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INDOLPYRUVIC ACID ADMINISTRATION INCREASES THE BRAIN CONTENT OF KYNURENIC ACID

IS THIS A NEW AVENUE TO MODULATE EXCITATORY AMINO ACID RECEPTORS *IN VIVO*?

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Abstract—The possibility of changing the tissue content of kynurenic acid (KYNA), a tryptophan metabolite which acts as an antagonist of the excitatory amino acid receptors, was investigated by measuring its concentration in the brain, blood, liver and kidney of rats using a specific method based on ion exchange chromatography and HPLC. The administration of tryptophan (TRP) or of its keto analogue, indolpyruvic acid (IPA) (50–500 mg/kg i.p.), significantly increased, in a dose-dependent manner, the content of KYNA in various organs, including the brain. The increased brain content of KYNA after IPA administration could not be completely explained by considering that IPA may be transaminated to TRP and that the enzymes leading from TRP to KYNA are known. An alternative pathway of KYNA synthesis from IPA was therefore proposed. These findings indicate that it is possible to change the brain content of an endogenous glutamate antagonist. This could be a new avenue to modulate *in vivo* excitatory amino acid receptors.

In the last few years it has been shown that kynurenic acid (4-hydroxyquinoline-2-carboxylic acid, KYNA), once considered an inactive metabolic compound originating from tryptophan (TRP) metabolism, antagonizes, in a non-competitive manner, the excitatory amino acid receptors, both *in vivo* and *in vitro* [1–3]. It has also been shown that KYNA is present in the rat and human brain [4, 5] and that its interaction with the excitatory amino acid receptors probably involves a modulatory site which recognizes glycine as a positive modulator [6–8].

In view of the potential pharmacological interest of antagonizing the excitatory amino acid receptors in different pathological situations [9, 10], we thought it interesting to investigate if and how the administration of possible precursors of KYNA could result in an increased brain content and utilization of this compound. Among the possible KYNA precursors, we focused our attention on TRP and on indolpyruvic acid (IPA) (Fig. 1). The last one is a natural compound, present in biological fluids and metabolized to tryptophan (TRP) through the action of the aromatic amino acid transaminase [11–13]. It has been previously demonstrated that TRP administration to rats increases the brain content of most of its metabolites including kynurenine, 5-HT, 5-HIAA, and quinolinic acid [14, 15] and that IPA administration increases the brain content of 5-HT, 5-HIAA and TRP [16]. In the present study we describe experiments showing that the intraperitoneal administration of both TRP and IPA causes an increased content of KYNA not only in peripheral organs, but also in the brain and we suggest that this could be a new avenue to modulate *in vivo* the excitatory amino acid receptors.

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MATERIALS AND METHODS

Materials. Kynurenic acid, xanthurenic acid, tryptophan, 5-HT-creatin-sulfate and 5-HIAA were purchased from Sigma Chemical Co. (St Louis, MO). Dowex AG1 Wx8 (100–200 mesh, acetate form) and Dowex AG50 Wx8 (80–100 mesh, H^+ form) from Biorad; acetonitrile and ethanol spectrograde were obtained from Merck. Male rats, Wistar strain, were obtained from Charles River, Como, Italy.

Determination of kynurenic acid. Kynurenic acid was identified and measured according to Carlà *et al.* [4] with the minor modification reported in Moroni *et al.* [5]. Briefly, the animals were killed by decapitation, their blood was collected and their brains, livers and kidneys rapidly removed and placed in ice-cold saline. Approximately 1 g of tissue was homogenized in 4 ml of a mixture (3:1) of ethanol and NaOH 0.1 N. After a first centrifugation (10 min, 5000 *g*) the pellet was resuspended in 5 ml of 90% ethanol. The collected supernatants were placed overnight at −80° to precipitate fatty materials which were discarded. Then, 250–300 mg of an ion exchange resin, Dowex AG1 Wx8 (acetate form 100–200 mesh) were added to the supernatant. The mixture was carefully mixed for 5 min and centrifuged. The supernatants were discarded while the resin was resuspended in 2 ml of water and placed in Pasteur pipettes in which a pellet of glass wool had been previously inserted. The columns thus formed were further washed with 5 ml of water and with 10 ml of 1 N formic acid. Kynurenic acid was eluted with 5 ml of 10 N formic acid. This was directly passed through similarly prepared Pasteur pipettes containing 250–300 mg of another Dowex resin (AG50 Wx8 H^+ form). These small columns were then washed with 0.01 N formic acid (2 ml) until the eluate reached pH 6. Kynurenic acid was then eluted

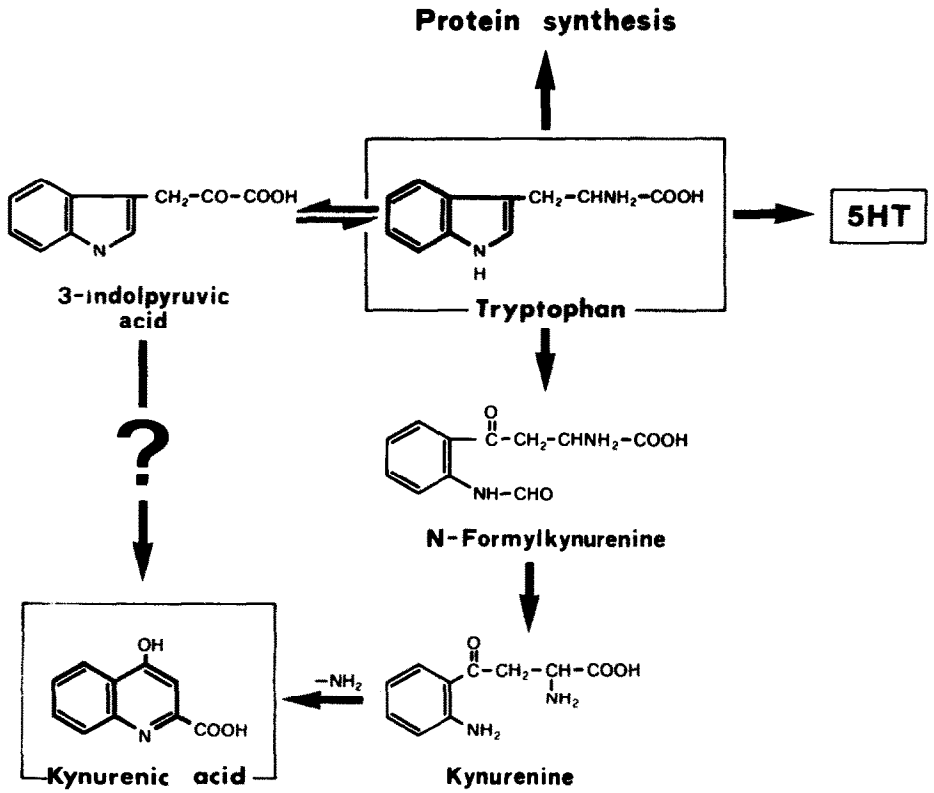

Fig. 1. The proposed pathways of tryptophan metabolism.

with 5 ml of 3 N NH_4OH. The collected eluate was dried under vacuum. The dry residue was resuspended in 300 μl of water and an aliquot of it (70 μl) was injected into an HPLC apparatus. This consisted of a Perkin–Elmer pump (model 10) with a syringe loading sample injection valve (model 7125 Rheodyne), a 3 cm length reverse phase pre-column and a 10 cm identical column (RP 18 Spheri 5 ODS; Brownlee Lab.). The detection was performed using a Perkin–Elmer Model LC 90 UV spectrophotometric detector operated at 340 nm. The mobile phase used on a routine basis was a mixture of sodium formiate (0.02 N), citric acid (0.02 N), NaH_2PO_4 (0.02 N) and acetonitrile 2%. Under those conditions, utilizing a flow-rate of 2 ml/min, authentic kynurenate and the material extracted from the brain had a retention time of 6 min 30 sec.

The sensitivity limit of the described HPLC method (signal/noise ratio:3) was 6 pmol of kynurenate per injection. Standard curves of authentic kynurenate passed through every step of the purification procedure gave linear responses in the range of 6–600 pmol per injection. The inter-assay variability was less than 7%. When a known amount of kynurenate was added to rat brain ethanol homogenates, its recovery, taking into account the documented loss in the ion exchange columns, was 95 ± 7%.

Determination of tryptophan. TRP was measured according to Lombardi *et al.* [17]. This procedure also allows the measurement of 5-HT and 5-HIAA not reported here. Briefly, the brain tissue was homogenized in $HClO_4$ 0.4 M. After centrifugation, the supernatant was filtered and placed in vials fitting a Perkin–Elmer LC 600 autoinjector connected to a liquid chromatography apparatus. This consisted of: the previously quoted autosampler, a Waters pump Mod. 6000 A, a RP8 guard column, a reverse-phase column (Brownlee–Spheri-50 S) and an electrochemical detector (BAS Model LC-4B) operated at 0.65 V. The mobile phase was selected according to Kilts *et al.* [18] and consisted of a solution (pH 2.5) containing Na_2HPO_4 75 mM, citric acid 100 mM and methanol 10%. Data were analyzed by ANOVA and the Dunnett *t*-test.

RESULTS

Effects of TRP or of IPA administration on the content of KYNA in the brain and other organs

Table 1 shows that TRP or IPA administration caused a dose-dependent increase in the content of KYNA in the brain and other organs. The kidneys had the largest increase in the concentration of KYNA. Time-course studies showed that the increased brain KYNA content, after 250 mg/kg i.p. of IPA, reached its maximum in 1 hr and it was still significantly higher than in controls 4 hr later (Fig. 2). Repeated administration of IPA (250 mg/kg/day for 7 days), however, did not further increase the accumulation of KYNA in the brain: 1 hr after the last injection of the precursors the animals had concentrations of KYNA in their brains that were not significantly different from those obtained in animals

Table 1. The effects of different doses of tryptophan or of indolpyruvic acid on the content of KYNA in the brain and other organs

	Brain	Liver	Kidney	Heart	Blood
Saline	16 ± 3	89 ± 10	262 ± 20	67 ± 8	20 ± 3
IPA 100	22 ± 2*	480 ± 35**	–	70 ± 5	–
IPA 250	30 ± 1**	827 ± 41**	2593 ± 180**	181 ± 2**	130 ± 9**
IPA 500	96 ± 10*	1980 ± 160**	–	–	–
TRP 100	23 ± 3*	455 ± 40**	1250 ± 110**	95 ± 15*	–
TRP 250	33 ± 4**	–	–	–	–

Values are pmol/g wet wt and are the mean ± SE of at least seven animals. IPA and TRP were administered intraperitoneally at the reported dose (mg/kg).

* $P < 0.05$.

** $P < 0.01$.

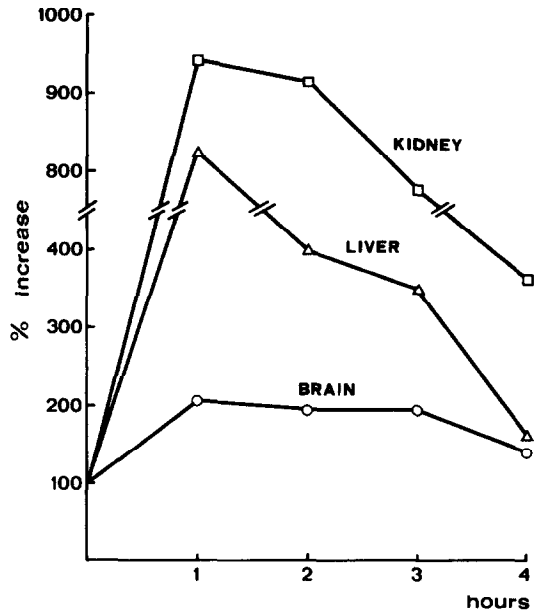

Fig. 2. Time-course of the changes of KYNA content in various organs after IPA (250 mg/kg i.p.) administration. Each point represents a mean value of at least six animals. Standard errors were within 10% (see Table 1).

treated once with an identical dose of the precursors (data not reported).

Effects of probenecid on the brain accumulation of KYNA

In order to clarify whether or not the observed increase of brain KYNA content after IPA administration was due to an increased rate of its synthesis or to a decreased rate of its disposal, rats were treated with a large dose of probenecid, a procedure often used to obtain an index of the rate of synthesis of acidic neurochemicals [19]. Probenecid (200 mg/kg i.p.) increased the content of brain KYNA by 43 pmol/g/hr (from 19 ± 3 to 62 ± 7 pmol/g mean ± SE of six animals) in control rats; this increase was 137 pmol/g/hr (from 12 ± 4 to 179 ± 20 pmol/g in rats pretreated with IPA (250 mg/kg i.p. 1 hr before probenecid).

Differential actions of TRP and of IPA on brain KYNA content

The increased formation of brain KYNA after the administration of IPA was able to occur because IPA is metabolized to TRP and subsequently to KYNA. By measuring, in the same brains, the content of both TRP and KYNA and by calculating the ratio of TRP/KYNA after the administration of the two precursors, it was possible to show that IPA administration resulted in an increased brain content of both TRP and KYNA, but that the ratio of TRP/KYNA was significantly lower after IPA than after TRP administration (Table 2).

DISCUSSION

The experiments reported here show, for the first

Table 2. Relationship between the brain content of tryptophan and KYNA

	Brain TRP	Brain KYNA	Ratio
Saline	21.6 ± 2.4	28.4 ± 2.9	760
TRP i.p. 250 mg/kg	321 ± 28**	56.4 ± 4.2**	5700
IPA i.p. 250 mg/kg	160 ± 10**	72.2 ± 6.1**	2200

Values are the means ± SE of at least six animals and are nmol/g wet wt for TRP and pmol/g wet wt for KYNA. The brain KYNA content in these experiments was higher than those reported in Table 1 because the animals were slightly older. The relationship between age and brain KYNA content has been previously reported [28].

time, that the administration of TRP or of its keto-analogue IPA, results in a dose-dependent increase of the concentration of KYNA in different organs. We focused our attention on the brain, which is one of the organs containing the lowest concentrations of KYNA, because in mammals, the excitatory amino acid receptors have been mainly studied in the central nervous system. In this organ IPA caused a large increase of KYNA synthesis. This was demonstrated not only by measuring brain KYNA content in animals pretreated with different doses of IPA, but also by measuring the apparent turnover rate of IPA after the inhibition of its disposal though a probenecid sensitive mechanism [19]. Under those circumstances, IPA administration (250 mg/kg) increased by approximately three times the rate of KYNA synthesis.

At least two metabolic pathways (Fig. 1) could explain this observation: IPA could be transaminated to TRP through the action of aromatic amino acid transaminases, enzymes that have been repeatedly demonstrated in the brain and in other organs [13, 20, 21]. These enzymes and the subsequent metabolism of TRP [22] therefore, could explain the increased concentration of KYNA after IPA administration. The experiments described in Table 2, however, suggest that in the brain a portion of IPA is transformed into KYNA without being transaminated to TRP (Fig. 1). In fact, if the transamination was the only pathway through which IPA could be metabolized to KYNA, the ratio between the brain concentration of TRP and that of KYNA should not be different in animals treated with the keto-acid or TRP. These experimental results, therefore, suggest that, at least in the brain, the metabolic pathway represented in Fig. 1 by a question mark is probably present.

A definitive demonstration for this metabolic pathway requires the measurement of the specific activity of TRP and of KYNA after the administration of labelled IPA [15].

Unfortunately, the experiments reported here cannot clarify whether or not the concentrations of KYNA reached in the brain after IPA administration are able to affect excitatory amino acid-mediated neurotransmission. *In vitro* experiments suggest that concentrations of KYNA in the range of 10^{-4} M are necessary to antagonize the actions of glutamic acid or to reduce synaptic excitation [3, 10, 23, 24]. These concentrations are certainly much larger than those obtained after IPA administration. It should be considered, however, that KYNA is an allosteric modulator of L-Glu receptors and that it probably acts at the level of the strychnine insensitive glycine receptor [7, 8]. Its action, therefore, is dependent not only upon its concentration or that of L-Glu, but also upon the local concentration of glycine. Low concentrations of KYNA could therefore be, in particular situations, functionally active. In support of this concept stands the observation that some of the pharmacological actions of IPA *in vivo* could be explained by considering its metabolism to KYNA and a consequent negative modulation of L-Glu receptors [16, 25]. Finally it should be noted that KYNA and glycine-sensitive excitatory amino acid receptors are present also in peripheral neurons [3, 26, 27]. Their physiological role is not known, but it is reasonable to suppose that their function is reduced by KYNA accumulation in the blood and in other peripheral organs. This accumulation occurs after IPA administration (Table 2) and is several times larger than that described for the brain.

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