Pharmacology Biochemistry & Behavior, Vol. 30, pp. 665–668. © Pergamon Press plc, 1988. Printed in the U.S.A. 0091-3057/88 $3.00 + .00

The Putative Endogenous Convulsant 3-Hydroxykynurenine Decreases Benzodiazepine Receptor Binding Affinity: Implications to Seizures Associated With Neonatal Vitamin B-6 Deficiency

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Received 21 July 1987

GUILARTE, T. R., L. D. BLOCK AND H. N. WAGNER, JR. *The putative endogenous convulsant 3-hydroxykynurenine decreases benzodiazepine receptor binding affinity: Implications to seizures associated with neonatal vitamin B-6 deficiency.* PHARMACOL BIOCHEM BEHAV **30**(3) 665–668, 1988.—The kynurenines, endogenous tryptophan metabolites with convulsant properties, have been postulated to play a role in the genesis of seizure disorders. We have previously reported that concentrations of 3-hydroxykynurenine (3-HK) higher than 0.2 mM are present in the brains of neonatal rats perinatally deprived of vitamin B-6. At a 1 mM concentration 3-HK significantly decreased the affinity of ^{3}H-flunitrazepam for benzodiazepine receptor sites in rat brain membrane preparations. Furthermore, lower concentrations (K_i=250 μM) of 3-HK antagonized the enhancing effect of GABA on ^{3}H-flunitrazepam binding. These results suggest that 3-HK may have a modulatory effect on the GABA/benzodiazepine/barbiturate receptor complex.

3-Hydroxykynurenine Putative endogenous convulsant Benzodiazepine receptors Seizures Vitamin B-6 deficiency

THE kynurenines, endogenous metabolites of tryptophan, have been shown to be neuronally active compounds in the mammalian central nervous system. Presently, a major motivation for the study of the kynurenines is their possible relevance to seizure disorders. This interest arises from recent evidence showing that L-kynurenine (L-Kyn), 3-hydroxykynurenine (3-HK), and quinolinic acid (QA) produce convulsions when injected into the cerebral ventricles or hippocampus of adult rodents [10, 12, 15, 25–27, 29]. Since kynurenines are normal constituents of the mammalian brain [3, 4, 30] it has been postulated that the kynurenines may be involved in the genesis of epileptic seizures [10–12]. In the normal brain, kynurenines are found in much lower concentrations than those needed to induce seizures (compare [3] and [12]). Under abnormal metabolic conditions, however, kynurenines in brain may reach high enough concentrations to precipitate seizure activity.

We have recently reported for the first time that increased concentrations of endogenously produced 3-HK are present in the brains of neonatal rats perinatally deprived of vitamin B-6 [6–8]. Mean concentrations of 3-HK in vitamin B-6 deficient cerebellum, corpus striatum, pons/medulla, and frontal cortex ranged from 9.7–18.6 and 102–142 nmoles/g tissue at 14 and 18 days of age. The highest concentration of 3-HK measured was 213 nmoles/g frontal cortex tissue from an 18-day-old vitamin B-6 deficient rat brain. Normal levels of 3-HK in rat brain are in the order of 1 nmole/g or less [3]. We believe this finding was significant since it was the first time that a putative endogenous convulsant was found in such high concentrations in the brains of rats under an experimental condition known to produce seizures (i.e., neonatal vitamin B-6 deficiency). We have suggested that the increased levels of 3-HK measured in brain may be responsible, at least in part, for the seizures observed in these animals [6–8]. Since there is no known biochemical mechanism for L-kynurenine- or 3-HK-induced seizures, the present investigation was undertaken in an effort to determine the potential role of 3-HK in seizures associated with neonatal vitamin B-6 deficiency and to try to elucidate a mechanism of action of kynurenine and 3-HK induced seizures.

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TABLE 1

INHIBITION OF 1 nM ^{3}H-FLUNITRAZEPAM BINDING TO BENZODIAZEPINE RECEPTORS BY KYNURENINES, INOSINE, AND L-TRYPTOPHAN AT A 1 mM CONCENTRATION

Compounds	% Inhibition*
Inosine	47.8 ± 2.5
3-Hydroxykynurenine	35.3 ± 6.3
Xanthurenic Acid	28.4 ± 6.3
L-Kynurenine	26.5 ± 7.6
Nicotinamide	24.4 ± 2.0
L-Tryptophan	11.2 ± 1.9
Quinolinic Acid	4.6 ± 6.3

*Mean ± 1 SD of 3–5 experiments each done in triplicate.

METHOD

Materials

Inosine, 3-hydroxy-DL-kynurenine, L-kynurenine (free base), xanthurenic acid, nicotinamide, L-tryptophan, and quinolinic acid were obtained from Sigma (St. Louis, MO).

Membrane Preparation and ^{3}H-Flunitrazepam Binding Assay

Long-Evans male rats (150–200 g) were killed by decapitation and the brain rapidly removed and dissected on an ice-chilled plate. All procedures involving tissue preparation were carried out in ice. Whole brain except cerebellum and brain stem was weighed, homogenized in 10 volumes 0.32 M sucrose using a motor-driven teflon pestle homogenizer, and centrifuged for 10 minutes, 1000×g at 4°C. The supernatant was spun at 30,000×g for 20 minutes at 4°C and the resulting pellet was homogenized using a PT-10 polytron (6 setting) for 15 seconds in 50 volumes of 50 mM Trizma base adjusted to pH 7.3 with 1 M citric acid (this was called Tris-citrate buffer). This tissue suspension was centrifuged at 30,000×g for 20 minutes at 4°C and the resulting pellet was washed three more times and frozen at −20°C overnight. The following day, the pellet was rehomogenized and washed 4 more times with buffer. The final pellet was resuspended in buffer to give a final volume in ml equal to 50 times the original tissue weight in grams and frozen at −70°C until used.

The assay for BDZ receptor binding was performed as follows: Triplicate assay tubes received tissue preparation (0.080–0.090 mg protein), 150 mM NaCl (final concentration), ^{3}H-flunitrazepam (NEN, spec. act. 78 Ci/mmole), and freshly made solutions of the compounds to be tested. Total assay volume was brought to 1 ml with 50 mM Tris-citrate buffer. For single point assay, the final ^{3}H-flunitrazepam concentration was 1.0 nM (ranged from 1.0–1.2 nM) and for saturation isotherms the concentration ranged from 16.0–0.25 nM. One micromolar nonradioactive flunitrazepam was used to assess nonspecific binding. Assay tubes were incubated at 0°C for 30 minutes, their contents filtered under vacuum through GF/B microfibre filters and rinsed with 2×5 ml of cold Tris-citrate buffer. Radioactivity in filters was determined using liquid scintillation spectrometry in 10 ml of Budget-Solve (Research Products International, Mount Prospect, IL) scintillation cocktail after overnight storage. Specific binding was usually 80–90% of total binding. GABA-stimulation of ^{3}H-flunitrazepam binding was performed as described above with the exception that 1.0 or 10.0 μM GABA (final concentration) was included in the test tubes. The 1 μM GABA was used for the determination of the K_i of 3-HK for the GABA-stimulated binding of ^{3}H-flunitrazepam. The 10 μM GABA was used on the determination of 3-HK effects on BDZ receptor B_{max} or K_d in the presence of GABA.

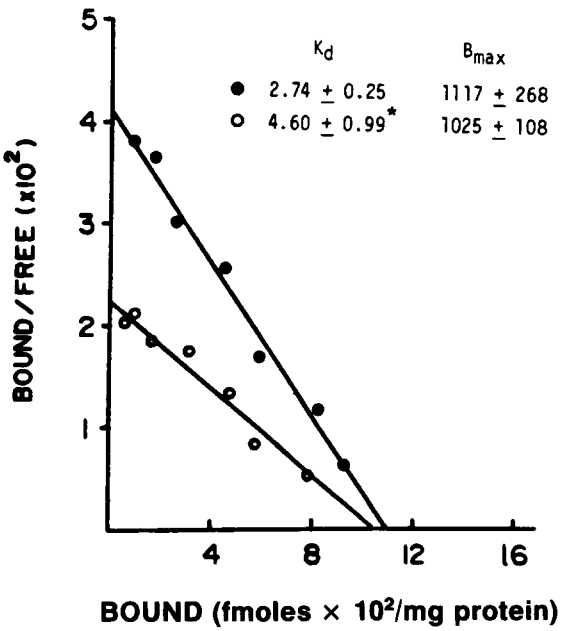

FIG. 1. Scatchard analysis of ^{3}H-flunitrazepam binding to benzodiazepine receptors in the presence (○) and absence (●) of 1 mM 3-hydroxykynurenine. Each point represents the average of 3 different experiments each done in triplicate. K_d values are nM and B_{max} are fmoles/mg protein. Asterisk represents significance at $p \leq 0.05$.

RESULTS

Table 1 shows the relative potencies of 3-HK and other kynurenines as well as inosine and L-tryptophan in inhibiting the specific binding of ^{3}H-flunitrazepam to a rat brain membrane preparation in the absence of GABA (basal binding). At a 1 mM concentration, 3-HK was 0.74 times as potent as inosine and 1.5 times more potent than nicotinamide, compounds which are putative endogenous ligands of the BDZ receptor [18, 19, 22, 28]. In comparison to other kynurenines, 3-HK was 1.33 and 1.22 times more potent than L-Kyn and xanthurenic acid (XA), respectively. Quinolinic acid and L-tryptophan had no significant effect on ^{3}H-flunitrazepam binding at the concentration tested.

To determine how 3-HK affected the binding of ^{3}H-flunitrazepam to BDZ receptors, saturation isotherms were performed in the absence and presence of 1 mM 3-HK. Figure 1 shows that the reduction in ^{3}H-flunitrazepam binding was the result of a change in the affinity constant (K_d) and not in the number of receptors (B_{max}), demonstrating competitive inhibition.

Convulsant BDZ's as well as putative endogenous ligands of the BDZ receptor are able to inhibit GABA-stimulated BDZ binding with a potency higher than for basal binding [20,23]; therefore, 3-HK was tested to determine if it could inhibit the GABA-enhancement of ^{3}H-flunitrazepam binding.

TABLE 2

EFFECT OF 3-HYDROXYKYNURENINE ON THE GABA-ENHANCED BINDING OF ^{3}H-FLUNITRAZEPAM TO BENZODIAZEPINE RECEPTORS*

	K_d (nM)	B_{max} (fmoles/mg protein)
10 μM GABA	1.98 ± 0.43	1499 ± 237
10 μM GABA + 400 μM 3-HK	3.12 ± 0.72†	1474 ± 147

*Mean ± 1 S.D. of 3–4 different determinations.
†$p<0.05$.

The mean±1 S.D. 3-HK IC_{20} and IC_{50} values for GABA-stimulated (1 μM GABA) binding of ^{3}H-flunitrazepam to BDZ receptors was 222±43 μM (n=3) and 359±48 μM (n=3), respectively. The average increase in the binding of 1 nM ^{3}H-flunitrazepam to BDZ receptors in the presence of 1 μM GABA was 30%. The IC_{20} of 3-HK for basal ^{3}H-flunitrazepam binding was 700±70 μM (n=3). An IC_{50} value for basal binding could not be obtained because of the poor solubility of 3-HK at high concentrations. These results show that 3-HK is a more potent inhibitor of GABA-stimulated ^{3}H-flunitrazepam binding to BDZ receptors than as an inhibitor of basal binding. Since a 3-HK IC_{50} for GABA-stimulated binding was obtained, a K_i was calculated using the formula $K_i = IC_{50}/(1 + [L]/K_d)$. Based on an IC_{50} of 359 μM, a GABA-stimulated ^{3}H-flunitrazepam K_d of 2.31 nM (K_d in the presence of 1 μM GABA) and a ligand (^{3}H-flunitrazepan; [L]) concentration of 1 nM, the K_i of 3-HK for GABA-stimulated (1 μM) binding of ^{3}H-flunitrazepam was 250 μM. This 3-HK concentration is in the same range as the 3-HK concentrations measured in the brains of neonatal rats perinatally deprived of vitamin B-6 [6–8]. The inhibition of GABA-stimulated ^{3}H-flunitrazepam binding by 3-HK was the result of a change in K_d and not on B_{max} (Table 2).

DISCUSSION

The convulsant properties of the kynurenines when injected intracerebroventricularly have been demonstrated by Lapin [10–16] and confirmed by Pinelli *et al.* [26]. However, electrophysiological and neurochemical studies have not been able to determine a biochemical mechanism for kynurenines-induced seizures with the possible exception of QA [5, 17, 29]. Our recent finding that extremely high concentrations of 3-HK are present in the brains of vitamin B-6 deficient neonatal rats prompted our search for a biochemical mechanism. The present study indicates that 3-HK inhibits the binding of ^{3}H-flunitrazepam to benzodiazepine receptors in rat brain membrane preparations with a potency similar to inosine (Table 1), a putative endogenous ligand of the BDZ receptor. Although 3-HK has a low potency for displacing the basal binding of ^{3}H-flunitrazepam, it is more potent in displacing the GABA-stimulated binding of ^{3}H-flunitrazepam to the BDZ receptor (Table 2). The GABA-stimulated binding has been suggested to be a more physiological relevant state of the BDZ receptor than in the absence of GABA [19]. Furthermore, we have shown that 3-HK concentrations which can occupy 20% of BDZ receptor sites (in vitro) are achieved in the brains of vitamin B-6 deficient neonatal rats, an experimental condition known to produce seizures. This level of BDZ receptor occupancy has been associated with pharmacological effects of diazepam and β-carbolines [9,24].

In our hands, 3-HK did not inhibit the binding of ^{3}H-spiperone or ^{125}I-LSD to dopamine or serotonin receptors, respectively (data not shown). Unequivocal results were not obtained with the sodium-independent binding of ^{3}H-GABA to its receptor. 3-HK appeared to inhibit the binding of ^{3}H-GABA to rat brain membrane preparation with an IC_{50} in the μM range in some but not all membrane preparations. Further studies are necessary to elucidate the GABA binding results and to determine if 3-HK interacts with other neurotransmitter receptor sites or uptake sites. Based on these in vitro binding studies, one may propose that the putative endogenous convulsant 3-HK may be producing its effects by modulation of the GABA/BDZ receptor complex. The results are consistent with the finding that putative endogenous ligands of the BDZ receptor, i.e., inosine, nicotinamide, and hypoxanthine, antagonize kynurenine-induced seizures in rodents [14].

The results presented in this communication may also help in the elucidation of a biochemical mechanism of seizures associated with neonatal vitamin B-6 deficiency. It is well known that vitamin B-6 deficiency reduces the activity of the B-6 dependent enzyme glutamic acid decarboxylase (GAD) and the levels of GABA in brain [2]. Since experimental treatments or drugs which attenuate or inhibit GABAergic neurotransmission promote seizure activity [21], it has been postulated that vitamin B-6 deficiency during the neonatal period produces seizures on the basis of decreases in brain GAD activity and GABA concentrations [2]. However, seizures have not been observed in vitamin B-6 deficient adult rats despite significant reductions in brain GAD activity and GABA concentrations [1]. Therefore, it appears that other factors are essential for producing seizures in vitamin B-6 deficient neonatal rats. Our previous finding that endogenously produced 3-HK accumulates to extremely high concentrations in the brains of vitamin B-6 deficient neonatal rats but not in B-6 deficient adult rats [7,8] is consistent with the seizures observed in the neonatal but not in the adult rats. The present finding that 3-HK decreases the affinity of ^{3}H-flunitrazepam binding to BDZ receptors may be another factor which increases the seizure susceptibility of vitamin B-6 deficient neonatal rats. The effects of 3-HK on the GABA/BDZ receptor complex combined with the decreased concentrations of brain GABA known to occur in neonatal vitamin B-6 deficiency may provide a working hypothesis for the elucidation of a biochemical mechanism of seizures associated with this condition.

To conclude it should also be pointed out that in the original studies on the identification of increased concentrations of 3-HK in vitamin B-6 deficient neonatal rat brain, a second yet unidentified peak was present in high concentrations in the HPLC chromatograms of brain tissue from B-6 deficient neonatal rats but not from control rats [7,8]. We also showed that this unidentified peak was a metabolite of 3-HK since intraperitoneal injections of 3-HK (100 mg/kg) to 13-day-old control rats generated the appearance of the unidentified peak as a function of time postadministration [8]. It is possible that this unidentified metabolite of 3-HK may also play a role in kynurenine-induced seizures and those associated with neonatal vitamin B-6 deficiency. Further studies are required to determine the chemical structure of this metabolite.

ACKNOWLEDGEMENTS

This work was supported by grant HD 20939 to T.R.G from the National Institute of Child Health and Human Development. Non-radioactive flunitrazepam was a gift from Dr. Michael Kuhar.

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