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Effect of aromatic ring–containing drugs on carnitine biosynthesis in rats with special regard to *p*-aminomethylbenzoic acid

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Abstract

Secondary carnitine deficiencies are associated with metabolic disorders or may be the consequence of the side effects of some drugs. The mechanisms may be either a facilitated urinary excretion or an inhibited biosynthesis. Based on our earlier findings with drugs and benzoic acid analogue metabolites, in the present study, we studied the possible inhibitory effect of some benzoic acid analogue drugs. In the pathway of carnitine biosynthesis, we tested the last step, the hydroxylation of γ-butyrobetaine (Bu) to carnitine in the liver. (Liver is the only organ in rats where this step takes place.) Of the 5 tested compounds, the *p*-aminomethylbenzoic acid (PAMBA) was found to be inhibitory. In tracer experiments with radioactive Bu, PAMBA (a single injection of 1.2 mmol/kg) reduced the conversion of [Me-^{3}H]Bu to [Me-^{3}H]carnitine from 62.6% ± 5.11% to 46.8% ± 5.02% (means ± SEM, $P < .02$). This single dose also markedly reduced the conversion of loading amount of exogenous unlabeled Bu, as measured by enzymatic analysis of carnitine. The conversion of endogenous Bu was also hampered by long-term administration of PAMBA, as indicated by increased Bu and decreased carnitine levels. Furthermore, single injection of PAMBA markedly reduced the Glu level in the liver from 2.87 ± 0.17 to 1.42 ± 0.11 μmol/g ($P < .001$). Trying to get closer to a mechanism by which the flux through the Bu hydroxylase was depressed, we supposed that alfa-ketoglutarate (α-KG), an obligatory cofactor of the enzyme, was also be depressed. It was expected because α-KG is a reversible copartner of L-glutamate through the Glu-dehydrogenase reaction. We found that PAMBA reduced the α-KG level from 207 ± 17.5 to 180 ± 19.1 nmol/g (means ± SEM, $P < .02$). Considering the conditions of the enzyme in vitro and in vivo, this decrease may contribute to the decreased in vivo flux through the butyrobetaine hydroxylase enzyme.

1. Introduction

L-Carnitine (3-OH-4-*N*-trimethylaminobutyrate), by transporting long-chain fatty acids through the inner mitochondrial membrane, is an obligatory factor for the mitochondrial β-oxidation of fatty acids [1]. All mammals are able to synthesize carnitine in a well-established metabolic pathway. Human carnitine deficiencies are classified as primary [2] and secondary according to their origin. The former is caused by a mutation of the sodium-dependent carnitine transporter, as it has been recently clarified [3,4]. A part of the latter class is associated with metabolic disorders, for example, in organic acidurias, whereas the other part is artificial, mostly caused by pharmacological treatments [5].

Dealing with drugs affecting carnitine levels and biosynthesis, we reported earlier that antidiabetic biguanides (such as buformin and metformin) enormously increased the carnitine content of rat liver [6]. By promoting fatty acid oxidation and ketone body formation, the increased carnitine level may contribute to some beneficial effects of biguanides, for example, prevention of hepatic steatosis. In contrary to biguanides, valproic acid (dipropyl-acetate, a commonly used anticonvulsive drug) inhibited carnitine biosynthesis in rats [7]. In the latter study, we observed that benzoic acid was also inhibitory. This observation led us to study benzoic acid analogue compounds in this respect. Testing some compounds accumulating during phenylketonuria (PKU), we found phenylacetic acid to be inhibitory [8]. In that study, we identified phenacetyl-carnitine, a novel compound in the rat liver and in the urine of patients with PKU. Interestingly, the effect of benzoic acid therapy on carnitine metabolism and the existence of benzoil-carnitine in humans were also reported earlier [9]. Extending the

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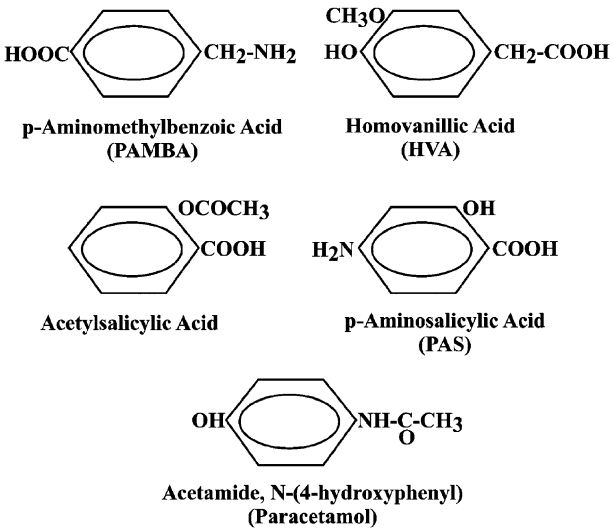

Fig. 1. Chemical structure of the benzoic acid analogue drugs involved in the investigation.

above conception, in the present study, we tested commonly used drugs analogue to benzoic acid, listed with structures in Fig. 1. We targeted the last step in the biosynthetic pathway of carnitine, in which γ-butyrobetaine (Bu, 4-*N*-trimethylaminobutyrate) undergoes a stereospecific hydroxylation by the Bu hydroxylase (EC 1.14.11.1.) enzyme, producing L-carnitine. This enzyme is located exclusively in the liver in rats, whereas it is located in the liver and kidney in man, rendering these organs the carnitine supplier for the whole organism [10].

2. Materials and methods

2.1. Materials

4-(Dimethylamino)butyric acid (nor-butyrobetaine), (3-carboxypropyl)trimethylammonium chloride (Bu), coenzyme A (CoA), heptanesulfonic acid, and other fine chemicals and enzymes were purchased from Sigma-Aldrich (Budapest, Hungary). The generics of the tested drugs, such as *p*-aminomethylbenzoic acid (PAMBA), homovanillic acid, acetylsalicylic acid, *p*-aminosalicylic acid, and paracetamol were also from Sigma-Aldrich. [^{3}H]Methyl iodide was from Amersham (Little Chalfont, UK). L-Carnitine was a kind gift from Sigma-Tau (Rome, Italy). Acetyl-CoA was prepared from acetic anhydride and CoA as described [11]. [Me-^{3}H]Bu with approximately 10^6 cpm/nmol specific activity was synthesized from nor-butyrobetaine and [^{3}H]methyl iodide as described [12].

2.2. Animals and treatments

Male Wistar rats weighing 200 to 250 g were used. The experiments were performed between 9:00 and 12:00 AM. The conversion rate of Bu to carnitine was measured either by using tracer amount of radioactive Bu or by giving loading amount of unlabeled Bu. Animals were given 1.2 mmol/kg of the putative of carnitine synthesis inhibitor intraperitoneally in 1.0 mL of 0.9% NaCl solution, whereas controls were injected with saline. (This dose falls in the range used in the experiments with rodents [13].) After 30 minutes, animals in the tracer experiment were injected with approximately 1.5×10^7 cpm [Me-^{3}H]Bu, whereas the animals in the loading experiment were given 200 μmol of unlabeled Bu. Thirty minutes later (by the 60th minute after the administration of the putative inhibitor), all animals were killed by decapitation, blood was collected into heparinized tubes, and the livers were rapidly frozen and stored in liquid nitrogen until analyses. In the tracer experiment, the liver samples were analyzed for isotope distribution between Bu and carnitine. (Because of the high specific activity of the radioactive Bu preparation, the Bu loading was negligible.) At the end of experiment (by the 30th minute after the administration of [Me-^{3}H]Bu), 30% to 35% of the injected radioactivity ([4.5-5.0] $\times$ 10^6 cpm) was recovered in the whole liver. Usually, in a control animal, 60% to 70% of total activity was found in carnitine.

2.3. Analyses

After alkaline hydrolysis of carnitine esters, total carnitine was determined by radioisotope method [14] using small columns [15]. Alfa-ketoglutarate (α-KG) [16] and L-glutamate [17] were determined by standard enzymatic analyses. The Bu in liver was determined as previously described [18]. Distribution of radioactivity between carnitine and Bu was evaluated by reversed-phase, ion-pairing, high-performance liquid chromatography (HPLC), basically as previously described [18]. In this case, all carnitine esters were hydrolyzed during the sample processing. The computerized HPLC system (TOSOH, TSK-6011, TOSOH Bioscience, Supracont, Ltd, Budapest, Hungary) consisted of a low-pressure gradient pump, Supelcosil LC-18 column with 5 μm particle size (Supelco Sigma-Aldrich Co, Budapest, Hungary), and a TSK 6040 UV/VIZ detector. The separation of Bu from carnitine was performed by isocratic elution, using 0.01 mol/L Na-heptanesulfonate adjusted to pH 2.5 with phosphoric acid and containing 7% methanol as eluent. The flow rate was 1.0 mL/min, the elution was monitored at 210 nm, and 1.0-mL fractions were collected into scintillation vials.

2.4. Statistical analysis

The values are presented as means $\pm$ SEM and were subjected to a Student paired *t* test.

3. Results and discussion

3.1. Selecting the inhibitory drug, PAMBA, by radioactive tracer experiment

To screen the putative inhibitory drugs, the tracer method was used, that is, measuring the conversion of [Me-^{3}H]Bu to [Me-^{3}H]carnitine. The structures of drugs

tested are shown in Fig. 1. These are as follows: the PAMBA, a hemostatic agent; acetylsalicylic acid (aspirin) with anti-inflammatory and analgesic effects; *p*-aminosalicylic acid, an antitubercular agent; and the antipyretic paracetamol. The homovanillic acid is the final metabolite of dihydroxyphenylalanine, commonly used for the therapy for Parkinson disease [19]. Homovanillic acid derives from dihydroxyphenylalanine and dopamine by the combined action of monoamine oxidase and catechol-*O*-methyltransferase.

Of the 5 compounds, it was PAMBA that significantly inhibited the conversion rate by reducing the ratio of [Me-^{3}H]carnitine from 62.6% to 46.8% (Fig. 2). Therefore, in the further investigations in this study, PAMBA was selected to obtain more evidence for its inhibitory action and to elucidate the mechanism.

3.2. Inhibitory effect of PAMBA on the conversion of unlabeled Bu

To confirm the above effect of PAMBA, we measured its effect on the Bu conversion when molecular loading amount of unlabeled Bu was applied; thus, the formed carnitine could be measured by enzymatic analysis. A single dose of 200 μmol Bu in 30 minutes increased the carnitine level from 216 ± 18.1 nmol/g (control group) to 469 ± 32.2 nmol/g (control + Bu group) in the liver and from 35.8 ± 2.22 to 94.1 ± 11.2 nmol/mL in the serum, respectively (Fig. 3). When PAMBA was added 30 minutes before Bu administration (Bu + PAMBA group), the carnitine level in the liver increased only to 329 ± 31.4 vs 496 ± 32.2 nmol/g ($P < .005$). In the serum, the corresponding levels were 58.7 ± 4.71 vs 94.1 nmol/mL ($P < .005$).

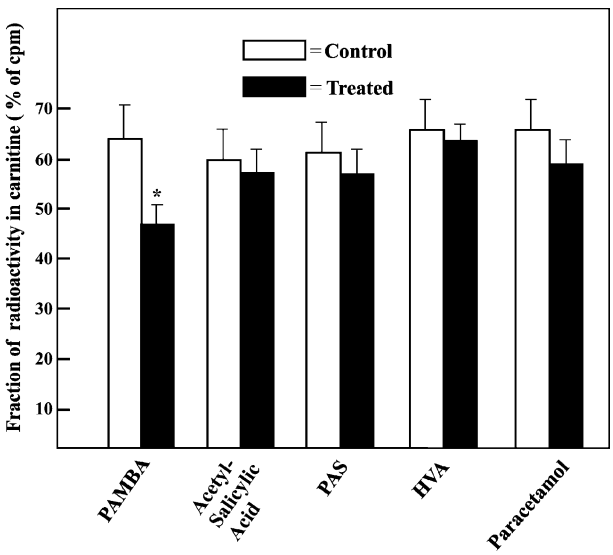

Fig. 2. Effect of benzoic acid analogue drugs on the conversion of [Me-^{3}H]Bu to [Me-^{3}H]carnitine in the liver of rats. The animals were given 1.2 mmol/kg of the indicated drugs 60 minutes and 1.5 × 10^7 cpm tracer [Me-^{3}H]Bu 30 minutes before killing them. The samples were processed for the separation of [Me-^{3}H]Bu from [Me-^{3}H]carnitine by HPLC. A 100-μL HPLC solvent A containing approximately 1 × 10^5 cpm radioactivity was injected to the HPLC equipment. Of the injected radioactivity, 95% was recovered under the peaks of carnitine and Bu. Bars indicate percentage of total radioactivity in carnitine (means ± SEM) for 5 animals. *$P < .01$.

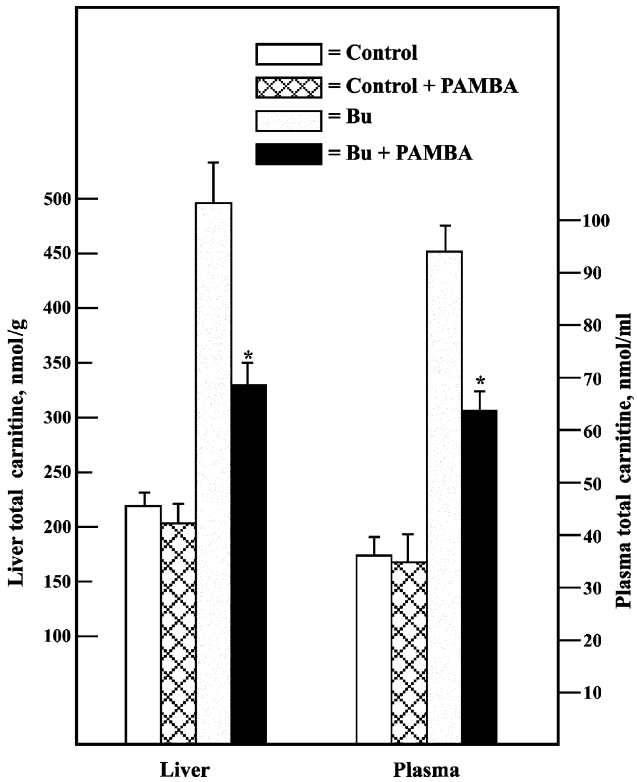

Fig. 3. Effect of PAMBA on the conversion of loading amount of unlabeled γ-butyrobetaine to carnitine. The animals were given either 1.0 mL 0.9% saline (control and Bu groups) or 180 mg/kg PAMBA (control + PAMBA and Bu + PAMBA groups). Thirty minutes later, the control and control + PAMBA groups were given saline again, and the Bu and Bu + PAMBA groups were given 200 mmol unlabeled Bu. By the 60th minute after the application of PAMBA and by the 30th minute after the addition of Bu, the experiment was terminated. Liver and plasma samples were subjected to enzymatic analysis for total carnitine. Bars indicate nanomoles per gram of carnitine in liver and nanomoles per milliliter in plasma (means ± SEM) for 6 or 5 animals. *$P < .005$ when compared with the group given Bu without PAMBA.

As Fig. 3 also shows, the short-term administration of 180 mg/kg PAMBA did not decrease significantly the endogenous carnitine levels (control + PAMBA group). To reveal if PAMBA can affect the endogenous Bu and carnitine levels, half-dose (90 mg/kg) was used for 4 consecutive days (Table 1). As it is seen, a slight but significant increase of Bu

Table 1
Effect of long-term administration of PAMBA on carnitine and Bu content of rat liver

Animals (n)	Bu (nmol/g tissue)	Total carnitine (nmol/g tissue)
Control (5)	4.6 ± 0.51	211 ± 17.2
Treated (6)	7.8 ± 0.91*	183 ± 15.2**

For 4 consecutive days, the animals were given 90 mg (60 mmol)/kg IP PAMBA.

* $P < .05$.

** $P < .01$.

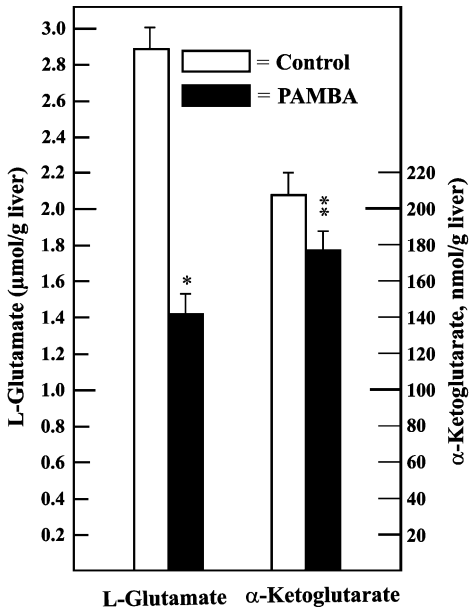

Fig. 4. Effect of PAMBA on the L-glutamate and α-KG levels in rat liver. The control and treated animals were given saline or 180 mg/kg PAMBA, respectively, 60 minutes before killing them. Samples were subjected to enzymatic analyses for L-glutamate and α-KG. Bars indicate means ± SEM for 5 animals. *$P < .001$; **$P < .02$.

was accompanied by a significantly reduced carnitine level. This effect strongly suggests a hampered flux through the Bu hydroxylase. The same trend was observed earlier with valproate [7].

3.3. Elucidating the mechanism of action of inhibitory effect of PAMBA

The assay used here to estimate the rate of carnitine biosynthesis measured the flux through Bu hydroxylase, the last enzyme in the pathway, immediately forming carnitine. Considering the mechanism of inhibitory effect of PAMBA, some possibilities can be raised. First, PAMBA might have inhibited directly the enzyme. A study [20] found direct inhibition with succinate semialdehyde and with 3-trimethylaminopropyl-1-sulfonate, where the latter is a structural analogue to Bu. PAMBA with its aromatic ring, however, did not inhibit Bu hydroxylase under the condition of enzyme activity measurement in Reference [20]. Earlier, we observed that valproic acid did not inhibit directly the Bu hydroxylase enzyme either [7]. Another possibility is if PAMBA had decreased the uptake of Bu by inhibiting its transport. However, in the tracer experiments with [Me-^{3}H]Bu (Fig. 2), (4.8 to 5.0) × 10^6 cpm was found in the livers both in the control and in the treated animals. In addition, on the effect of long-term PAMPA treatment (Table 1), the Bu level was even increased in the liver, excluding the possibility of inhibition of Bu uptake by PAMBA.

Searching further for a mechanism of inhibition observed in vivo, we supposed that an inhibitory agent, such as PAMBA, might exert its action by affecting the levels of some cofactors necessary for the enzyme. The Bu hydroxylase uses ascorbic acid, Fe^{2+}, and α-KG as cofactors [20,21]. This mechanism was also suggested by our earlier data showing that valproic acid [7] and phenylacetic acid [8], which although inhibited the hydroxylation of Bu, decreased markedly the Glu and, to a lesser degree, the α-KG level. It seems obvious because the 2 compounds are partners in the transaminase and Glu-dehydrogenase reactions, thus reflecting each other's level. In the present study (Fig. 4), PAMBA, under the given conditions, markedly reduced the L-glutamate level from 2.87 ± 0.17 to 1.42 ± 0.11 μmol/g ($P < .001$) in the liver. As expected, α-KG level also decreased, namely, from 207 ± 17.2 to 180 ± 14 2 nmol/g ($P > .02$). The change of α-KG level corresponds to 276 vs 240 μmol/L concentration. To judge if the presented decrease of α-KG is enough to hamper the flux through the Bu hydroxylase, we have to take into account K_m value of α-KG. There is controversy about this value in the literature. The first article [20] reported 0.5 mmol/L K_m for α-KG (using 40 mmol/L K^+ ion concentration), whereas a recent one [22] calculated 0.05 mmol/L (using 100 mmol/L K^+ ion). The latter authors attributed the difference to the K^+ ion concentration. However, the question arises, how can this change in potassium cause 1 order of magnitude decrease in K_m value of α-KG? Doubt is raised by the data provided by the authors, which show that the enzyme activity at 40 mmol/L K^+ reached the maximum at 100 mmol/L [22]. It should also be considered that a lesser part of total cellular α-KG is located in the cytosol, at the location of the Bu hydroxylase enzyme. In our measurement of 234 nmol/g, total cellular α-KG of 86.7 nmol was located in the cytosol (data not shown), suggesting that a decrease in total cellular α-KG results is more pronounced in the cytosol. After all, we propose that the lowered α-KG level may be the mechanism by which PAMBA hampers the flux through the Bu hydroxlase. In this context, it is noteworthy that in a related work by us, we found that exogenously added α-KG could partly protect the Bu-carnitine conversion from the inhibitory effect of valproic acid [7].

In the pathway of carnitine biosynthesis there is another hydroxylation step, converting 6-*N*-trimethyllysine to 3-OH-6-*N*-trimethyllysine [23]. Not surprisingly, trimethyllysine hydroxylase needs the same cofactors (eg, α-KG) as Bu hydroxylase. Therefore, an inhibitor of Bu hydroxylase, such as PAMBA, probably affects the other hydroxylase too.

Our earlier finding that valproic acid inhibited carnitine biosynthesis [7] was extended to clinical relevance. Namely, children subjected to long-term valproic acid therapy had lower serum carnitine level, and carnitine therapy was applied to supplement carnitine [24]. In patients with PKU that have extremely high phenylacetic acid level (the metabolite shown to be inhibitory for carnitine biosynthesis), we found lower serum carnitine levels. Searches for drugs affecting carnitine levels and for the mechanisms and

their clinical relevance, including the effect of PAMBA, are ongoing.

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