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(*R,S*)-3,4-dichlorobenzoylalanine (FCE 28833A) causes a large and persistent increase in brain kynurenic acid levels in rats

Carmela Speciale [a,*], Hui-Qiu Wu [b], Massimo Cini [a], Marina Marconi [a], Mario Varasi [a], Robert Schwarcz [b]

[a] *Pharmacia & Upjohn, R & D – CNS, Via Giovanni XXIII 23, 20014 Nerviano, Milano, Italy*
[b] *Maryland Psychiatric Research Center, University of Maryland School of Medicine, Baltimore, MD, USA*

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Abstract

Kynurenic acid is an endogenous excitatory amino-acid receptor antagonist with neuroprotective and anticonvulsant properties. We demonstrate here that systemic administration of the new and potent kynurenine 3-hydroxylase inhibitor (*R,S*)-3,4-dichlorobenzoylalanine (FCE 28833A) causes a dose-dependent elevation in endogenous kynurenine and kynurenic acid levels in rat brain tissue. In hippocampal microdialysates, peak increases of 10- and 80-fold above basal kynurenic acid concentrations, respectively, were obtained after a single oral or intraperitoneal administration of 400 mg/kg FCE 28833A. After intraperitoneal treatment with FCE 28833A, extracellular brain kynurenic acid levels remained significantly elevated for at least 22 h, rendering this compound a far more effective enhancer of kynurenic acid levels than the previously described kynurenine 3-hydroxylase blocker *m*-nitrobenzoylalanine. FCE 28833A and similar molecules may have therapeutic value in diseases which are linked to a hyperfunction of excitatory amino-acid receptors.

Keywords: Anticonvulsant; Kynurenine; Microdialysis; Neurodegenerative disorder; Neuroprotection

1. Introduction

Pharmacological manipulation of the kynurenine pathway of tryptophan metabolism has been successfully utilized to increase the brain concentration of kynurenic acid, a broad spectrum excitatory amino-acid antagonist with neuroprotective and anticonvulsant properties (Perkins and Stone, 1982; Foster et al., 1984). For example, substantial elevations of brain kynurenic acid levels can be achieved in vivo through the systemic or intracerebral administration of its bioprecursor, kynurenine (Speciale et al., 1990; Swartz et al., 1990). These increases are probably responsible for the resulting enhanced resistance of the brain to hypoxic-ischemic insults during the perinatal period (Nozaki and Beal, 1992) and for the raised threshold to convulsant stimuli (Vécsei et al., 1992).

An alternative strategy to increase brain kynurenic acid levels was introduced by Moroni, Pellicciari and coworkers (Carpenedo et al., 1994; Pellicciari et al., 1994). Based on earlier work by Decker et al. (1963), their studies demonstrated that inhibition of kynurenine 3-hydroxylase, the enzyme responsible for the conversion of kynurenine along a metabolic route leading to the endogenous excitotoxin quinolinic acid (Stone, 1993; Guidetti et al., 1995), causes cerebral kynurenic acid concentrations to rise several-fold. Moreover, the authors showed that these elevations occurred not only in brain tissue homogenate but could also be measured by in vivo microdialysis, and suggested that sufficient extracellular kynurenic acid may have been produced to allow blockade of the glycine co-agonist site of the NMDA receptor (ED_{50} for kynurenic acid: 15 μM; Kessler et al., 1989). Indeed, the inhibitor *m*-nitrobenzoylalanine (mNBA; IC_{50}: 3 μM) not only raised extracellular kynurenic acid levels up to 12-fold but also displayed anticonvulsant activity against sound-induced seizures in DBA/2 mice and against maximal electroshock-induced convulsions in rats (Carpenedo et al., 1994).

During our efforts to develop more efficacious kynurenine 3-hydroxylase inhibitors, we recently synthesized a series of analogs and derivatives of kynurenine. As compared to mNBA, one of the compounds, (*R,S*)-3,4-dichlorobenzoylalanine (FCE 28833A), showed significant improvement in potency both as an enzyme inhibitor and in

* Corresponding author. Tel.: (39-2) 4838-3225; Fax: (39-2) 4838-3937.

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its ability to raise cerebral kynurenic acid levels in vivo. Some of these data have been communicated in abstract form (Molinari et al., 1995).

2. Materials and methods

2.1. Animals and chemicals

Male Wistar rats (180–220 g), housed under standard laboratory conditions on a 12 h:12 h light:dark cycle with free access to food and water, were used in all experiments. All reagents were purchased from Sigma (St. Louis, MO, USA). mNBA and FCE 28833A were synthesized in the Pharmacia&Upjohn laboratories (Nerviano, Italy), according to Pellicciari et al. (1994) and Giordani et al. (in press).

2.2. Administration of mNBA and FCE 28833A

Both test compounds were suspended in methocel at a concentration of 400 mg/5 ml and administered either orally (p.o.) or intraperitoneally (i.p.). Control animals received an identical volume of methocel via the same route.

2.3. Biochemical analyses

Kynurenine 3-hydroxylase activity was measured in a crude mitochondrial preparation of whole forebrain according to the method of Carpenedo et al. (1994). Kynureninase and kynurenine aminotransferase activities were determined in forebrain homogenates as described by Takikawa et al. (1986) and Okuno et al. (1991), respectively. Brain tissue levels of kynurenine were determined in a perchlorate extract by HPLC with UV detection (340 nm), using a C_{18} reverse-phase column and a mobile phase containing 3.6 mM heptanesulfonic acid in 50 mM ammonium acetate buffer/methanol (97:3), pH 6.0. Kynurenic acid was measured in brain tissue homogenate or in microdialysate as described previously (Swartz et al., 1990; Wu et al., 1992).

2.4. Microdialysis

Microdialysis was performed as detailed by Wu et al. (1992). Dialysate samples were collected every 60 or 90 min using a CMA/142 fraction collector (Carnegie Medicin, Stockholm, Sweden). Baseline values for kynurenic acid in the microdialysate were established by averaging the results from three 60-min samples collected prior to the administration of the test compounds. Data were not corrected for recovery through the dialysis probe (approximately 20%). Proper microdialysis probe placement was ascertained in every animal by histological means.

3. Results

3.1. Enzyme assays in vitro

In rat forebrain preparations, mNBA and FCE 28833A inhibited kynurenine 3-hydroxylase activity with IC_{50} values of 3.0 ± 0.2 and 0.2 ± 0.02 μM, respectively (inhibition curves not illustrated). In contrast, both compounds did not significantly affect kynureninase and kynurenine aminotransferase activities (less than 50% inhibition at 100 μM; data not shown).

3.2. Behavioral observations

Cursory observations revealed no apparent behavioral abnormalities during the period of microdialysis following the oral administration of either mNBA or FCE 28833A, or after the i.p. injection of mNBA. However, animals treated with 400 mg/kg FCE 28833A (i.p.) displayed obvious sedation starting 10 min after administration of the compound and lasting approximately 4 h.

3.3. Effect of p.o. administration of FCE 28833A on brain kynurenine and kynurenic acid levels

The effect of the oral administration of 400 mg/kg FCE 28833A on brain tissue kynurenine and kynurenic acid is shown in Table 1. A significant elevation in the levels of both metabolites was observed after 2 h, and accumulations kept increasing with time to reach more than 10 times control values after 8 h. The dose depen-

Table 1
Time- and dose-related effects of oral FCE 28833A treatment on kynurenine and KYNA levels in the rat brain

	Kynurenine (pmol/g tissue)	Kynurenic acid (pmol/g tissue)
Time (h)		
Control		
0	319 ± 14	5 ± 2
2	2067 ± 362 **	23 ± 8 **
4	3560 ± 119 **	33 ± 5 **
6	3912 ± 324 **	56 ± 12 **
8	5711 ± 508 **	69 ± 5 **
Dose (mg/kg)		
Control		
0	432 ± 40	2 ± 0
50	1300 ± 347 **	10 ± 2 **
100	2922 ± 500 **	23 ± 3 **
200	4104 ± 411 **	41 ± 7 **
400	6089 ± 895 **	69 ± 10 **

Time-related effects were studied by treating the animals orally with 400 mg/kg FCE 28833A. For dose dependency experiments, rats were killed 8 h after the administration of the compound. Tissue kynurenine and kynurenic acid levels were measured as described in Materials and methods. Data are the means ± S.E.M. of at least 5 animals per group. ** $P < 0.01$ as compared to untreated control rats (Dunnett's test).

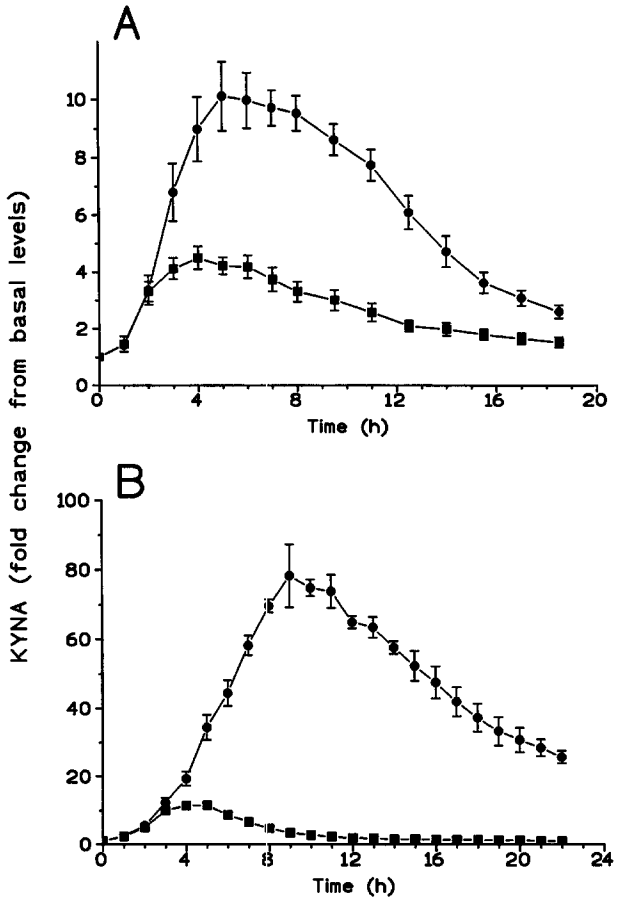

Fig. 1. Extracellular levels of kynurenic acid in the hippocampus of unanesthetized rats following the oral (A) or intraperitoneal (B) administration of 400 mg/kg mNBA (squares) and FCE 28833A (circles) at time 0. Experiments were conducted as described in Materials and methods. Basal kynurenic acid concentrations were 144.7±6.5 fmol/60 μl dialysate. Data are the means ± S.E.M. of 6–7 animals per group (see text for statistical analysis: two-way ANOVA followed by Newman-Keuls post-hoc analysis).

dency of the FCE 28833A effect was subsequently studied at the 8 h time point. 50 mg/kg FCE 28833A caused a 3–5-fold increase in the brain concentrations of both kynurenine and kynurenic acid, and progressively larger effects were noted up to a dose of 400 mg/kg (Table 1).

3.4. Effect of FCE 28833A or mNBA on extracellular kynurenic acid levels in the hippocampus

Extracellular kynurenic acid levels were measured by in vivo microdialysis for several hours following the oral application of FCE 28833A and mNBA (400 mg/kg each) (Fig. 1A). Comparison of the effects of the two kynurenine 3-hydroxylase inhibitors revealed that FCE 28833A caused more pronounced and longer lasting increases in kynurenic acid concentrations. After FCE 28833A administration, a significant elevation in kynurenic acid levels was observed within 2 h, peak increases of 10-fold basal kynurenic acid levels were seen between 5 and 8 h, and the elevation remained statistically significant up to 14 h ($F(17,170) = 24.85$; $P < 0.001$). The kynurenic acid-enhancing effect of mNBA was more modest, but also reached statistical significance within 2 h. Kynurenic acid levels returned to baseline values by 10 h ($F(17,170) = 28.23$; $P < 0.001$; Fig. 1A). The effect of FCE 28833A was significantly greater than that of mNBA between 4 and 11 h after administration of the compounds ($F(14,154) = 12.75$; $P < 0.001$).

More potent effects of the two compounds, and a quantitatively greater dissociation between FCE 28833A and mNBA, were observed when hippocampal microdialysis was performed after an i.p. injection of the two inhibitors (Fig. 1B). Both compounds raised kynurenic acid levels significantly by 2 h, but a marked difference in peak values was obtained for FCE 28833A and mNBA. Moreover, the effect of mNBA peaked earlier and was much shorter lasting than that of FCE 28833A, returning to baseline values by 11 h ($F(21,210) = 39.07$; $P < 0.001$). In contrast, kynurenic acid levels were still elevated by approximately 30-fold 22 h after FCE 28833A administration, i.e., at the end of the microdialysis period ($F(21,210) = 70.27$; $P < 0.001$). Throughout the entire 22 h observation period, FCE 28833A-induced elevations in kynurenic acid levels were substantially more pronounced than those elicited by mNBA ($F(21,210) = 73.29$; $P < 0.001$).

4. Discussion

The present study demonstrated that the systemic administration of FCE 28833A to rats causes a large increase in the brain levels of the endogenous excitatory amino-acid receptor antagonist kynurenic acid and its bioprecursor kynurenine. These increases, which lasted for several hours following a single application of FCE 28833A, were measurable in brain tissue homogenate and, in the case of kynurenic acid, by in vivo microdialysis. The presence of elevated levels of kynurenic acid in the extracellular compartment is particularly noteworthy since it is expected to improve the ability of kynurenic acid to reduce excitability and, in particular, to antagonize neuronal NMDA receptors through blockade of the glycine co-agonist site (Kessler et al., 1989). Our data also showed that i.p. injection of FCE 28833A was more effective than oral administration of the compound, and that by either route FCE 28833A was far more potent than mNBA in its ability to elevate brain kynurenic acid levels (cf., Carpenedo et al., 1994).

In contrast to the mode of action of kynurenine, which is transaminated enzymatically and thereby raises the levels of kynurenic acid directly (Turski et al., 1989; Speciale et al., 1990; Swartz et al., 1990; Stone, 1993), FCE 28833A-induced increases in brain kynurenic acid occur indirectly. Most likely, the compound acts by inhibiting kynurenine 3-hydroxylase activity and thus diverts kynurenine pathway metabolism away from the quinolinic

acid/NAD-producing branch towards the synthesis of kynurenic acid. This is in line with the greater potency of FCE 28833A than mNBA both as an enzyme inhibitor (IC_{50} values of 0.2 μM vs. 3 μM, respectively) and as a kynurenic acid-enhancing agent, but differential resorption or other pharmacokinetic differences may also have accounted for the superior effectiveness of FCE 28833A over mNBA. Notably, kynurenine 3-hydroxylase inhibition by FCE 28833A causes a rapid accumulation of kynurenine not only in the brain but also in the blood (Speciale et al., 1996). Our data therefore suggest that FCE 28833A acts primarily in the periphery and effects a secondary, massive influx of kynurenine into the brain (Fukui et al., 1991). However, it remains to be determined if some of the compound's influence on kynurenic acid synthesis may also be due to its penetration through the blood-brain barrier and subsequent inhibition of cerebral kynurenine 3-hydroxylase. Regardless of the peripheral or central origin of the FCE 28833A-induced surge in brain kynurenine, cerebral transamination of kynurenine, rather than kynurenic acid entry from the blood, underlies the long-lasting, pronounced elevation in cerebral kynurenic acid concentrations. This was reinforced in a recent study where the intrahippocampal application of the non-specific kynurenine aminotransferase inhibitor aminooxyacetic acid greatly attenuated the rise in kynurenic acid levels in hippocampal microdialysate following the systemic administration of FCE 28833A (Speciale et al., 1996).

Two aminotransferases with distinct biochemical properties (Guidetti et al., manuscript in preparation) have recently been cloned (Mosca et al., 1994; Buchli et al., 1995). Their relative contribution to the cerebral synthesis of kynurenic acid is still unknown and may change with varying physiological and pathophysiological conditions. Notably, ultrastructural immunohistochemical studies with antibodies against kynurenine aminotransferase I demonstrated immunoreactive deposits in astrocytic processes engulfing positively identified glutamatergic synapses (Schwarcz et al., 1996). This implies that the anatomical arrangement is in place for the optimal delivery of de novo produced kynurenic acid for interaction with neuronal excitatory amino-acid receptors.

In spite of the enduring and quantitatively substantial increase in kynurenic acid levels and the advantageous neuroanatomical microenvironment, it remains to be seen if the FCE 28833A-induced elevations in brain kynurenic acid concentration suffice to attenuate NMDA receptor function. While the answer may depend in part on possible concomitant changes in the levels of the endogenous NMDA receptor agonist quinolinic acid, preliminary evidence suggests that this may indeed be the case. Thus, the weaker congener of FCE 28833A, mNBA, possesses anticonvulsant properties (Carpenedo et al., 1994; Pellicciari et al., 1994), and FCE 28833A shows anti-ischemic effects in the gerbil global ischemia model (Speciale et al., 1996) and has sedative effects after i.p. administration in rats (this communication). Tests in several other relevant rodent models are currently under way, primarily to study the compound's anti-excitotoxic potency. If in vivo efficacy as a functional excitatory amino-acid receptor antagonist can be ascertained, FCE 28833A will not only be of use as a superior experimental probe for the study of kynurenic acid neurobiology, but may also have therapeutic value in diseases which are linked to a hyperfunction of excitatory amino-acid receptors.

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