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SHORT COMMUNICATIONS

Presence and biosynthesis of phenylacetic acid in the rabbit brain

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The growing body of evidence suggesting a role for phenylethylamine (PEA) in neuropsychiatric disorders [1–5] has raised interest in clinical and neurochemical studies of its major metabolite, phenylacetic acid (PAAc) [4]. This substance, which is excreted in human urine in rather large quantities, mostly as phenylacetylglutamine (PGA) [6], was first shown to be an endogenous neuroacid in rabbit brain [7, 8] and human cerebrospinal fluid (CSF) [9].

Brain PAAc is mostly an *in situ* product of L-phenylalanine (PhAl) metabolism, formed both via phenylpyruvic (PPY) acid [10] and in a biochemical pathway involving PhAl decarboxylation to PEA which is then converted by monoamine oxidase (MAO) to phenylacetaldehyde with further oxidation of this substance to PAAc [11]. Some PAAc is also formed in brain from peripherally borne PEA, a substance that readily crosses the blood–brain barrier [4]. This study is an attempt to quantitate the contribution of these different pathways to brain PAAc levels, thus opening the possibility of using this acid as an index of brain PEA activity.

Materials and methods

Radioactive phenylacetic acid ([1-^{14}C]PAAc, sp. act. 0.12 mCi/mmole, and [^{3}H-(G)]PAAc, sp. act. 18.5 Ci/mmole) was obtained from ICN, Irvine, Ca, and CEA, France, respectively. Its purity, checked by thin-layer chromatography (TLC) followed by radioscanning, was found to be over 95% pure.

White male New Zealand rabbits (2.0 to 2.5 kg) were decapitated and the brains (~8 g each) were removed immediately. After separation of the main surface blood vessels and blood clots, the tissue was rapidly rinsed in chilled saline (pH 9.0, NaOH), blotted dry, weighed, and placed in an ice-cold saline solution (pH 9.0, 1:3, w/v) containing [^{3}H-(G)]PAAc (as internal standard for recovery; ~10,000 dpm/g brain tissue) and pargyline (2×10^{-5} M final concn). This mixture was homogenized and centrifuged (10,000 *g*; 15 min), and the pellet, after being suspended in fresh saline (pH 9.0, 50 ml), was centrifuged again. The pH of the pooled supernatant fractions was brought to 2.0 (conc. HCl), and PAAc was taken up into chloroform (15 ml × 2). The acid was extracted into a pH 9.0 solution (0.1 N NaOH), followed by pH adjustment (2.0, HCl) and its subsequent extraction into fresh chloroform (10 ml × 2). The organic layers were pooled, chloroform was gently evaporated, and the residue was dissolved in 100 μl of fresh chloroform. In some experiments (six controls), an aliquot of the dried residue was mixed with 6 N HCl, transferred to a glass tube, and heated at 100° for 1 hr to hydrolyze any conjugated PAAc (PAG) present (for details see Ref. 12). PAAc was extracted into chloroform and analyzed as described below. The organic solution was placed on a Quanta/Gram cellulose TLC plate, along with a separate spot containing 5 μg [1-^{14}C]PAAc (~10,000 dpm), and run (95% ethanol: conc. amonia, 20:1) for 2 hr at room temperature. After TLC radioscanning (radioactivity present in R_f 0.25–0.32 area), the section containing the standard PAAc was sprayed and developed (for details see Refs. 7 and 13). The area containing the brain PAAc was scraped off the plate and extracted into chloroform, and this solution was gently concentrated to 40 μl. After the addition of benzoic acid [5 μl containing 200 ng; gas–liquid chromatography (GLC) reference purposes], *N*,*N*-bis-trimethylsylilacetamide (BSA; 3:1, v/v) was added to the solution and it was allowed to stand for 5 min at room temperature. Samples were analyzed in a Beckman GLC model 65 equipped with a flame ionization detector (H_2 and air; N_2 as carrier gas). Aliquots were separated for counting (5–10 μl) and for injection (5–10 μl) into a column system packed with 10% OV 17 on Diaport S (80–100 mesh) (Beckman Instrument Co.); initial column temperature was held at 100° for 5 min, followed by programming (2.5°/min) to a final temperature of 250°. PAAc concentration was estimated by comparing the average area under the peak response (5–10 μl injections; two to five determinations for each biological sample showing a variation range of 3 to 7%) with a standard curve obtained by plotting areas under the peak response as a function of known amounts of PAAc (linear in the 5–500 ng range). In each case, the identity of the peak obtained from brain samples was confirmed as the trimethylsilyl ester derivative of PAAc (TMSP) (R_t of 10.4 min) by comparing its retention time to that of the BSA derivative of benzoic acid (R_t 8.3 min), and by analyzing a sample aliquot enriched with known amounts of standard TMSP. In some samples the presence of PAAc in the TLC chloroform-concentrated extract was confirmed by mass spectrometry [MS-902 double focus spectrometer (Associated Electrical Ind. Scient. App. Ltd., Manchester, England) with a solid probe attachment]. Brain PAAc levels reported represent the average of at least three experiments (sensitivity limit of about 3 ng) and are corrected for the percentage of derivatized [^{3}H(G)]PAAc that was recovered (range 36–68%).

Results

Table 1 shows that the levels of free rabbit brain PAAc were in a range of concentrations similar to those of other neuroacids formed from the metabolism of *p*-tyramine and the catecholamines [14]. Total rabbit brain PAAc levels, obtained after hydrolysis of any PAG present, did not differ significantly from values obtained for the free acid itself (Table 1). PAAc levels were increased significantly by the i.p. administration of its precursors, PhAl and PEA (Fig. 1). As would be expected, previous treatment of the animals with either the MAO inhibitor pargyline or probenecid, a substance known to block PAAc transport mechanisms [15], significantly decreased or increased, respectively, brain PAAc levels. Injection of the type B (deprenyl) or type A and B (pargyline) MAO inhibitors resulted in lower than control PAAc brain levels, whereas they remained unchanged after the administration of harmaline, a type A MAO inibitor. Probenecid, given either to untreated animals or in conjunction with the aromatic L-aminoacid decarboxylase inhibitor α-methyldopa, amphetamine or MAO inhibitors resulted in higher than control brain PAAc concentrations. α-Methyldopa decreased and *d*-amphetamine, 13.5 mg/kg injected 90 min prior to sacrifice, increased brain PAAc levels. Similar doses of *d*-amphetamine given 24 hr prior to sacrifice did not modify significantly the brain concentration of PAAc.

Table 1. Phenylacetic acid levels and biosynthetic pathways in rabbit brain*

Pretreatment (drug†, dose, and time prior to sacrifice)	Phenylacetic acid (ng/g wet tissue)		
	Saline	Pargyline‡	Probenecid§
Saline	24 ± 1.6	17 ± 1.5‖	78 ± 7.6‖
Pargyline, 15 mg/kg, 24 and 2 hr	17 ± 1.5‖		48 ± 3.9‖
Deprenyl, 0.5 mg/kg, 24 and 2 hr	15 ± 1.5‖		43 ± 3.4‖
Harmaline, 5 mg/kg, 24 and 2 hr	22 ± 1.5¶		68 ± 4.3‖
Phenylethylamine, 100 mg/kg, 5 min	2700 ± 87.2‖	37 ± 3.1‖	4328 ± 163.9‖
Phenylethylamine, 50 mg/kg, 5 min	2610 ± 144.2‖	43 ± 6.3‖	3984 ± 125.1‖
Phenylethylamine, 25 mg/kg, 5 min	2300 ± 132.0‖	36 ± 6.4‖	3789 ± 129.1‖
Phenylethylamine, 5 mg/kg, 5 min	320 ± 32.2‖	31 ± 3.8‖	976 ± 51.4‖
L-Phenylalanine, 100 mg/kg, 10 min	104 ± 9.1‖	74 ± 8.1‖	485 ± 12.1‖
α-Methyldopa, 200 mg/kg, 24 and 4 hr	16 ± 1.5‖	18 ± 1.5‖	51 ± 2.5‖
α-Methyldopa hydrazine, 200 mg/kg, 24 and 4 hr	20 ± 1.8¶	16 ± 2.1‖	64 ± 11.8‖
Probenecid, 200 mg/kg, 90 min	78 ± 7.6‖	48 ± 3.9‖	
d-Amphetamine · SO_4, 13.5 mg/kg, 24 hrs	29 ± 3.1¶	22 ± 2.5¶	87 ± 4.6‖
d-Amphetamine · SO_4, 13.5 mg/kg, 90 min	41 ± 4.0‖	25 ± 1.4¶	131 ± 6.4‖

* Values represent mean ± S.E.M. of at least three experiments; control rabbit brain saline–saline PAAc was obtained from seventeen different animals (seventeen determinations).

† Drugs were dissolved and injected in saline (i.p.).

‡ Dosage: 15 mg/kg, 24 and 2 hr prior to sacrifice.

§ Dosage: 200 mg/kg, 90 min prior to sacrifice.

‖ $P < 0.01$, compared to saline–saline control (Student's *t*-test).

¶ $P > 0.05$ (NS).

Discussion

Clinical and basic studies are increasingly supporting a role for the noncatecholic phenylethylamines in the pathophysiology of a number of neuropsychiatric diseases [4, 16]. In this respect, PEA has been of particular interest as its chemical structure and pharmacological and behavioral properties closely resemble those of amphetamine [5]. Attempts to use tissue, plasma, CSF or urinary PEA levels as a biological marker of disease states have been difficult and controversial as there are still technical problems in the development of relatively simple and reproducible methodologies for PEA determination in biological samples [4]. The use of urinary levels of PAAc, the main metabolite of PEA, has been suggested as an index of PEA neuroactivity [17]. This proposition, however, has to be viewed with great caution as only a small proportion of urinary PAAc is the result of brain PEA metabolism [6]. Most of the acid arises from amine degradation in peripheral tissues, other metabolic routes, and dietary sources [4, 6, 10]. Recent suggestions of the use of brain and CSF PAAc levels as an indicator of brain PEA activity appear more reasonable [18]. This acid is formed *in situ* in brain (PAAc crosses the blood–brain barrier only with great difficulty) [15], both from peripherally originating PEA and from PhAl [4, 11]. This aminoacid yields PAAc via transamination or oxidative deamination to PPY followed by decarboxylation and further oxidation to PAAc [7, 10] (Fig. 1), and by decarboxylation to PEA followed by conversion by MAO to phenylacetaldehyde and subsequent oxidation to PAAc [7, 1] (Fig. 1). Only a very small portion of brain PAAc appears to derive from tyrosine and dopamine, via pathways involving their respective conversion to *p*-tyramine [19] followed by *p*-dehydroxylation of this amine to PEA [20]. Although PEA is converted in brain to several substances [4, 5, 11], under physiological conditions PAAc mostly as the free acid [this paper and work in progress (rabbits); F. Karoum, personal communication, as deduced from preliminary work on human brain, Ref. 12 for methodological details] accounts for more than 95% of the brain metabolites of this amine [21].

Results obtained after the i.p. injection of PhAl (to naive or pargyline-treated animals) indicate that a significant portion of brain PAAc is derived from this aminoacid via the PhAl → PEA → PAAc metabolic pathway. This is corroborated by the α-methyldopa inhibition of PhAl decarboxylation to PEA, which results in a significant decrease of brain PAAc levels to values similar to those reached in animals pretreated with the MAO inhibitors deprenyl or pargyline. As expected, harmaline did not modify significantly the brain PAAc content. Although pretreatment with α-methyldopa hydrazine failed to produce a statistically significant decrease in brain PAAc levels, the smaller values obtained could be viewed as a measure of the relative contribution of peripherally borne PEA to the total brain PAAc concentration. Further corroboration of these results was obtained after the i.p. administration of this amine. PEA (5, 25, 50 or 100 mg/kg) resulted in dramatically increased brain PAAc levels to

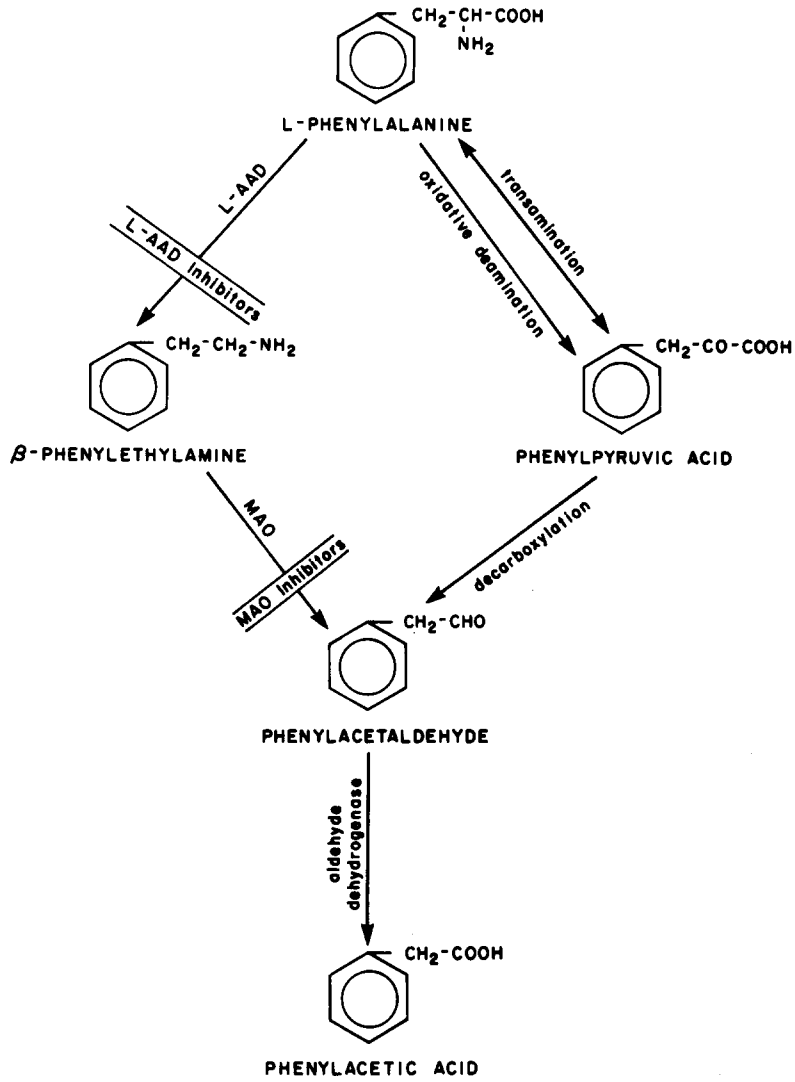

Fig. 1. Main pathways for the *in vivo* biosynthesis of phenylacetic acid in the rabbit brain. L-AAD = L-aminoacid decarboxylase.

values expected from its ability to readily cross the blood–brain barrier and the kinetics of rabbit brain MAO [22] [5 min after its i.p. injection to mice, 0.16 to 160 mg/kg (containing labeled amine), about 0.5 to 1.2% of PEA is in the brain; S. Huprikar and A. D. Mosnaim, unpublished results]. However, supporting the proposed PAAc biosynthetic pathway, pretreatment with pargyline resulted in PAAc brain levels only slightly higher than those of control animals.

The increase in brain PAAc levels 90 min after the injection of *d*-amphetamine (an effect which disappeared at 24 hr) was expected, as under this condition there is some evidence that this drug increases brain PEA levels [4], possibly by competitive inhibition of its conversion to *p*-tyramine [23]. Comparison of the brain PAAc levels or probenecid-treated animals with that of control animals supports previous reports that this drug inhibits PAAc transport mechanism [15]. It also illustrates the rate of brain PAAc formation. As expected, probenecid pretreatment dramatically increases the already high PAAc levels obtained after the injection of PhAl, *d*-amphetamine or PEA. Values obtained for the animals treated with 25, 50 and 100 mg/kg PEA appear to be lower than expected, indicating perhaps that at such high brain PAAc concentrations some acid leaves the brain by a passive diffusion mechanism.

Changes in the levels of free rabbit brain PAAc after the administration of either α-methyldopa or MAO inhibitors (pargyline, deprenyl) to controls or probenecid-pretreated animals (Table 1) provide an indication of both the relative contribution of the PhAl → PPY → PAAc pathway to free brain PAAc concentration and the rate of brain PAAc formation via this metabolic route. Results obtained after the administration of α-methyldopa hydrazine, which eliminates most peripherally borne PEA from brain [4], and of probenecid could be viewed as an approximation to the combined *in situ* brain turnover rate of both the PhAl → PPY → PAAc and the PhAl → PEA → PAAc pathways.

Interpretation of the results described in this paper, suggesting that brain PAAc levels could be used as an indication of brain PEA metabolism, are based on results showing that under physiological conditions (control animals) brain PAAc exists mostly as the free acid. Further work is needed, however, to ascertain the possible effects of the different pharmacological manipulations described in Table 1 on the formation and levels of conjugated PAAc in rabbit brain.

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*Departments of *Pharmacology and ‡Psychiatry University of Health Sciences/The Chicago Medical School, and §Psychiatry Service North Chicago Veterans Administration Medical Center North Chicago, IL 60064, U.S.A.*

ARON D. MOSNAIM*†
MARION E. WOLF‡§
UZOMA P. MADUBUIKE*

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* Send correspondence to: Aron D. Mosnaim, PH.D., Department of Pharmacology, UHS/The Chicago Medical School, 3333 Green Bay Road, North Chicago, IL 60064.

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Effect of cimetidine on paracetamol activation in mice

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Cimetidine is now known to inhibit the cytochrome P-450 mediated metabolism of a range of drugs, both in animals and in man [1]. Since paracetamol toxicity results from the metabolic conversion of the drug to a reactive, arylating intermediate by cytochrome P-450, it is possible that administration of cimeditine may result in a reduction in the hepatotoxicity associated with paracetamol overdose. A number of reports [2–5] have recently demonstrated a protective effect of cimetidine against paracetamol toxicity in animals and inhibition of the oxidative metabolism of paracetamol was implicated as the mechanism. However, it has recently been shown that one week pretreatment with cimetidine (1 g/day) [6] or co-administration of cimetidine (2 g) [7] did not alter the metabolism of a therapeutic dose of paracetamol in healthy human volunteers. Thus, the effect of larger doses of cimetidine on the metabolism of a potentially hepatotoxic dose of paracetamol has been investigated in the C3H mouse, which we have previously shown [8] to be a good model for paracetamol metabolism studies.

Materials and methods

Paracetamol was obtained from the Sigma Chemical Co. (St. Louis, MO), cimetidine from Smith, Kline and French (Sydney, Australia) and piperonyl butoxide from Chemical Dynamics Corp (South Plainfield, N.J.). Experiments were carried out using male C3H mice (23–27 g). Food and water were allowed *ad libitum*. Paracetamol, 200 mg/kg, (30 ml/kg in 0.9% saline) was administered by gavage. Cimetidine, either 50 mg/kg or 100 mg/kg (0.3 ml/kg in 0.9% saline), was administered intraperitoneally (i.p.) 30 min prior to and 1 hr after the paracetamol dose. Piperonyl butoxide (1 g/kg, i.p.) was administered as a single dose 0.5 hr before the paracetamol. The control group of animals was administered a similar volume of saline i.p. Groups of 6 mice received each treatment and animals were placed in individual metabolic cages for the collection of urine for 24 hr after the paracetamol dose. Urine was analysed for unchanged paracetamol and its glucuronide, sulphate, cysteine and mercapturic acid conjugates by high performance