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Note

Quantification of kynurenic acid in cerebrospinal fluid: effects of systemic and central L-kynurenine administration

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Kynurenic acid (KYNA) is a well established metabolite of L-tryptophan (L-TRP) in systemic tissues and has recently been shown to be present in brain tissue [1,2]. On local application to neurons of the central nervous system, KYNA may act as a non-selective antagonist of excitatory amino acid receptors and attenuate the neuronal excitation induced by agonists of N-methyl-D-aspartate (NMDA), kainate and quisqualate receptors [3–5]. In cerebral cortex, hippocampus and spinal cord, KYNA can block excitation of post-synaptic neurons in response to stimulation of presynaptic neurons [3–6]. Disturbances in the concentrations of KYNA in the extracellular fluid space of brain may influence the activity of excitatory amino acid receptors. Another L-TRP metabolite, quinolinic acid (QUIN), is an agonist of NMDA receptors [6], a convulsant [7] and a neurotoxin [8]. Importantly, KYNA can attenuate these "excitotoxic" effects of QUIN [9]. It is possible that increased concentrations of QUIN in the extracellular fluid space of brain may have neuropathologic consequences and that KYNA may modulate the excitotoxic effects of QUIN in pathologic states.

Recently we have demonstrated marked increases in the concentrations of QUIN in the cerebrospinal fluid (CSF) of patients infected with the human immunodeficiency virus (HIV-1) [10], the cause of the acquired immune deficiency syndrome (AIDS). CSF QUIN concentrations are also increased in macaques infected with simian retrovirus type D (including D/1/California [11]) and macaques with septicemia [12]. It is possible that QUIN and the balance between QUIN and KYNA are of relevance in the neuropathology of the AIDS dementia complex [10]. However, measures of KYNA in the CSF in these disorders have not been determined, essentially because quantitative methods are not available.

In the present paper, a simple high-performance liquid chromatographic (HPLC) method is described to quantify the concentrations of KYNA in the CSF. The method is based on the separation of KYNA by HPLC and the quantification of KYNA by fluorescence following post-column mixing with zinc acetate, which enhances the fluorometric signal for the quantification of KYNA [13].

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EXPERIMENTAL

KYNA and zinc acetate were obtained from Sigma (St. Louis, MO, U.S.A.) and the remaining compounds were obtained from Fischer (Fair Lawn, NJ, U.S.A.). Pooled samples of CSF from normal human (lumbar) and normal macaque (*Macaca mulatta*) (cisternal) were prepared from six individuals and six animals. Additional pooled samples of CSF from either six human patients with clinical AIDS and six macaques infected with D/1/California and clinical simian acquired immune deficiency syndrome (SAIDS) were also studied.

HPLC system

The HPLC system consisted of an LKB 2150 pump, a 5-μm Ultrasphere ODS column (150 mm × 4.6 mm I.D.; Altech, Berkeley CA, U.S.A.), an LKB 2249 pump which added a 1 mol/l zinc acetate solution to the column eluent and a Hewlett-Packard 1046A fluorescence detector. For the routine CSF KYNA assay and determinations of assay variabilities, the mobile phase consisted of 10 mmol/l sodium acetate and 50 ml/l acetonitrile at a pH of 6.50 and a flow-rate of 0.7 ml/min, and KYNA eluted at 4 min 15 s. The flow-rate of the zinc acetate solution always equaled the flow-rate of the mobile phase. KYNA was monitored at an excitation wavelength of 254 nm and quantified at an emission wavelength of 404 nm. A 50- or 100-μl volume of CSF was injected using a Gilson Model 231 autosampler. Peak heights were compared to authentic standards.

Assay verification

The identity of the KYNA peak in the four pooled CSF samples was verified by varying the concentrations of acetonitrile in the mobile phase between 30 and 70 ml/l and changing the pH between 4.00 and 7.40, and demonstrating that the peak-height ratio of KYNA in the samples and authentic KYNA standard remained stable and that the peak tracked with authentic KYNA standards. Slight variations of the buffer composition shifted the order of elution of adjacent peaks allowing careful tracking of the other peaks in the cromatograms with respect to KYNA. The times of elution using these buffers ranged from 2 min 55 s to 7 mm 55 s. The intra-assay coefficient of variation was determined by injecting aliquots of the same sample six times within the same assay over a 6-h period. The inter-assay coefficient of variation was determined by injecting one aliquot of the same sample in six separate assay runs performed over a nine-day period.

To investigate whether the KYNA signal in the CSF samples is influenced by constituents of CSF, known amounts of KYNA (130–400 fmol) were added to samples of CSF and the resultant increases in the KYNA signal determined. To investigate sample stability, aliquots of a pooled sample of CSF were repeatedly frozen and thawed ten times and the KYNA concentration was measured. Samples of CSF were left at room temperature for 24 h and the KYNA concentration compared to an initial aliquot kept at −70°C.

Effects of intraperitoneal and intracisteral L-*kynurenine administration on CSF KYNA concentrations in the rat*

Twenty-eight male Sprague–Dawley rats (300–330 g) received either an intraperitoneal injection of sterile saline as control ($n=7$), L-kynurenine (L-KYN) ($n=7$; 75 μmol/kg) or KYNA ($n=7$; 75 μmol/kg). One hour later, rats were anaesthetized by an intraperitoneal injection of sodium pentobarbital (90 mg/kg) and placed in a stereotaxic frame. A cannula made out of a 1 cm length of cut 25-gauge stainless-steel hypodermic needle attached to a 30 cm length of PE20 tubing was inserted into the cisternal space after removal of the overlying muscle. A sample of CSF (90–120 μl) was collected into a 1-ml syringe under negative pressure and the concentrations of KYNA were measured.

In a second study, twelve male rats were anaesthetized by an intraperitoneal injection of sodium pentobarbital and placed in a stereotaxic frame. A cannula made from a 0.7 cm length of 30-gauge needle attached to a 10 cm length of PE10 polypropylene tubing was inserted into the cisternal space by hand and an 80 μl sample of CSF collected into a 1-ml syringe with a 30-gauge needle inserted into the PE10 tubing. The syringe, needle and most of the PE10 tubing containing CSF were removed and replaced by a 100-μl Hamilton syringe containing either sterile saline alone as control or sterile saline with 1 nmol per 50 μl of L-KYN. A total of 50 μl of the solution (accounting for the 5-μl dead volume of the cannula system) was infused into the cisternal space by hand over a 1-min period. The cannula was left in place for a further 5 min and then removed. One hour later, a second sample of CSF was collected (70–120 μl) and the concentrations of KYNA were measured.

Statistical analyses

Results were analyzed by paired t-test or analysis of variance and Dunnett's t-test. Correlation coefficients were calculated by the method of least squares. Results are expressed as the mean ± one standard error of the mean. A p value of <0.05 was considered significant.

RESULTS

The fluorescence detection method had a minimum sensitivity of 33 fmol at a signal-to-noise ratio of 10:1, which gives a minimum sensitivity of 0.33 nmol/l of CSF based on a 100-μl injection. With authentic KYNA, the standard curve was linear over the range of CSF KYNA concentrations encountered in CSF samples ($r=0.99$). Fig. 1 shows typical chromatograms of (A) authentic KYNA and (B–E) CSF from pools of either normal macaques (B), normal man (C), macaques infected with D/1/California (D) and patients with AIDS (E) based on 50-μl injections. In all four CSF samples, the peak identified as KYNA tracked with authentic KYNA over the different acetonitrile/pH combinations tested (ten buffers) and maintained their peak heights compared to a KYNA standard run with the same buffer.

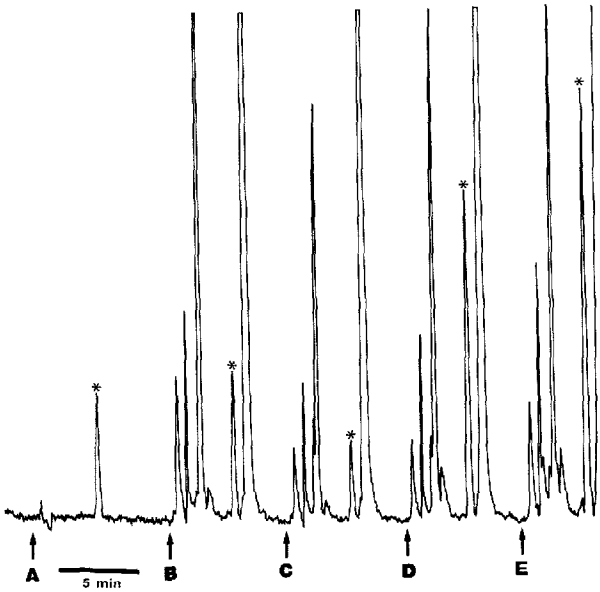

Fig. 1. Chromatograms of (A) authentic KYNA standard, (B) pooled cisternal CSF samples of six normal macaques, (C) pooled lumbar CSF samples from six normal humans, (D) pooled cisternal CSF samples of macaques infected with D/1/California and (E) pooled lumbar CSF samples of humans with clinical AIDS, all based on 100-μl injections. The arrow marks the time of injection and the time of elution of KYNA (asterisk) was 4 min 15 s. The ordinate is the relative intensity of emission at 404 nm and the chart speed was 0.5 cm/min.

Addition of known amounts of KYNA to each of the pooled samples increased KYNA peak heights to within ± 3% of expected values, demonstrating no enhancement or attenuation of the KYNA signal in the CSF samples tested. No change in KYNA peak height followed repeated thawing or keeping CSF samples at room temperature. Repeated injections of the same sample of CSF resulted in an intra-assay coefficient of variation of 5.03%. The inter-assay coefficient of variation in CSF was 9.8%.

Typical values of the concentrations of KYNA in CSF were: normal man, 3.6 ± 0.21 nmol/l; macaque (*Macaca mulatta*), 5.20 ± 0.32 nmol/l; and rat, 1.31 ± 0.22 nmol/l. Significant increases in the concentrations of KYNA in CSF of rats followed systemic administration of either L-KYN (4.86 ± 1.59 nmol/l; $p < 0.01$) and KYNA (7.44 ± 2.65 nmol/l, $p < 0.001$) compared to saline-injected controls (1.52 ± 0.64 nmol/l; Fig. 2). Intracisternal administration of L-KYN also increased CSF KYNA concentrations from a pre-injection control value of 1.19 ± 0.60 nmol/l to a post-L-KYN injection value of 7.73 ± 1.63 nmol/l ($p < 0.001$; Fig. 3). In contrast, in rats that received an intracisternal injection of saline, CSF KYNA did not change from a pre-injection value of 1.30 ± 0.39 nmol/l to a post-injection value of 1.45 ± 0.40 nmol/l.

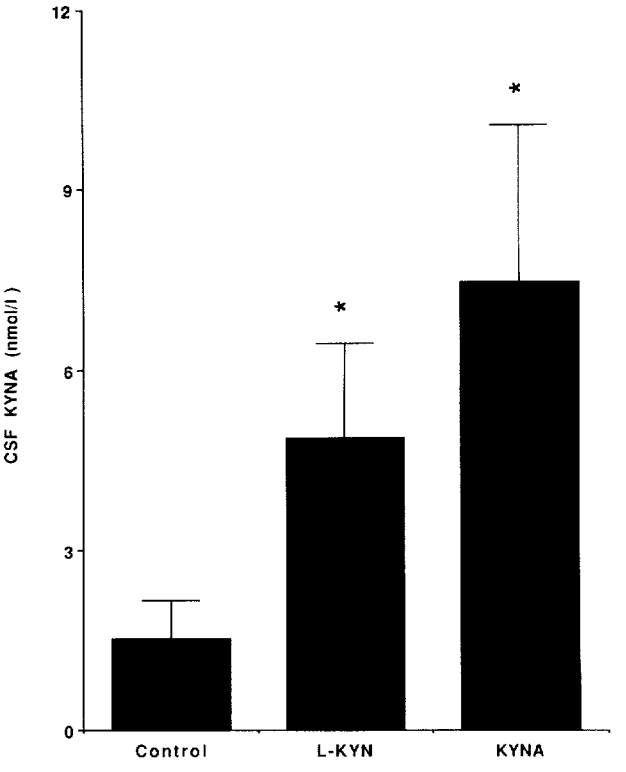

Fig. 2. Increased CSF KYNA concentrations of rats 60 min after a systemic injection of either sterile saline, L-KYN (71 μmol/kg) or KYNA (75 μmol/kg). Values are mean ± one standard error of the mean (error bar). *$p < 0.05$.

DISCUSSION

The results demonstrate that KYNA is present in CSF and can be quantified accurately and reproducibly by HPLC and fluorescence detection following post-column mixing of the column eluate with zinc acetate. The post-column mixing of the column eluate with zinc acetate [13] appreciably magnified the KYNA signal in CSF to quantifiable concentrations which obviated the need for extraction procedures. Addition of known amounts of KYNA to samples of CSF produced appropriate increases in the KYNA signal. The tracking experiments demonstrated that there were no interfering peaks with the buffer used for quantitative measures of KYNA in the CSF samples tested. KYNA was identified in the CSF of normal man and macaques as well as human patients with clinical AIDS and macaques infected with D/1/California. The tests employed verified that the concentrations of KYNA in these retrovirus-infected patients and macaques were increased.

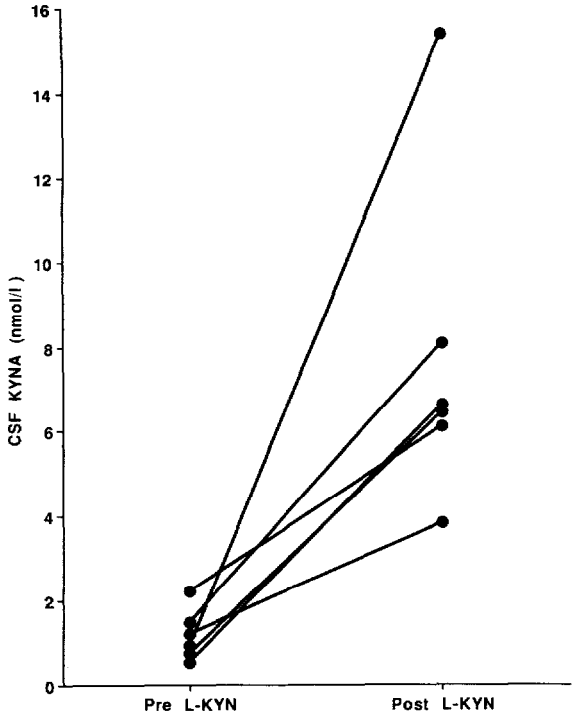

Fig. 3. Increased CSF KYNA concentrations of rats 60 min after an intracisternal injection of 1 nmol of KYNA. Samples of CSF were collected prior to L-KYN administration (Pre L-KYN; 1 nmol per rat) and 1 h later (post L-KYN).

In man, macaque and rat, KYNA concentrations were in the low nanomolar range (<8 nmol/l). The concentration in man is appreciably lower than the 140-1580 nmol/l concentration of KYNA in human brain tissue extracts reported by Turski *et al.* [14] and relatively close to the concentrations of 14 nmol/l reported by Carla *et al.* [1] in rat brain. The mechanisms responsible for maintaining a relatively large gradient between tissue and CSF of man are unclear. In man, macaque and rat, CSF QUIN concentrations typically range from 12 to 30 nmol/l [12,15,16]. Therefore, the concentrations of QUIN are higher than the concentrations of KYNA in normal man, macaque and rat CSF.

The source of KYNA in the CSF and brain is unclear, although activity of the enzyme which converts L-KYN to KYNA, kynurenine transaminase, has been detected in brain [17]. The present observation that L-KYN administered into the brain side of the blood–brain barrier markedly increased the concentrations of KYNA in rat CSF (Fig. 3) suggests that L-KYN may be a source for KYNA both in brain and CSF and is consistent with the conversion of L-KYN to KYNA in rat brain tissue slices with liberation of KYNA into the incubation medium reported by Turski *et al.* [14]. The present observation that systemically adminis-

tered L-KYN increased the concentrations of KYNA in rat CSF indicates that blood may supply substrate for the synthesis of KYNA in the brain, at least at times when blood levels of L-KYN are increased, such as in HIV-1-infected patients [18,19] and in macaques infected with D/1/California [11]. No study has reported quantitative data on the permeability of the blood–brain barrier to KYNA, although the small increases in CSF KYNA concentrations that followed systemic administration of KYNA suggest that CSF KYNA concentrations are also sensitive to increased availability of KYNA in blood. Some of the increases in CSF KYNA concentrations that followed systemic L-KYN administration (Fig. 3) may reflect conversion to KYNA in systemic tissues and entry of KYNA into the CSF albeit to a small degree.

The increased concentrations of KYNA and QUIN in the CSF of patients with clinical AIDS and macaques infected with D/1/California [11] are also consistent with the increases in blood L-KYN concentrations that are observed in patients [11,18,19] and infected macaques and may reflect activation of indoleamine-2,3-dioxygenase in extrahepatic tissues [20]. Studies in experimental animals have established that local administration of QUIN to neurons of the central nervous system increases neuronal firing rates and may induce either seizures or neurodegeneration [6–8]. Co-administration of KYNA with QUIN attenuates the excitotoxic effects of QUIN [9]. The observations that KYNA concentrations are increased in the conditions where the concentrations of QUIN are also increased raise the possibility that while QUIN may have neurologic effects in infectious disease KYNA may modulate the effects of QUIN and other ligands of excitatory amino acid receptors.

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