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Differential modulation of brain benzodiazepine receptor subtypes by ricinelaidic acid *in vitro*

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Abstract—The C-18 hydroxy fatty acids ricinelaidic acid and ricinoleic acid diminish the oleic acid-stimulated agonist benzodiazepine binding in the rat brain *in vitro*. The oleic acid-induced enhancement of [^{3}H]diazepam binding was completely abolished in membranes from the cerebellum, but only partially decreased in membranes from the hippocampus, cortex and the whole brain from 7-day-old rat pups. Related hydroxy fatty acids as well as hydroxy fatty acid esters had no effect on the oleic acid-stimulated diazepam receptor binding.

Key words: ricinelaidic acid; benzodiazepine receptors; fatty acids; rat brain; *in vitro*

Unsaturated fatty acids, e.g. oleic, arachidonic and docosahexaenoic acid, have been shown to increase the binding of agonist benzodiazepine and γ-aminobutyric acid (GABA) receptor ligands to rat brain membranes *in vitro* [1–3]. Binding of the benzodiazepine receptor antagonist flumazenil (Ro 15-1788) and the GABA receptor antagonist 2-(3-carboxypropyl)-3-amino-6-(4-methoxyphenyl)-pyridazin (SR 95531) was not influenced by the unsaturated fatty acids *in vitro* [2]. Originally, binding studies using derivatives of β-carbolines and the triazolopyridine, 3-methyl-6-(3-trifluoromethyl)phenyl-1,2,4-triazolo[4,3-*b*] pyridazine (CL 218872), indicated the presence of two subtypes of benzodiazepine receptor, BZ1 and BZ2 [4, 5]. There is a high density of BZ1 receptors in the cerebellum of the rat, while BZ2 receptors are present in high concentrations in the hippocampus [6, 7]. Furthermore, the BZ1 receptor subtype represents only a small percentage of benzodiazepine receptors in the brain tissue of rats under 7 days old [8]. The recent discovery of a variety of receptor subunits (α_{1-6}, β_{1-4}, γ_{1-3}, δ and ρ_{1-2}), which in various compositions may form the GABA/benzodiazepine receptor complex, suggests a multiplicity of benzodiazepine receptors [9]. The presence of an α_1 subunit seems to mediate the BZ1 receptor subtype pharmacology [10].

We report here that the hydroxy unsaturated fatty acid, ricinelaidic acid, reduces the increase in benzodiazepine receptor binding by oleic acid in rat brain membranes *in vitro*. Almost complete inhibition of enhancement is found on binding of [^{3}H]diazepam to membranes from rat cerebellum. Ricinelaidic acid partially inhibits the oleic acid-stimulated increase in [^{3}H]diazepam binding to membranes from the cortex, hippocampus and whole brain of 7-day-old rat pups.

Materials and Methods

Ricinelaidic acid ([*R*-(*E*)]-12-hydroxy-9-octadecenoic acid); ricinoleic acid ([*R*]-12-hydroxy-*cis*-9-octadecenoic acid); oleic acid (*cis*-9-octadecenoic acid); recinelaidic acid methyl ester and ricinelaidic acid ethyl ester were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). [^{3}H]Diazepam (85 Ci/mmol) and [^{3}H]muscimol (20 Ci/mmol) were from Dupont-NEN (Boston, MA, U.S.A.). A stock solution of the fatty acids (4×10^{-2} M) was obtained by dissolving in ethanol (93%, v/v). The solutions were kept at −20° in the dark until use. Freshly prepared dilutions were made in 93% ethanol and added to binding assays in a volume of 25 μL.

Neuronal membrane suspension was prepared from adult male Wistar rats (weighing approx. 200 g). The rats were decapitated and the cerebral cortex, hippocampus and cerebellum rapidly dissected. From rat pups, whole brain tissue was used. The tissue was homogenized in 20 vol. (w/v) of Tris–citrate buffer (50 mM, pH 7.1) at 0–4° by an Ultra Turrax homogenizer. The homogenate was centrifuged (25,000 *g*) for 10 min and the pellet resuspended in Tris–citrate buffer followed by centrifugation. The final pellet was either used directly in the assay of [^{3}H]diazepam binding or kept at −20° until use.

The binding assay of [^{3}H]diazepam consisted of a 1 mL membrane suspension (2 mg original tissue for adult rat brain, 5 mg original tissue for rat pups); 25 μL oleic acid, 25 μL ricinelaidic acid (or other fatty acid) and 25 μL of [^{3}H]diazepam (0.6 and 1 nM final concentration for adult and rat pup tissue, respectively) in Tris–citrate buffer (50 mM, pH 7.1). [^{3}H]Diazepam binding equilibrium was obtained after 40 min at 0–4°. Bound radioactive ligand was separated from free by filtration through Whatman GF/C glass fibre filters. Filters were washed twice with 5 mL ice-cold Tris–citrate buffer (50 mM, pH 7.1). Midazolam (5×10^{-6} M final concentration) was used to define non-specific binding (less than 10% of total binding). For saturation experiments seven concentrations (0.1–20 nM) of [^{3}H]diazepam were used.

[^{3}H]Muscimol binding was performed on membranes prepared from a frozen pellet which was thawed and washed once in Tris–citrate buffer. Conditions for the binding assay were as described for [^{3}H]diazepam binding. The concentration of [^{3}H]muscimol was 5 nM and GABA (10^{-4} M final concentration) was used to define non-specific binding (approximately 25% of total binding). Five per cent of ethanol (v/v) in the binding assays decreased the specific [^{3}H]diazepam binding by 10–15% and [^{3}H]muscimol binding by 15–20% compared with control. All binding assays were performed in duplicate.

Results and Discussion

Oleic acid (10^{-4} M) enhanced [^{3}H]diazepam binding *in vitro* to approximately 200% of control in three brain regions from adult animals and in whole brain membrane preparations from 7-day-old rats. Ricinelaidic acid alone (or ricinoleic acid—experiment not shown) slightly reduced [^{3}H]diazepam binding (10–20% of control) but completely abolished the oleic acid-induced stimulation (ED_{50} 30 μM, data not shown) of [^{3}H]diazepam binding to rat cerebellar membranes. In hippocampal, cortical and whole brain preparations from 7-day-old rats, the ricinelaidic acid only partially inhibited the oleic acid-induced enhancement. Accurate ED_{50} values on the effect of unsaturated fatty acids in hippocampal and cortical membranes could not be obtained due to the low solubility of the oleic acid/

Table 1. Effect of ricinelaidic acid (RA 10^{-4} M) on oleic acid (OA 10^{-4} M)-stimulated receptor binding in various rat brain regions and whole forebrain of 7-day-old rat pups *in vitro*

Brain region	[^{3}H]Diazepam binding (% of control)			[^{3}H]Muscimol binding (% of control)		
	OA	RA	OA + RA	OA	RA	OA + RA
Cortex	191 ± 20	90 ± 15	136 ± 12*	201 ± 36	133 ± 14	238 ± 24
Hippocampus	197 ± 18	102 ± 10	162 ± 10	207 ± 38†	140 ± 7†	246 ± 33†
Cerebellum	182 ± 28	79 ± 11	91 ± 20*	122 ± 5†	110 ± 5†	130 ± 8†
Whole brain 7-day-old rats	187 ± 14	96 ± 6	155 ± 18	263 ± 50†	127 ± 11†	341 ± 58†

* $P < 0.001$ when compared to OA (Student's *t*-test).
† Mean ± SD of four determinations; all other values are means ± SD of seven determinations.

ricinelaidic acid mixtures in the incubation buffer. A series of fatty acids (10^{-4} M) including 10-hydroxydecanoic acid, 12-hydroxydodecanoic acid, 16-hydroxyhexadecanoic acid, methyl and ethyl esters of ricinelaidic acid, and methyl ester of ricinoleic acid had no effect on oleic acid-stimulated [^{3}H]diazepam binding (data not shown). A similar effect of ricinelaidic acid was found using arachidonic acid or docosahexaenoic acid instead of oleic acid to stimulate [^{3}H]diazepam binding or [^{3}H]muscimol binding (data not shown). Scatchard plot analyses of [^{3}H]diazepam binding to cerebellar membrane preparations showed that ricinelaidic acid reversed the oleic acid-induced decrease in the affinity constant, K_d. B_{max} (pmol/g tissue) values and K_d (nM) values are means ± SEM of five independent experiments: control B_{max} 64.2 ± 6.6, K_d 8.5 ± 0.3; + oleic acid B_{max} 74.6 ± 6.7, K_d 6.0 ± 0.6*; + ricinelaidic acid B_{max} 58 ± 3.6, K_d 11 ± 0.6*; oleic acid + ricinelaidic acid B_{max} 69.6 ± 6.9, K_d 9.5 ± 1.0 (*$P < 0.01$ compared to control, Student's *t*-test).

Several benzodiazepine receptor subtypes exist in the brain. The first description of subtypes differentiated between a cerebellar type BZ1 (type 1) and a BZ2 (type 2) subtype abundant in limbic structures, e.g. the hippocampus [4, 5, 7]. Ontogenetic studies in rat have shown that the benzodiazepine receptors during the first 7 days after birth are mainly of the BZ2 subtype [8]. It is interesting that the ability of ricinelaidic acid to reverse the enhancement of [^{3}H]diazepam binding by oleic acid was stronger in cerebellar membranes (BZ1-enriched brain region) compared to tissue containing relatively few BZ1 receptors compared to BZ2 receptors (e.g. hippocampus or brain tissue from less than 7-day-old rats).

Oleic acid stimulated [^{3}H]muscimol binding to 200% of control in hippocampal and cortical membrane preparations of adult animals and in whole brain preparations of 7-day-old rats. Interestingly, the oleic acid stimulation of [^{3}H]-muscimol binding was much smaller in the cerebellum compared to the cortex and hippocampus (Table 1). Ricinelaidic acid alone slightly stimulated [^{3}H]muscimol binding in all regions studied and—in contrast to the effect on [^{3}H]diazepam binding—this effect was additive to the stimulation of [^{3}H]muscimol binding induced by oleic acid.

The mechanism is unknown by which unsaturated fatty acids enhance the agonist GABA/benzodiazepine receptor ligand binding as well as the reversal of this effect by ricinelaidic and ricinoleic acid. It is well documented that fatty acids modulate membrane structure and the function of membrane-bound enzymes and receptors (see e.g. Ref. 11). It is tempting to speculate that certain fatty acids (oleic acid and arachidonic acid) modulate the lipid microenvironment of the GABA/benzodiazepine receptor complex in the membrane leading to a higher affinity of benzodiazepine agonists for binding to their receptors. It seems unlikely that this effect is due to general membrane effects such as change in viscosity, since: (a) reversibility of the oleic acid-induced enhancement by hydroxy fatty acids only for the benzodiazepine site; (b) differential sensitivity of benzodiazepine receptor subtypes to ricinelaidic acid as shown by the regional and ontogenetic differences; (c) structural specificity of the hydroxy fatty acids that are able to abolish the effect of oleic acid on the benzodiazepine binding site.

The physiological relevance of the possible *in vitro* effects that the hydroxy fatty acids might have is unknown. We have found no reports on the presence of endogenous ricinelaidic or ricinoleic acid in the brain or other tissue. However, a large number of hydroxy derivatives of arachidonic acid have been found to be present in brain tissue [12].

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