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RENAL UPTAKE OF *p*-AMINOHIPPURIC ACID *IN VITRO*

EFFECTS OF PALMITATE AND L-CARNITINE

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SUMMARY

1. The uptake of *p*-aminohippuric acid by rat renal cortical slices and its relationship to the renal uptake of non-esterified fatty acids was studied.

2. 1 mM palmitate competitively inhibits renal *p*-aminohippuric acid uptake.

3. 1 mM carnitine stimulates renal *p*-aminohippuric acid uptake at low palmitate concentration but has no effect when 1 mM palmitate is present in the incubation media.

4. It is suggested that the renal organic acid transport system participates in the uptake of palmitate by renal cells. Furthermore, palmitate and *p*-aminohippuric acid may share common intracellular binding sites in renal cortical cells.

5. Factors affecting the affinity of intracellular binding sites, may regulate uptake of *p*-aminohippuric acid and uptake and metabolism of non-esterified fatty acids in renal cortical cells.

INTRODUCTION

Experiments in the dog *in vivo*[1] indicate that certain dicarboxylic acids (α-ketoglutarate) gain access to renal tubular cells through the organic acid renal transport system. However, the circulating blood level of α-ketoglutarate is small under most experimental circumstances and thus, the overall contribution of this substrate to the renal energy production (as judged by the renal Q_{O_2}) is quantitatively of minor significance. In contrast, *in vivo*[2] and *in vitro*[3] studies of renal substrate uptake have shown that non-esterified fatty acids are major renal substrates. In addition, our previous experiments in the dog *in vivo*[4], have suggested that substrates of the organic acid transport system (probenecid, chlorothiazide) inhibit the renal uptake of non-esterified fatty acids. Also, CROSS AND TAGGART[5] had previously shown that medium chain fatty acids (octanoate) inhibit the uptake of *p*-aminohippuric acid by rabbit kidney cortical slices.

It is the purpose of this work, to establish in kinetic terms the relationship between the renal organic acid (*i.e. p*-aminohippuric acid) transport system and the renal uptake of a major renal substrate, palmitate. It also attempts to determine the mechanism of the interaction between the renal *p*-aminohippuric acid and palmitate uptake systems.

METHODS

15 experiments were performed in each of which 3–5 white rats of either sex, weighing 200–250 g each were used. After ether anesthesia, both kidneys were excised through a midline abdominal incision and decapsulated. The kidneys were separately weighed and placed in ice-cold Krebs–Ringer bicarbonate under CO_2–O_2 (6.2:93.8, by vol.) (P_b = 683 mm Hg). The kidneys were sliced perpendicularly to their pole to pole axis, obtaining 6 chunks of tissue per kidney. The two chunks at the poles were discarded. With the remaining four chunks, the cortex was separated from the medulla along the demarcation line corresponding to the arcuate vessels. The medullary portions were discarded, and slices of cortical tissue 0.3–0.5 mm thick, were obtained with a Steady Riggs microtome. The outermost slices (*cortex cortices*) were discarded. The cortical slices were preincubated for 60–120 min in Krebs–Ringer bicarbonate under CO_2–O_2 (6.2:93.8, by vol.) at 37° until taken for final incubation. Slices weighing 50–100 mg (wet) were incubated in 25 ml erlenmeyer flasks containing 2 ml of either Krebs–Ringer bicarbonate with 2.5 % defatted[6] albumin and varying concentrations (0.05–2 mM) of *p*-aminohippuric acid or in Krebs–Ringer bicarbonate with 2.5 % albumin complexed[7] to 1 mM palmitate and varying concentrations of *p*-aminohippuric acid. Incubations were carried at 37° and lasted 1.25–60 min, depending on the experiment. Each flask was run in duplicate. Upon the end of incubation, the slices were quickly taken out of the media, carefully blotted on filter paper and weighed to the nearest 1/10 mg on a torsion balance.

The weighed slices and 1-ml aliquots of the incubation media were extracted overnight with 4 % trichloroacetic acid at room temperature (25°). Analysis of *p*-aminohippuric acid was made in duplicate aliquots of the protein free filtrates of tissue and medium samples according to the method of BRATTON AND MARSHALL[8].

Recoveries of *p*-aminohippuric acid varied between 95 and 102 % at *p*-aminohippuric acid concentrations of 0.05–2 mM. The coefficient of variation for triplicate determinations was 1.7 %. 1-ml aliquots of the incubation media were used for analysis in triplicate of the medium non-esterified fatty acid concentrations. Non-esterified fatty acid extraction was performed according to the double extraction procedure of TROUT *et al.*[9] Titration of the non-esterified fatty acid extracts was done by the manual method of KELLEY[10]. Thus, the concentration of non-esterified fatty acid in the incubation media was directly checked by titration.

In some experiments, *p*-amino[^{14}C]hippuric acid (spec. act., 1.29 μC/umole) was used. The final (2.5 min of incubation) ^{14}C content of renal slices and of the incubation media were determined in duplicate, 1-ml aliquots of neutralized protein free filtrates prepared as described above. ^{14}C counting was done in the solution of BRAY[11] with a Nuclear Chicago, Unilux II liquid scintillation counter. Counting efficiency (approx. 50 %) was exactly determined by internal standardization with [^{14}C]toluene standard.

RESULTS

Uptake of *p*-aminohippuric acid by the rat kidney cortical slice occurred against a chemical concentration gradient, establishing slice to medium concentration ratios greater than unity at all medium *p*-aminohippuric acid concentrations tested.

Time-course of *p*-aminohippuric acid accumulation was studied with 0.05 mM

p-aminohippuric acid in the incubation medium (Fig. 1). *p*-Aminohippuric acid uptake occurred linearly with time only in the initial 1–3 min of incubation. Thus, the rate of *p*-aminohippuric uptake during the first 2.5 min of incubation was taken in subsequent experiments as an estimate of the initial velocity of *p*-aminohippuric acid uptake.

The rate of *p*-aminohippuric acid uptake during the first 2.5 min of incubation varied with medium *p*-aminohippuric acid following the pattern of a saturable transport system (Fig. 2). Inhibition by 1 mM palmitic acid of rat kidney cortical *p*-aminohippuric acid uptake occurred at all medium *p*-aminohippuric acid concentrations tested. However, the inhibition was statistically significant ($P < 0.05$) only with 0.05 mM *p*-aminohippuric acid in the incubation media. The significance of the inhibitory effect of palmitate on the initial velocity of *p*-aminohippuric acid uptake at varying *p*-aminohippuric acid concentrations in the incubation media was also tested with the variance ratio (F) test[19]. The difference between the two regression populations was significant at $P < 0.01$ (Fig. 2).

The inhibition of renal *p*-aminohippuric acid uptake *in vitro* by palmitate was characterized by an increase in the apparent "K_m" of the renal *p*-aminohippuric acid transport system with no significant change in the maximal velocity of *p*-aminohip-

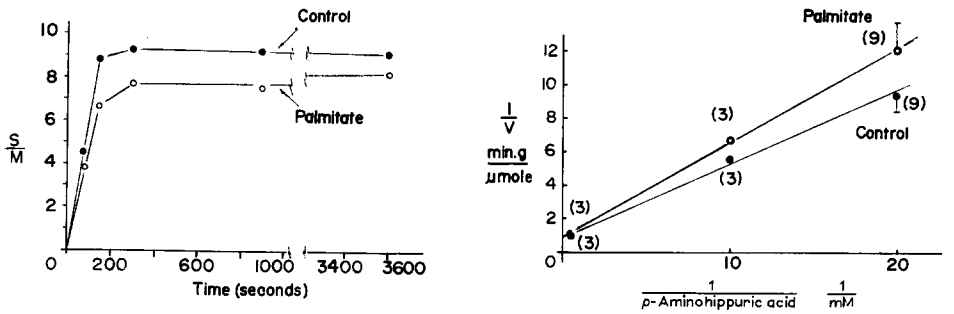

Fig. 1. Time-course of *p*-aminohippuric acid uptake by rat renal cortical slices in the presence and absence of palmitate. Uptake of *p*-aminohippuric acid by slices (50–100 mg) was measured after 75, 150, 300, 900 and 3600 sec of incubation in 2 ml Krebs–Ringer bicarbonate containing 0.05 mM *p*-aminohippuric acid, at 37° and a P_{CO_2} of 40 mm Hg. Control media (●) had 2.5% defatted albumin. Experimental media (○) had 2.5% albumin complexed to 1 mM palmitate. The ratio of the *p*-aminohippuric acid content of the slices (μmoles/g, wet) to the *p*-aminohippuric acid concentration in the incubation media (μmoles/ml) at the end of incubation (S/M) is plotted against time of incubation (sec). Each observation is the mean of duplicate flasks.

Fig. 2. Effect of palmitate on the initial velocity of *p*-aminohippuric acid uptake by rat renal cortical slices with varying concentrations of *p*-aminohippuric acid in the incubation media. Rat renal cortical slices, (50–100 mg wet weight) were incubated in 2 ml Krebs–Ringer, bicarbonate at 37° and a P_{CO_2} of 40-mm Hg. Control media (●) contained 2.5% albumin. Experimental media (○) contained 2.5% albumin complexed to 1 mM palmitate. The *p*-aminohippuric acid concentration in the media was varied between 0.05 and 2 mM. Incubation was carried out for 2.5 min. Uptake of *p*-aminohippuric acid by the slices was not corrected for *p*-aminohippuric acid trapped in the extracellular fluid. The number of observations at each medium *p*-aminohippuric acid concentration is indicated in parenthesis. Each observation is the mean of duplicate flasks. One standard error of the mean is indicated by the bars above or below the points. Equations of the regression lines were calculated by the least squares method: Control: $1/v = 0.72\ 1/S + 0.5$; $b = 0.72 \pm 0.16$ (mean ± S.E.); $a = 0.5 \pm 0.2$ (mean ± S.E.); $r = 0.86$. Palmitate: $1/v = 0.56\ 1/S + 0.26$; $b = 0.56 \pm 0.11$ (mean ± S.E.); $a = 0.26 \pm 0.10$ (mean ± S.E.); $r = 0.78$. Variance ratio (F) test for statistical significance of the difference between the two regression populations[19]: $F = 13.5$; degress of freedom: (1.28); $P < 0.01$.

TABLE I

SLICE TO MEDIUM (S/M) *p*-AMINOHIPPURIC ACID CONCENTRATION RATIOS IN RAT KIDNEY CORTICAL TISSUE INCUBATED *in vitro* WITH AND WITHOUT PALMITIC ACID

Slices (50–100 mg wet weight) were incubated at 37° in 2 ml Krebs–Ringer bicarbonate solution with 0.05 mM *p*-aminohippuric acid under O_2–CO_2 (93.8:6.2, by vol.) (P_b = 683 mm Hg) for 2.5 min. The media also contained 2.5% defatted albumin alone or 2.5% defatted albumin complexed to 1 mM palmitic acid. *p*-Aminohippuric acid was analysed in protein free filtrates of tissue and media. Each observation is the mean of duplicate flasks. Control and palmitate-containing flasks were always handled in pairs.

Expt.	*S/M at 2.5 min*		*Δ S/M at 2.5 min (control − palmitate)*
	Control	*Palmitate (1 mM)*	
4	8.76	6.65	2.41
5	6.15	5.48	0.67
	4.02	1.72	2.30
6	3.17	2.52	0.65
	3.90	3.51	0.34
7	4.67	3.78	0.87
	4.78	3.55	1.23
7a	3.11	2.85	0.26
	4.13	3.73	0.40
Means ± 1 S.E.	4.77 ± 0.56	3.75 ± 0.52	1.02 ± 0.27
P			< 0.01

TABLE II

EFFECTS OF L-CARNITINE ON RENAL *p*-AMINOHIPPURIC ACID SLICE TO MEDIUM CONCENTRATIONS RATIOS UPON INCUBATION WITH OR WITHOUT PALMITIC ACID

Rat kidney cortical slices (50–100 mg, wet) were incubated for 2.5 min at 37° in 2 ml Krebs–Ringer bicarbonate solution with 0.075 mM *p*-amino[1-^{14}C]hippuric acid (spec. act., 1.29 μC/μmole) under O_2–CO_2 (93.8:6.2, by vol.) (P_b = 683 mm Hg). The media also contained: (1) 2.5% defatted albumin, or (2) 2.5% defatted albumin and 1 mM L-carnitine; (3) 2.5% albumin complexed to 1 mM palmitate; and (4) 2.5% albumin complexed to 1 mM palmitate *plus* 1 mM L-carnitine. (The numbers above the first 4 columns correspond to each medium composition.) ^{14}C was determined in aliquots of protein-free filtrates (4% trichloroacetic acid) of tissues and media, in Bray's solution. Counting efficiency was determined by internal standardization. Each observation is the mean of duplicate flasks. Statistical significance was determined by Student's *t* test. S/M, slice to medium *p*-aminohippuric acid concentration ratio.

Expt.	*S/M at 2.5 min*				*Δ S/M at 2.5 min*			
	(1) *Control*	(2) *Carnitine*	(3) *Palmitate*	(4) *Palmitate, carnitine*	(5) (2) − (1)	(6) (3) − (1)	(7) (4) − (1)	(8) (4) − (3)
1	1.56	2.87	—	1.71	1.31	—	0.15	—
2	1.34	2.43	—	1.55	1.10	—	0.22	—
3	2.09	3.25	2.05	2.12	1.16	−0.04	0.03	0.07
4	2.99	3.35	2.85	2.37	0.36	−0.14	−0.62	−0.48
5	2.59	2.47	2.47	2.66	−0.12	−0.12	0.07	0.19
6	2.54	3.78	2.11	3.08	1.24	−0.43	0.54	0.97
Mean ± S.E	2.18 ±0.26	3.02 ±0.22	2.37 −0.32	2.25 ±0.22	0.84 ±0.24	−0.18 ±0.17	0.06 ±0.10	0.18 ±0.29
P	(2) *vs.* (1) < 0.05		(4) *vs.* (3) > 0.05		(5) < 0.05		(8) > 0.05	

puric acid uptake, a finding consistent with an inhibition of the competitive type (Fig. 2).

In an attempt to determine the site of the inhibition of renal *p*-aminohippuric acid uptake by palmitate, the effects of L-carnitine on renal *p*-aminohippuric acid accumulation were studied. 1 mM carnitine significantly stimulated (38.5 %) the initial uptake of *p*-aminohippuric acid by rat kidney cortical slices incubated in Krebs–Ringer bicarbonate solution containing 2.5 % defatted albumin (Table II, columns 1, 2 and 4). In contrast, L-carnitine produced a slight (7.6 %) but statistically insignificant effect on renal *p*-aminohippuric acid uptake in slices incubated in Krebs–Ringer bicarbonate solution containing 2.5% albumin complexed to 1 mM palmitate (Table II, columns 1, 3 and 5).

DISCUSSION

Our results indicate that in addition to the inhibitory effect that medium chain fatty acids have on renal *p*-aminohippuric acid uptake[5], non-esterified fatty acids of longer chain length, such as palmitic acid, also inhibit the renal *p*-aminohippuric acid uptake *in vitro*. Since long chain fatty acids are found at significant concentrations in most mammalian tissues and fluids our finding indicates that the rate of renal *p*-aminohippuric acid transport (and hence *p*-aminohippuric acid *in vivo* clearance and maximum renal tubular *p*-aminohippuric acid transport rate) may be influenced by the endogenous levels of long chain fatty acids.

Our present results are consistent with our previous observation on the inhibitory effects that substrates of the organic acid transport system have on renal non-esterified fatty acids uptake in the dog *in vivo*[4]. The competitive nature of the inhibition of renal *p*-aminohippuric acid uptake by palmitate shown here, indicates that there is a common step in the renal handling of non-esterified fatty acids, a metabolizable substrate, and *p*-aminohippuric acid a non-metabolizable, actively secreted substance. Thus, the renal organic acid transport system may serve not only in the transcellular transport of non-metabolizable organic acids and other end products of metabolism such as glucuronides and sulfates, but may also participate in the cellular uptake of major renal substrates such as non esterified fatty acids.

The observation that carnitine stimulates renal *p*-aminohippuric acid uptake when there was no palmitate in the incubation media while it stimulated much less the rate of renal *p*-aminohippuric acid uptake in the presence of 1 mM palmitate in the media, may give some insight into the nature of the site of interaction of the non-esterified fatty acid and *p*-aminohippuric acid renal uptake systems. Carnitine promotes fatty acid oxidation more at high than at low medium non-esterified fatty acid[3,12]. In contrast, carnitine stimulated *p*-aminohippuric transport significantly only in the absence of palmitate in the media. In addition, carnitine does not increase the oxygen uptake of rat renal cortical slices[3]. It is thus unlikely that the effects of carnitine on *p*-aminohippuric acid transport are mediated by changes in the energy available to the cells. It should be noted that palmitate[3] and other substrates[5] increase renal cortical slice oxygen consumption but inhibit *p*-aminohippuric acid accumulation. Thus, there is no correlation between the amount of energy available to the cells from aerobic metabolism and from fatty acid oxidation specifically, and the rate of *p*-aminohippuric acid uptake.

It is possible that carnitine has a stimulatory effect on renal *p*-aminohippuric acid transport, while palmitate has an inhibitory effect on it but there is at present no explanation for the mechanism of a possible direct stimulatory effect of carnitine on *p*-aminohippuric acid transport. It is also possible that the effects of carnitine on *p*-aminohippuric acid transport are mediated through changes in the tissue non-esterified fatty acid concentration. Carnitine addition could stimulate *p*-aminohippuric acid transport by decreasing tissue non-esterified fatty acids concentration[12] thus removing the inhibitory effect of tissue non-esterified fatty acids on *p*-aminohippuric acid transport.

It is also likely that in the presence of a supply of non-esterified fatty acid in the incubation media, the tissue non-esterified fatty acid concentration could be maintained or could decrease to a lesser extent, upon carnitine addition and *p*-aminohippuric acid transport could remain under the inhibition of tissue non-esterified fatty acids.

The mechanism by which tissue non-esterified fatty acid could inhibit *p*-aminohippuric acid transport deserves some consideration. There is evidence that some non-esterified fatty acid[13] and also that acyl-CoA esters[14] inhibit certain intracellular enzymes. Thus intracellular non-esterified fatty acids (or acyl-CoA) may competitively inhibit enzymatic steps of *p*-aminohippuric acid transport. On the other hand, there is also evidence for the existence of intracellular non-esterified fatty acid-binding particles in several tissues[15]. In addition, intracellular binding of *p*-aminohippuric acid in renal tissue has been described[16]. It is thus possible that common intracellular binding sites exist in renal tissue for non-esterified fatty acid and *p*-aminohippuric acid. It might be pertinent to point out that both non-esterified fatty acids[17] and *p*-aminohippuric acid[18] bind to extracellular albumin.

A similar line of reasoning could account for changes in renal non-esterified fatty acidsup take produced by substrates of the *p*-amino hippuric acid transport system[4]. The rate of non-esterified fatty acids uptake and metabolism depends largely on the relative affinities and the relative concentrations of extracellular albumin and intracellular non-esterified fatty acids binding sites[15]. Thus, changes in the affinity of intracellular non-esterified fatty acid binding sites which may result from competition with other binding substances may influence the rate of non-esterified fatty acid uptake and metabolism and thus the rate of energy production in the kidney.

In conclusion, our results are consistent with the hypothesis that a competitive interaction between *p*-aminohippuric acid and non-esterified fatty acid for uptake by renal cortical tissue may result from competition for common intracellular binding sites or from competitive inhibition of enzymatic steps of *p*-aminohippuric acid transport by either intracellular non-esterified fatty acid or long chain acyl-CoA esters. The effects of carnitine on renal *p*-aminohippuric acid uptake could be accounted for more simply by assuming that its effects are mediated through changes in intracellular non-esterified fatty acids concentration.

Identity of binding sites either at the cell membrane or at an intracellular site does not mean identity of transporting systems. Further experiments are in progress in our laboratory to gain insight into these problems.

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