysed over time.

Cell movement, representing the chemokinetic effects of KYNA, was characterized by measurement of the following parameters: (1) migration (shortest distance between the starting point and the end point); (2) motility (actual path of a cell from the starting point to the end point) and (3) motility speed (ratio of actual path to time).

For the morphometry analysis, different basic and complex morphological parameters were displayed as a function of time. The (1) area, (2) thickness and (3) volume were grouped as basic metrics, while the more complex parameters relating to cell shape were: (4) the eccentricity (how elongated a cell is comparing to a circle), (5) the hull convexity (how different the 3D shape of a cell is compared to a perfect convex shape), (6) the irregularity (how different the circumference of a cell is compared to the circumference of a perfect circle) and (7) roughness (smoothness/roughness of a cell). The smoothness/roughness of a cell, characterized by the roughness parameter, indicates the viability of the treated cell (Lajkó et al. 2015; Tóth et al. 2014).

Statistical evaluation of data

The Delta CI and slope values were calculated using the integrated software (RTCA 1.2, Roche Applied Science, Indianapolis, IN, USA) of the xCELLigence SP System. The results of the experiments with holographic microscope were evaluated using the Holostudio™ M4 2.6.2 program. For further analysis of the data, the Origin Pro8.0 (OriginLab Corporation, Northampton, MA, USA) statistical program was used. Data shown in the figures represent mathematical averages of three parallels and ± SD values. Statistical analysis was done by application of ANOVA using Origin Pro8.0. Tukey's post hoc test was used to assess the statistical significance among means. The levels of significance are shown as follows: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

Results

Cell adhesion behaviour of GP8 endothelial cells

Both the collagen and gelatin + fibronectin coatings elicited an increased adhesion of the cells compared to the control (uncoated surface), but with different activity. The attachment rate of the GP8 cells proved to be higher with gelatin + fibronectin (308.5%, $p = 0.00011$) compared to collagen (132.1%, $p = 0.0196$). However, the difference was significant only in the first 5 h of the measurements; it then gradually decreased to level of collagen-induced adhesion. This pattern of adhesion curve suggests that the initial spreading of the cells—accompanied by a rapid, strong adhesion—is followed by a partial release of focal contacts. In contrast, the collagen coating resulted in a gradual increase of adhesion whereby spreading then reached a constant level (plateau phase) (Fig. 3a, b).

Cell adhesion modulator effect of kynurenic acid and its analogues

Following the characterization of cell adhesion in GP8 cells, KYNA and KYN—as reference molecules—and 4 KYNA analogues (Table 1) were investigated for their effects on cell adhesion of GP8 cells on a gelatin + fibronectin-coated surface.

In Fig. 4, the effects of KYNA and KYN are shown as a function of time (Fig. 4aa, ba) and concentration (Fig. 4ab, bb). The concentration-dependent effects of KYNA analogues are shown in Fig. 5 and their time-dependent effects are shown in Online Resource 1. The effects of KYNA derivatives were investigated in different experiments using

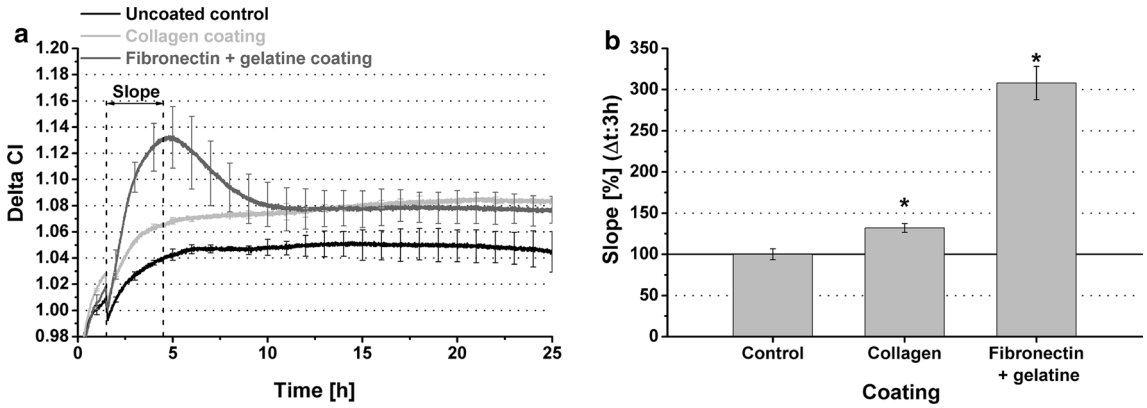

Fig. 3 Characterization of adhesion properties of GP8 endothelial cells. Time course study (**a**) and slope analysis (**b**) of the adhesion measurement by xCELLigence SP System. The Delta CI (Delta Cell Index) refers to the difference of the CI value at the point in time of cell inoculation and the CI value at another given point in time. The slope values are expressed as a percentage of the control and describe the changing rate of Delta CI in a 3-h time interval (between the point in time of cell inoculation and the point in time resulting in a maximum of cell adhesion). Data shown in the figures represent mathematical averages of three parallels and ± SD values. The levels of significance are shown as follows $*p < 0.05$

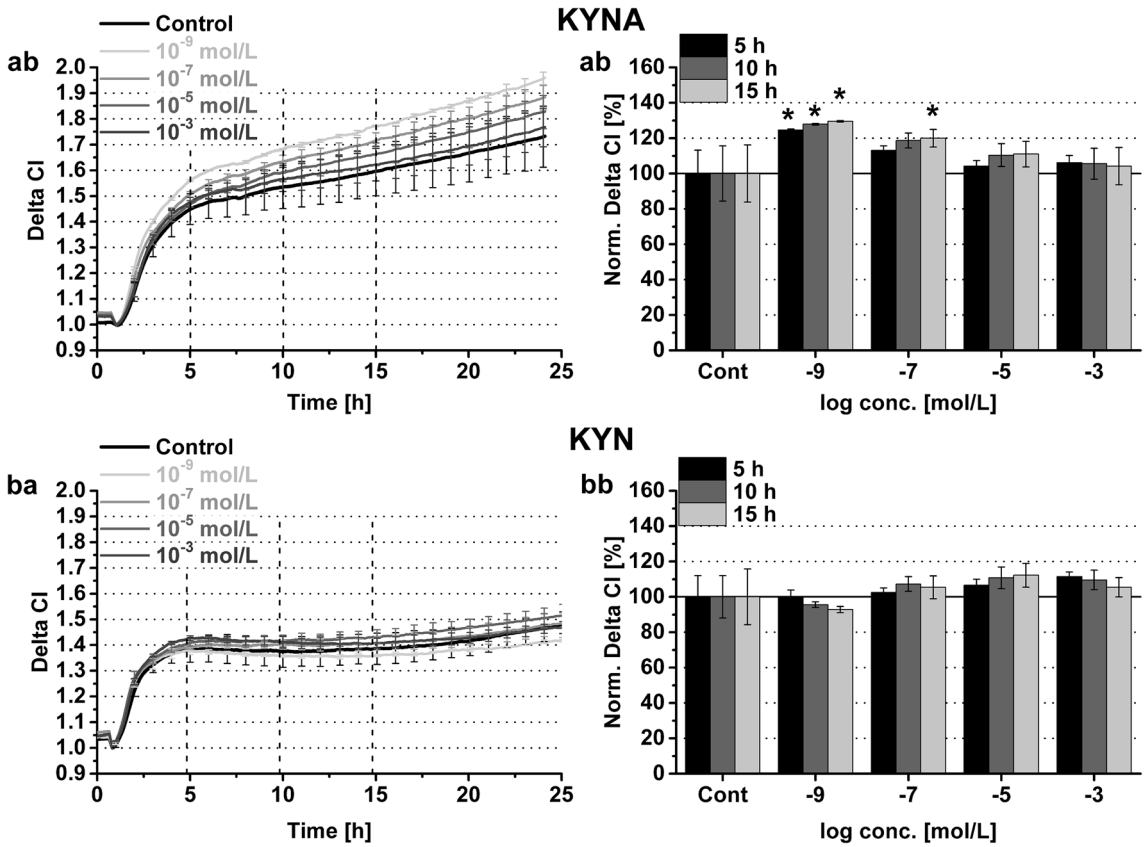

Fig. 4 Effects of KYNA and KYN on cell adhesion of GP8 endothelial cells. Time course study of cell adhesion modulator effects of KYNA (**aa**) and KYN (**ba**) as well as statistical evaluation of the effect of KYNA (**ab**) and KYN (**bb**) at individual time points (5, 10 and 15 h). The Delta CI (Delta Cell Index) refers to the difference of the CI value at the point in time of cell inoculation and the CI value at another given point in time. The Norm. Delta CI (Normalized Delta Cell Index) values were calculated at individual time points (5, 10 and 15 h), and were normalized to the control (control = 100%). Data shown in the figures represent mathematical averages of three parallels and ± SD values. The levels of significance are shown as follows: $*p < 0.05$. *KYNA* kynurenic acid, *KYN* L-kynurenine

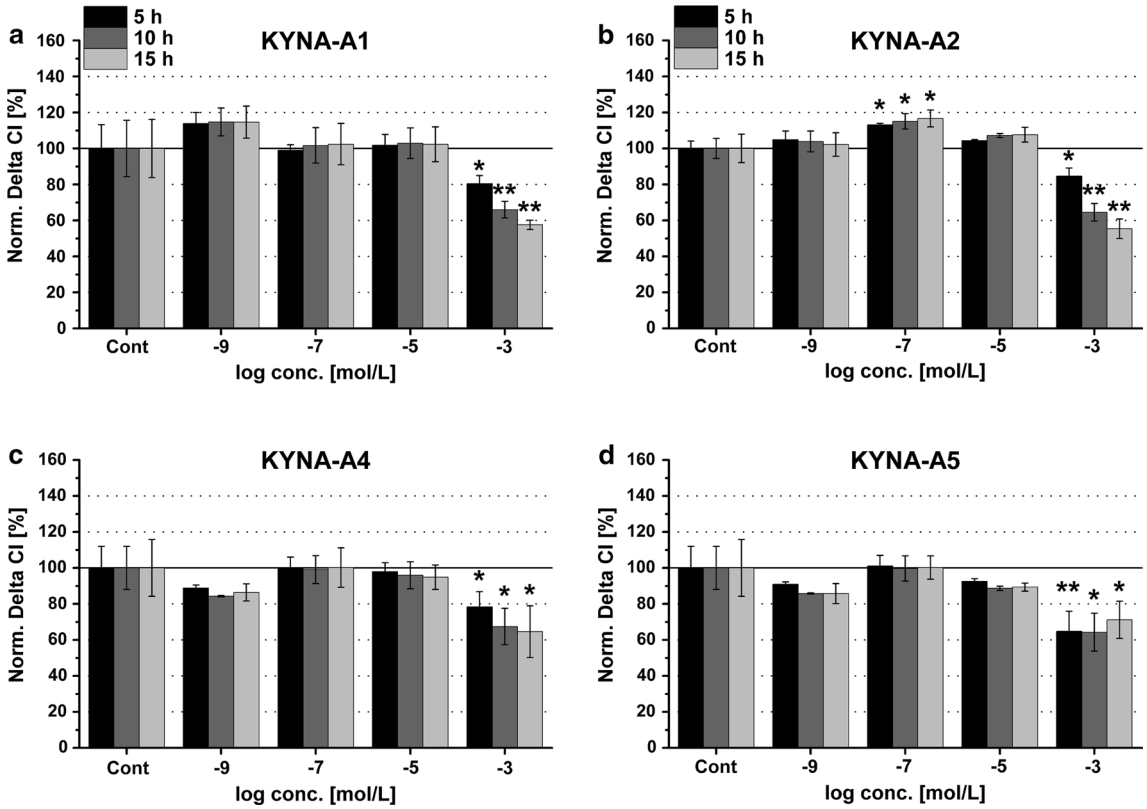

Fig. 5 Effects of KYNA amides (KYNA-A1, -A2, -A4 and -A5) on cell adhesion of GP8 endothelial cells. The Norm. Delta CI (Normalized Delta Cell Index) values were calculated at individual time points (5, 10 and 15 h) and were normalized to the control (control = 100%). Data shown in the figures represent mathematical averages of three parallels and ± SD values. The levels of significance are shown as follows *$p < 0.05$; **$p < 0.01$. *KYNA* kynurenic acid, KYNA derivatives—amides of C2-carboxylic function (KYNA-A1 and -A2) and amides of C2-carboxylic function + side-chains on C3 (KYNA-A4 and -A5)

an internal control. Although the experimental conditions (coating, cell number, maximum volume/well and cells with consecutive passage number) were the same in each case, the maximum Delta CI value of the control varied between 1.05 (Fig. 3), 1.7 (Fig. 4aa; Online Resource 1) and 1.45 (Fig. 4ba; Online Resource 1) in the time course cell adhesion studies. In the Delta CI vs. Time graphs (Figs. 3a, 4aa, ba; Online Resource 1), the time-dependent effect of KYNA derivatives are depicted and compared to the identical control. The different adhesion kinetics of GP8 cells could also be observed in Figs. 3a, 4aa, ba. Some examples of this kind of variation in adhesion kinetics of same cells between experiments are noted in literature (Backer et al. 2017; Leurs et al. 2012; Tang et al. 2015). The real-time impedance-based measurements that can record the impedance with the given time interval are sensitive enough to detect the actual status of the cells, which could not otherwise be revealed by classical end-point assay.

To determine that KYNA and its derivatives induced CI change was a result of endothelial reaction and not due to the physico-chemical properties of the compounds (e.g. acidity), some cell-free wells were loaded with only the tested ligands at 10^{-3} mol/L concentration. KYNA derivatives themselves did not cause any significant change in CI values.

KYNA itself was able to increase the adhesion of GP8 cells through an inversely proportional concentration-dependent manner, with maximum effect at a concentration of 10^{-9} mol/L. This positive effect of KYNA was more pronounced as time progressed (15 h: 129.5%, $p = 0.039$) (Fig. 4aa, ab). KYN itself differs considerably in structure from KYNA and failed to exert a cell adhesion modulator effect; it had neutral effects throughout the entire concentration range, even at the highest concentration of 10^{-3} mol/L (Fig. 4ba, bb).

All of the KYNA derivatives had a time-dependent inhibitory effect ($p < 0.05$) on cell adhesion of GP8 cells at the highest concentration (10^{-3} mol/L) tested, whereas KYNA's effects were neutral at this non-physiological concentration (Fig. 5 and Online Resource 1). KYNA-A1, KYNA-A2, KYNA-A4 and KYNA-A5 are amide derivatives. The KYNA-A1 analogue formed by coupling KYNA and 2-dimethylaminoethyl-amine (Nagy et al. 2011) increased the attachment of GP8 cells with a lower activity (15 h: 114.6%, $p_{control} = 0.081$; KYNA-A1/10^{-9} mol/L

vs. KYNA/10^{-9} mol/L: $p = 0.061$) as compared to KYNA at a concentration of 10^{-9} mol/L (Fig. 5a). The adhesion enhancement effect of KYNA-A2, containing a heterocycle (2-pyrrolidinoethylamine) in the side-chain (Nagy et al. 2011), was also lower (15 h: 116.7%, $p_{control} = 0.019$; KYNA-A2/10^{-7} mol/L vs. KYNA/10^{-9} mol/L: $p = 0.045$) and its activity was observed at a higher concentration (10^{-7} mol/L) compared to KYNA (Fig. 5b).

KYNA-A4 and KYNA-A5 differ from KYNA-A1; they contain a tertiary nitrogen in different ring systems as a second side-chain at the C3 position (Demeter et al. 2012). Both KYNA-A4 and KYNA-A5 had neutral effects at the 10^{-9}–10^{-7} mol/L concentration range (Fig. 5c, d).

Cell motion analysis

Single cells (50 cells/field) were tracked in time-lapse videos created by the HoloMonitor M4 microscope in order to obtain detailed information (migration, motility and motility speed) regarding cell locomotion. For this cell-tracking experiment, KYNA was chosen since it had the most significant cell adhesion inducer effects in GP8 cells.

Based on the results of the tested parameters, KYNA decreased the movement of GP8 cells in both tested concentrations (10^{-9}, 10^{-7} mol/L) (Fig. 6; Table 2). The migration (distance) and the motility (actual path) of KYNA-treated cells gradually decreased at the 2 h interval (migration: 10^{-9} mol/L—14.51 µm, $p = 0.0026$; 10^{-7} mol/L: 16.80 µm, $p = 0.0011$; motility: 10^{-9} mol/L—146.48 µm, $p = 0.0012$; 10^{-7} mol/L: 153.48 µm, $p = 0.0013$) compared to the control cells (migration: 18.36 µm; motility: 192.16) (Fig. 6a, b; Table 2). The motility speed was rather constant during this time frame (Fig. 6c; Table 2).

Morphometry

The time-lapse recordings provided several different morphological parameters (basic and complex ones; see the list and the definitions in "Digital holographic microscopy"), allowing for additional information on adhesion measurements and for analysis of viability.

The observed changes in the basic parameters (area, thickness and volume) (Online Resource 2) corresponded well with the adhesion inducer effects of KYNA. Due to the treatment (10^{-7} mol/L), the mean area was slightly but significantly larger (257.8 µm^2, $p = 0.0032$) and in parallel, the mean average thickness (2.17 µm, $p = 0.023$) was smaller than that of the control (area: 243.9 µm^2, thickness: 2.31 µm); these changes indicated an increase in the spreading of the treated cells (Online Resource 2). KYNA induced changes in two

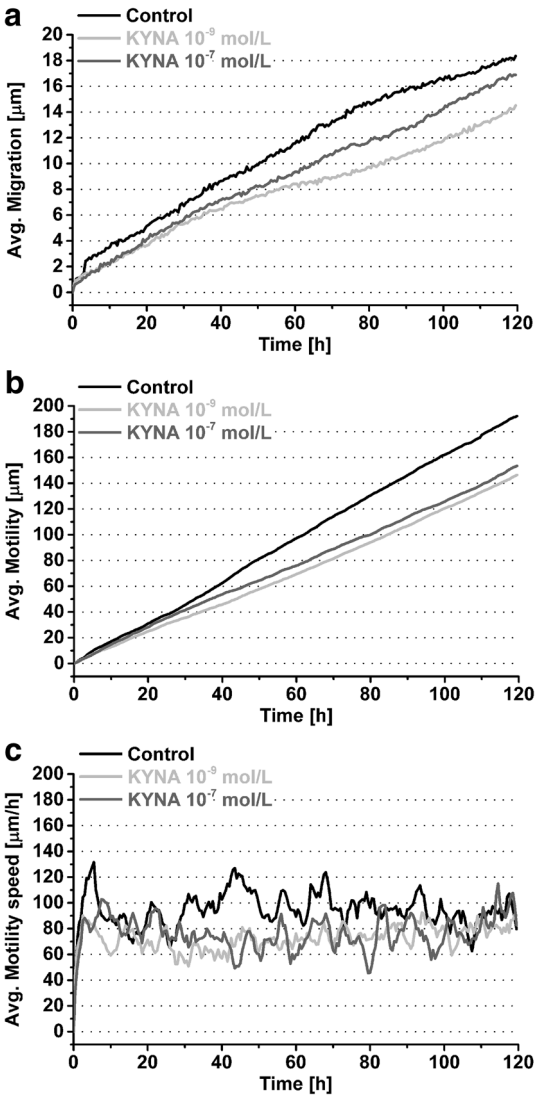

Fig. 6 Cell locomotory responses of GP8 endothelial cells induced by KYNA. Measurements of migration (**a**); motility (**b**); motility speed (**c**) by holographic microscopy. Data shown in the figures represent averages (Avg.) and ± SD values calculated for 50 cell/group in 240 consecutive frames. *KYNA* kynurenic acid

complex morphological parameters, irregularity and roughness (as viability markers). A healthy cell is characterized by lower roughness and higher irregularity, while a dying or dead cell has higher roughness and lower irregularity. There was an increase in the mean irregularity (0.42, $p = 0.0047$) and a decrease in the mean roughness (4.24, $p = 0.00205$) of KYNA (10^{-9} mol/L) of treated cells compared to that of the control (irregularity: 0.39, roughness: 4.68). KYNA failed to have any effect on the other observed parameters (volume, eccentricity, hull convexity) (Online Resource 2).

Table 2 Effect of KYNA on GP8 endothelial cell movement detected by holographic microscopy

Parameters	Control	KYNA 10^{-9} mol/L	KYNA 10^{-7} mol/L
Avg. migration (μm)	18.36 ± 5.01	14.51 ± 3.59**	16.80 ± 4.52**
Avg. motility (μm)	192.16 ± 56.32	146.48 ± 41.62**	153.48 ± 42.89**
Avg. motility speed (μm/h)	94.52 ± 14.28	72.67 ± 9.93*	76.25 ± 13.46*

Data represent averages (Avg.) and ± SD values calculated for 50 cell/group in 240 consecutive frames

The presented data were calculated by HoloStudio™ M4 2.5 and analysed by Origin Pro 8.0

The levels of significance are shown as follows: *$p < 0.05$; **$p < 0.01$

Discussion

In several CNS diseases, stimulation of the re-endothelization process is considered to be a promising approach towards the treatment or prevention of these pathological conditions (del Zoppo and Milner 2006; Lyros et al. 2014; Persidsky et al. 2006). Some clinically used pharmacological agents (e.g. statins, angiotensin-converting-enzyme inhibitor) have been shown to enhance endogenous re-endothelization capacity. These drugs can improve endothelial repair through various mechanisms including: (1) increasing the number of circulating endothelial progenitor cells through mobilization from the bone marrow; (2) inducing of endothelial proliferation, differentiation and migration; and (3) enhancement of adhesiveness of endothelial cells by upregulation of the expression of different cell adhesion molecules (e.g. β_1-, β_5-integrins, selectins) (Besler et al. 2008; Zampetaki et al. 2008).

KYNA and its synthetic analogues have been found to have therapeutic value in different neurological disorders due to their neuroprotective and anti-inflammatory activity (Vecsei et al. 2013). In the present work, our main purpose was to investigate the action of KYNA on GP8 endothelial cell behaviour and morphology and to compare the adhesion modulator effects of different synthetic KYNA derivatives.

For detailed characterization of a candidate drug, the kinetic analysis of cell physiological responses and of morphological changes by non-invasive and label-free methods (such as holographic microscopy or impedance-based techniques) represents a powerful approach.

Based on our results, KYNA itself was most effective in enhancing adhesion at 10^{-9} and 10^{-7} mol/L concentrations, which are well correlated to physiological levels [the plasma concentration of KYNA is 30–50 nmol/L ~ 3–5 × 10^{-8} mol/L (Mandi and Vecsei 2012)]. This adhesion inducer effect was well correlated with morphometry measurements done by holographic microscope. The morphological parameters indicated greater spreading of GP8 cells, which could explain the increased impedance and adhesion detected by the xCELLigence system. Based on the results of the morphometry (e.g. thickness, eccentricity irregularity), it could also be concluded that the KYNA did not have toxic effects; the cells showed typical flat and elongated shape during the time interval (Antal et al. 2014; Tóth et al. 2014). It was also shown by cell tracking that KYNA caused a slow and random movement of the GP8 cells, resulting in a decreased motility (actual path) and migration (shortest distance). This effect can be beneficial with respect to resting endothelium in order be able to stabilize the cell–ECM and cell–cell junctions; while for endothelial repair, high endothelial motility seems to be important and involves the migration of cells as a cohesive group rather than as individual cells. In our study, individual cells were monitored and thus, their individual migration cannot be considered as a clear indication of migratory response of the entire cell population. Based on a previously reported wound-healing assay accomplished using the Holomonitor M4 microscope, it is assumed that in a cell population, the effects of KYNA could be different depending on the cell number and on the position of cells in a monolayer (Phase Holographic Imaging PHI AB 2015). The link between KYNA and the endothelium has been previously investigated by Pawlak et al. In their studies, KYN metabolites generated by endothelial cells were shown to associate with endothelial dysfunction markers (e.g. increased serum level of circulating sICAM-1, sVCAM-1 adhesion molecules) and with the oxidative status of endothelial cells in patients having chronic kidney disease who were at high risk for atherosclerotic cardiovascular disease (Pawlak et al. 2009, 2010). From these studies, cause and effect could not be determined; nevertheless, the protective role of KYNA on vascular endothelium is believed to be likely. The endothelium seems to be an important source of KYNA (Stazka et al. 2002). Different risk factors (e.g. hyperhomocysteinemia) for endothelial dysfunction affect the endothelial production of KYNA both in vitro and in vivo (Pawlak et al. 2010; Stazka et al. 2005). Moreover, KYNA has shown to offer protection against hyperhomocysteinemia-caused endothelial damage (Wejksza et al. 2009).

Our results have raised the question concerning the molecular mechanisms underlying the effects of KYNA and the need to study how the observed changes in endothelial function are mediated throughout. Nevertheless, we would like to suggest some potential mechanisms that may explain the effects of KYNA. Several types of glutamate receptors, including NMDA receptors, have been demonstrated to be

expressed on brain endothelial cells (Krizbai et al. 1998). These receptors are involved in the glutamate-induced hyper-permeability of the BBB as well as alteration in the expression, distribution and phosphorylation of a tight junction protein, the occludin (Andras et al. 2007). Oláh et al. reported that KYNA as an NMDA receptor antagonist could reduce BBB permeability similarly to that of the positive reference compound, an NMDA receptor blocker (Oláh et al. 2013). These results indicate that KYNA might induce endothelial cell adhesion by regulating the expression of adhesion molecules either directly, or via NMDA receptors. The potential role of cell adhesion molecules in the actions of KYNA is further supported by a former study in an in vitro vascular flow model. It was reported that KYNA triggered the firm adhesion of monocytes to both fibronectin and ICAM-1 via β1 integrin- and β2 integrin-mediated mechanisms with activation of GPR35 (Barth et al. 2009).

Our current results regarding the most significant adhesion inducer activity of KYNA occurring at nanomolar concentrations appear to be compatible with the former theory such that KYNA in the concentration range between a nanomolar and micromolar displays different effects. This Janus-face activity could be explained by the presence of different receptors/binding sites with different affinities and the induction of different mechanisms (e.g. antagonist for glycine-site of NMDA receptor: IC50 = 0.01–10 µmol/L and agonist for GPR35: EC50 = 0.1–30 µmol/L) (Cosi et al. 2011; Moroni et al. 2012; Rozsa et al. 2008). The time-dependent effects of KYNA on endothelial cells are consistent with other studies, indicating the rapid up-regulation and long-term down-modulation of immune response induced by KYNA (Barth et al. 2009). In our adhesion measurements, the increasing effects of KYNA, as time elapsed, could be due to its possible effects on the de novo synthesis and expression of adhesion molecules. Based on our results, the effects of KYNA could also be advantageous in the cerebral endothelium through preservation of the integrity of both the endothelium and BBB. By facilitating the adhesion of endothelial cells, KYNA may play a role in endothelial repair mechanisms in the case of neurodegenerative and neuroinflammatory diseases. These results pave the way for further studies to help clarify the potential role of KYNA in the homeostasis of endothelium.

Interestingly, the precursor molecule KYN proved to be ineffective as a cell adhesion modulator. Once the KYN is locally synthesized or taken up from the blood, it can be further metabolized to KYNA by irreversible transamination (Vecsei et al. 2013). Therefore, KYN can be protective to the cerebral endothelial cells because it can be considered as a prodrug of KYNA (Vecsei et al. 2013).

Comparing our present findings on endothelial cells to our preliminary results (as yet unpublished) on two murine mammary tumour cell lines (LM2 and LM3) having different metastatic capabilities, it is suggested that the cell adhesion modulator activity of KYNA and KYN appears to be dependent on cell type, or on the invasive capacity of the cells. Both compounds had neutral effects in non-metastatic LM2 cells, while in the case of the metastatic LM3 cell line, KYN slightly increased cell adhesion and KYNA decreased it. Based on these impedance-based measurements, we may be able to exclude the possibility that KYNA exerts some unknown influence and induces morphological changes irrespective of cell type. Nevertheless, further studies need to be undertaken which can take into account, for example, intracellular [Ca^{2+}] or the changes of cytoskeleton.

KYNA is produced both in the brain and in peripheral tissues [e.g. heart (Baran et al. 1997), vasculature (Stazka et al. 2002), immune cells (Mandi and Vecsei 2012)]. Because KYNA has limited ability to cross the BBB, only the neuroprotective effects of locally synthesized KYNA can be taken into account in the CNS. The systemic administration of KYNA as a neuroprotective agent is restricted and indirect pharmacological approaches are needed to exploit its therapeutic potential (Nagy et al. 2011). Accordingly, several new KYNA analogues have been designed. KYNA amides synthesized in our laboratory are promising candidates because they have similar neuroprotective activity compared to KYNA [e.g. selective inhibition of NMDA receptors (Nagy et al. 2011), they protect against epileptiform seizures (Demeter et al. 2012), they diminish hippocampal cell loss and preserve long-term potentiation (Fülöp et al. 2012)]. In the present work, different KYNA amides were studied with respect to endothelial cell adhesion. These KYNA amides were formed through transformation of the C2-carboxylic function of KYNA with a (1) water-soluble side-chain containing a (2) new cationic centre with (3) side-chain substitution enhancing penetration into the brain.

According to the results of the KYNA derivatives, the original moieties at the C2 and C3 positions of KYNA seem to be required for cell adhesion inducer activity. The substitution of KYNA with a single amide side-chain of C2-carboxylic function in KYNA-A1 and KYNA-A2 has reduced cell adhesion inducer ability of the parent drug. However, the positive effects of KYNA-A1 and KYNA-A2 show that they could contribute to maintenance or restoration of the integrity of the cerebral endothelium. It has been demonstrated that the substitution of a side-chain at the C2 position enhances penetration of these compounds through the BBB (Nagy et al. 2011), whose properties also support the therapeutic significance of their adhesion enhancer effects. KYNA-A4 and KYNA-A5, each having a side-chain at position C3, had neutral effects on cell adhesion at physiological concentrations. In a recent study, it has been shown that the permeability of KYNA-A4 was remarkably high in an in vitro BBB model compared to that of other KYNA derivatives (Demeter et al. 2012). Considering our current results,

it is possible that KYNA-A4 can cross the BBB without any alterations to the endothelial barrier, which has a potential significance with respect to clinical applications as a derivative non-harmful to endothelial cells.

The strong adhesion decreasing effects of KYNA analogues on cell–ECM adhesion were observed at the 10^{-3} mol/L concentration, whose effect cannot be considered as an unfavourable effect of endothelial structure or function. This millimolar concentration is much higher than the normal plasma level of KYNA and even higher than its substantially elevated concentration in different pathological conditions (e.g. inflammation) (Mandi and Vecsei 2012). The negative effects of KYNA derivatives at 10^{-3} mol/L give rise to the question as to whether the KYNA derivatives could decrease the viability of endothelial cells. Based on the real-time data provided by the xCELLigence System, the cytotoxic/antiproliferative effects of KYNA derivatives can be excluded. This impedance-based assay is sensitive enough for cytotoxicity experiments. In the event of a cytotoxic compound, a drop in the CI value would be observed (Urcan et al. 2010). At high concentration, the decreased adhesion was followed by a plateau phase (between 5 and 15 h incubation) and later (after 15 h), a slow gradual increase in CI values, indicating proliferation of GP8 cells. We hope that further studies (e.g. measurements of adhesion protein expression and of morphometry with holographic microscope) will be able to explain the adhesion decreasing effects of KYNA derivatives.

This in vitro study, in which cells of one type are exposed to fixed concentrations of KYNA and its analogues, is a first step towards understanding the potential effects of KYNA; however, our experimental setup has some limitations as a potential model that could simulate the in vivo conditions of the BBB. Firstly, in the establishment and maintenance of the BBB; other elements of the neurovascular unit (in addition to the brain microvascular endothelial cells) are involved, including astrocytes, pericytes, neurons and basal lamin (Persidsky et al. 2006). Secondly, the concentration of the administered KYNA can be dynamically modulated by different cells in the CNS. Endothelial cells and astrocytes were demonstrated as an important source of KYNA (Dantzer et al. 2008; Stazka et al. 2002); while, for example, macrophages and microglia can uptake and metabolize KYN to form other types of kynurenines instead of KYNA (Vecsei et al. 2013). Blood flow can also dilute concentrations of KYNA produced locally. It should also be considered that different disease states could significantly influence KYNA concentrations, the functions of the BBB and endothelial cells. During inflammation (Mandi and Vecsei 2012) and in Alzheimer's disease (Dezsi et al. 2015), it was found that there is an enhanced degradation of tryptophan in parallel with an increase in KYNA. Thus, in order to predict the therapeutic significance of KYNA, it could be interesting to investigate the effects of KYNA and its analogues on endothelial cells in a laminar flow environment, as well as on other cell types of the neurovascular unit (e.g. astrocytes). Future work should also rely on a model that takes inflammatory and endothelial damage conditions into account, and could also represent a more suitable system for investigating the effects of KYNA as a potential drug that promotes endothelial repair. Such a model would surely require a preceding study on a basal/quiescent state of endothelial cells such as ours.

According to our data, kynurenines may be able to modulate the complex function of neurovascular unit by influencing cell–ECM adhesion and thus, they offer a new therapeutic option for diseases relating to dysfunction of the BBB.

Conclusions

In summary, based on our results, GP8 brain endothelial cells can be successfully used as a sensitive model to study the effects of KYNA derivatives (or other types of neuropharmacological drugs) on cell adhesion, morphology and movement. The results obtained on endothelial responses indicate that cell–ECM adhesion and locomotion of brain endothelial cells could be a new therapeutic target of KYNA and its amide derivatives. Based on their adhesion inducer effects, these compounds (KYNA, KYNA-A1 and A2) could play a potential role in facilitating endothelial repair and in maintaining the endothelial integrity of the BBB in different neurological disorders. However, in order to firmly assess this hypothesis, further experiments are needed to be able to clarify the molecular background behind the action of KYNA derivatives; they are also needed in order to investigate the influence of these compounds on other elements of the neurovascular unit, and in a model that takes endothelial dysfunction/damage into account. Nevertheless, our measurements with impedimetric and holographic methods suggest that these novel label-free biosensing methods can be especially useful for conducting high content analyses of the effects of a candidate drug on cell behaviour and morphology.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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