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Participation of P-dependent and P-independent Glutaminases in Rat Kidney Ammoniagenesis and Their Modulation by Metabolic Acidosis, Hippurate and Insulin

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Summary

The key regulatory enzymes of kidney ammoniagenesis appear to be P-dependent (PDG) and P-independent (PIG) glutaminases. While the participation of PDG has been satisfactorily elucidated, the significance of PIG remains doubtful. Rat kidney cortex slices synthesized ammonia even under basal conditions. Metabolic acidosis, hippurate and insulin stimulated ammonia production. Under basal conditions, PDG activity in kidney homogenate, was twice as high as PIG activity. Metabolic acidosis stimulated ammonia production by the stimulation of both PDG (100 %) and PIG (57 %) activities. Hippurate stimulated only PIG activity both under basal conditions (90 %) and in metabolic acidosis (52 %), while it inhibited PDG activity only insignificantly under basal conditions and markedly (53 %) in metabolic acidosis. Insulin stimulated both PIG and PDG activities under basal conditions as well as in metabolic acidosis and potentiated the PIG stimulation by hippurate while it potentiated the hippurate inhibition of PDG both under basal conditions and in acidotic rats. In conclusion, both PDG and PIG participate in ammoniagenesis and are stimulated by metabolic acidosis and insulin. Hippurate stimulates PIG, while it inhibits PDG in metabolic acidosis and even after insulin administration. The effect of hippurate appears to be of physiological interest.

Key words

Ammoniagenesis – P-independent glutaminase – P-dependent glutaminase – Metabolic acidosis – Hippurate – Insulin

Introduction

Metabolic acidosis, besides direct effects of increased hydrogen ion concentration, alters various metabolic pathways. Metabolic acidosis impairs enterocyte energy balance and the transport of amino acids and peptides (Dzúrik 1973), alters cellular immunity by inhibiting leukocyte, monocyte and lymphocyte metabolism and function (Ogle *et al.* 1994, Ballmer *et al.* 1995, May *et al.* 1996), inhibits the production of growth hormone (Dass *et al.* 1994) causing nephrogenic nanism (Tannen *et al.* 1996), modifies the balance of ureagenesis and glutamine production (Schoolwerth and Gesek 1990) and interferes with additional metabolic pathways (Halperin *et al.* 1996). As a result it is accepted as a major risk factor of kidney disease progression (Hoffsten and Klahr 1983, Spustová *et al.* 1997).

Two thirds of H^+ are excreted in NH_4^+ and ammoniagenesis appears to be the main adaptation mechanism; ammonia production depends on the acid base balance. In kidney diseases, ammoniagenesis correlates with the clearance of endogenous creatinine.

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However, the variability is high with decreased ammoniagenesis in some patients even with a minor kidney function alteration, i.e. in early phases of kidney diseases (Hoffsten and Klahr 1983, Fedelešová *et al.* 1987).

Ammoniagenesis is modulated at various steps (Dass and Kurtz 1990, Kurtz *et al.* 1990), the major one being glutamine (Gln)-deamidation cleavage by glutaminases, which is P-dependent glutaminase (PDG) is localized intramitochondrially in the cells of various nephron segments, is stimulated in acidosis (Dass and Kurtz 1990). It is generally thought to be of primary importance for acid base balance. On the contrary, P-independent glutaminase (PIG), is localized mainly at the luminal membrane of (proximal) tubular cells, but it is also present at the cellular membranes of other tubular cells and also in the interstitial microvascular compartment (Dass *et al.* 1981). PIG is identical with an isoenzyme of gammaglutamyl transferase, stimulated by hippurate and inhibited by acivicin (Dass and Kurtz 1990). Very little is known about its physiological significance. A recently developed methodology for the simultaneous determination of PIG and PDG activity (Lardner and O'Donovan 1994) made it possible to compare and evaluate the activity and significance of the two isoenzymes (Katunuma *et al.* 1966, Curthoys and Lowry 1973). The paper deals with this comparison.

Material and Methods

Rats

The experiments were performed on male Wistar rats (150–180 g). Acidosis was elicited by 1.5 % NH_4Cl in drinking water for at least 7 days. Insulin was administered intraperitoneally in a dose 0.6 IU/100 g body weight 15 min before rat sacrifice without anaesthesia.

Kidney cortex slices

Rat kidney cortex slices were prepared manually with razor blades and immersed in cold saline for several minutes. Kidney cortex slices (35–40 mg) were incubated in 1 ml Ringer bicarbonate medium at pH 7.4 (containing in mM: NaCl 119, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4.7H_2O$ 1.2, $NaHCO_3$ 25 and KH_2PO_4 1.2) with 5.0 mM glutamine and/or hippurate in an atmosphere of 0.95/0.05 O_2/CO_2 at 37 °C for 1 h. At the end of incubation, the slices were removed from the incubation medium which was immediately centrifuged 10 min at 3000 rpm at 4 °C and the supernatant was used for ammonia determination by the Nessler reaction.

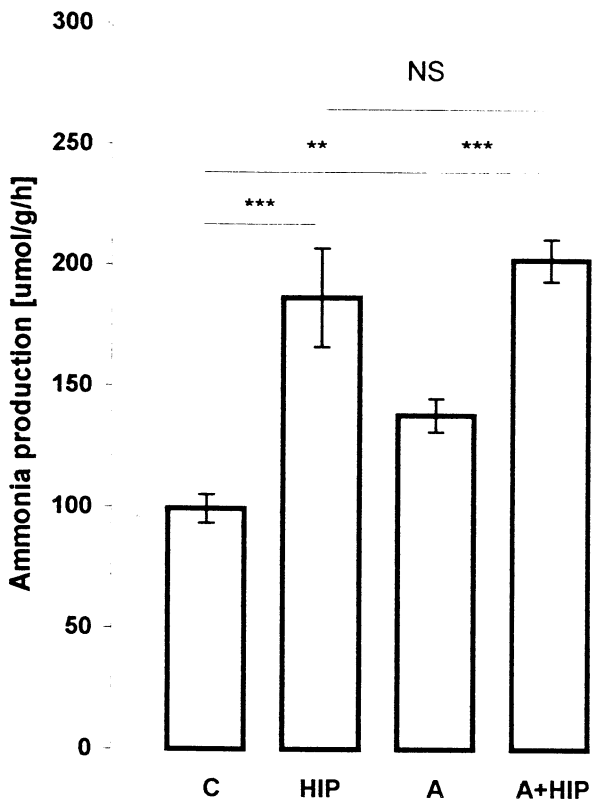

Fig. 1. *Effects of hippurate on ammonia production in kidney cortex slices under basal conditions and after acidification (n=6). Vertical bars represent S.E.M. C (control); Hip (hippurate); A (acidification). ** $p<0.01$; *** $p<0.001$; NS (non-significant).*

Determination of glutaminases activities (Lardner and O'Donovan 1994)

Kidney cortex homogenates were prepared after kidney decapsulation and excision of both the internal and external medulla. The tissue was diluted by adding 10 ml 0.9 % NaCl/g tissue and homogenized in tissue homogenizer OMNI TH. 0.5 ml homogenate was diluted with 1.0 ml 0.1 M glutamine with or without 0.5 ml 1.0 M phosphate buffer pH 8.0 and the volume of the incubation medium was supplemented by Tris-HCl buffer pH 8.0 up to 3.0 ml. The diluted and buffered medium was incubated in an atmosphere of 0.95/0.05 O_2/CO_2 at 37 °C for 30 min. The enzymatic reaction was evaluated against the blanks of spontaneous glutamine breakdown and inorganic phosphate interference. At the end of incubation the sample was deproteinized by 3 ml of 10 % TCA and after centrifugation at 3000 rpm for 10 min the supernatants of samples were neutralized with 0.2 ml saturated KOH and ammonia determined by Nessler's reaction. Proteins in the sediment were determined according to Lowry *et al.* (1951).

The amount of ammonia in the presence of phosphate in the incubation medium represents the activities of both glutaminases and the ammonia production in the absence of phosphate in the incubation medium just the activity of P-independent glutaminase. The method in principle depends on tissue homogenization, incubation in the presence/absence of inorganic phosphate and the determination of synthesized ammonia.

Calculations

Ammonia production was calculated *per* 1 g tissue/h in the case of kidney cortex slices and *per* 1 g protein/30 min in homogenates. Statistical evaluation was done by the Student's t-test.

Results

Ammoniagenesis in kidney cortex slices

Kidney cortex slices incubated in Krebs-Ringer bicarbonate containing 5 mM glutamine synthesized ammonia (Fig. 1). Ammoniagenesis was stimulated by metabolic acidosis and even more by hippurate. Metabolic acidosis and hippurate stimulation were partially additive. Ammoniagenesis was also stimulated by insulin and in this case hippurate did not increase ammoniagenesis further (Fig. 2) which indirectly pointed to the same localization of stimulation by both insulin and hippurate. In any case, the experimental model was adequate and the stimulation of ammoniagenesis by known modulators was present.

PDG and PIG participation in ammoniagenesis

PDG and PIG activities were determined in kidney homogenates of animals treated similarly as in the case of kidney cortex slices. PIG activity was stimulated by metabolic acidosis and even more by hippurate (Fig. 3A). The stimulation was additive. The basal PDG activity was twice as high as PIG activity (Fig. 3B). It was stimulated by metabolic acidosis, while it was inhibited by hippurate both under basal conditions (on the border of significance) and significantly in metabolic acidosis.

Insulin stimulation of both PIG and PDG basal activities was evident if the controls without (Fig. 3) and after insulin administration (Fig. 4) were compared. It was about twice as high in the presence of insulin. Moreover, insulin did not alter the stimulation of PIG by hippurate and metabolic acidosis in separate experiments and even increased their additive effect (Fig. 4A). Moreover, insulin intensified the inhibitory potential of hippurate under basal conditions and even in metabolic acidosis-activated PDG (Fig. 4B).

Because of the primary interest in PIG, the effects of insulin and hippurate were evaluated in an additional experiment (Fig. 5). Both insulin and hippurate stimulated the basal PIG activity and their stimulation was additive.

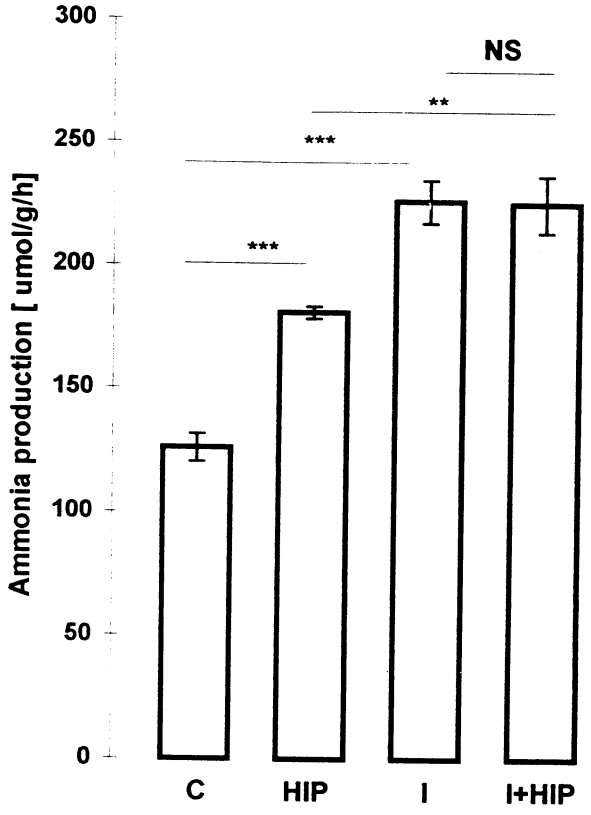

Fig. 2. *Effects of hippurate on ammonia production in kidney cortex slices under basal conditions and after insulin stimulation (n = 6). Vertical bars represent S.E.M. C (control); Hip (hippurate); I (insulin). ** $p < 0.01$; *** $p < 0.001$; NS (non-significant).*

Discussion

Kidney cortex slices methodology

The kidney cortex is functionally and even structurally highly non-homogeneous and its slices are correspondingly heterogeneous. The purpose of their use was to respect this heterogeneity, because of intensive cross-talk between various kidney cells. This is important especially in the case of PIG activity determinations. This activity is present not only in tubular cells but also in other parts of the kidney, such as microvascular structures. On the other hand, the difference caused in one area, i.e. proximal tubules, could be masked by the tissue not participating in ammoniagenesis or which is not stimulated by a modulator. Fortunately, remarkable changes in ammoniagenesis found in the present experiments are in good accordance with the experiments on isolated tubules and the direct determination of PIG and PDG activities. Thus, our experiments document the validity of the experimental model which enables the evaluation of PIG and PDG activities.

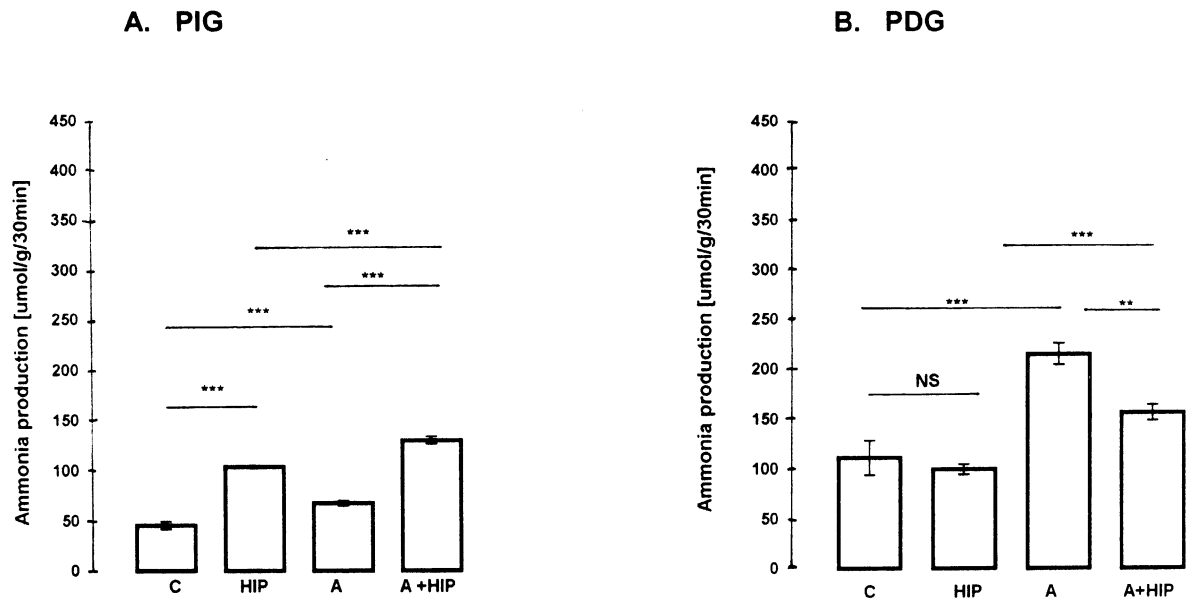

Fig. 3. *Effect of hippurate on PIG (A) and PDG (B) activity in kidney homogenates under basal conditions and after acidification (n=6). Vertical bars represent S.E.M. C (control); Hip (hippurate); A (acidification). ** p<0.01; *** p<0.001; NS (non-significant).*

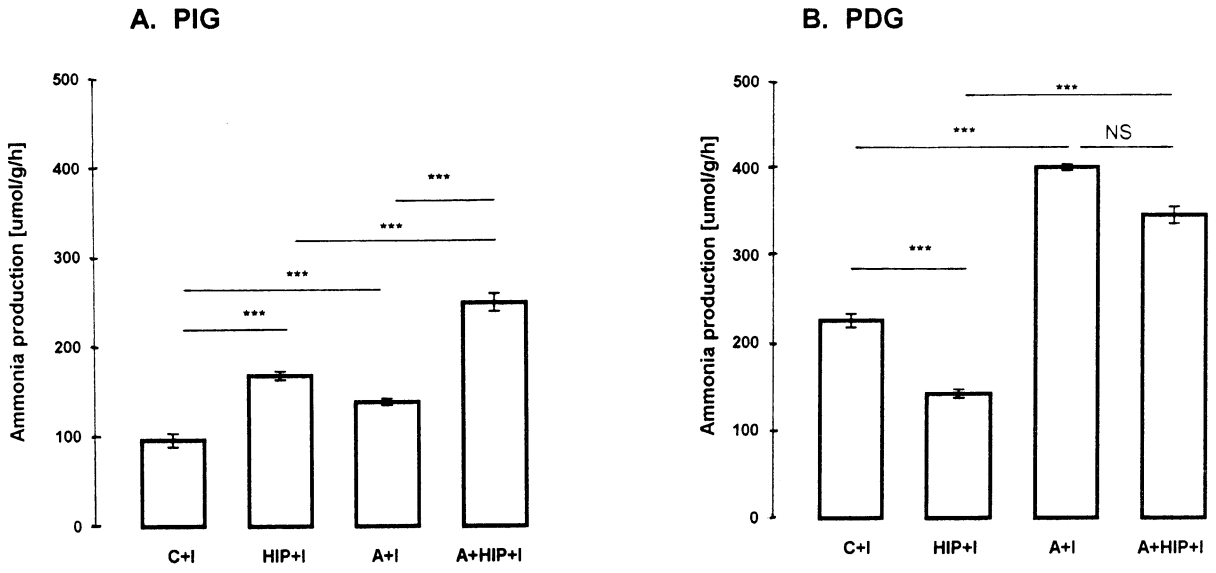

Fig. 4. *Effect of hippurate on PIG (A) and PDG (B) activity in kidney homogenates under insulin-stimulated conditions and after acidification (n=6). Vertical bars represent S.E.M. C (control); Hip (hippurate); A (acidification); I (insulin). (*** p<0.001); NS (non-significant).*

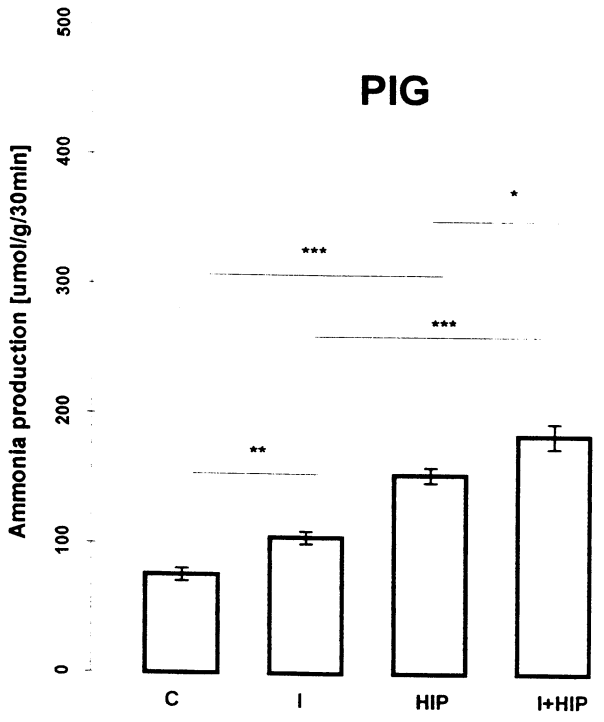

Fig. 5. *Effect of insulin and hippurate on PIG activity in kidney homogenates under basal conditions (n=6). Vertical bars represent SEM. C (control); Hip (hippurate); I (insulin). * $p<0.05$; ** $p<0.01$; *** $p<0.001$.*

Methodology of PIG and PDG activity determinations

At present two principles of PIG and PDG differentiation are available: structural distribution and enzyme activity dependence on inorganic phosphate. PDG activity is localized intramitochondrially with the highest activity in distal straight and convoluted tubules, intermediate in proximal convoluted tubules, and low in proximal straight tubules and glomeruli. The past procedure of its determination depended on the isolation of mitochondria and eventually on the assessment of monomer and polymer forms of PDG. However, because of enhanced ammonia production due to decreased K_m in both cases, it seems superfluous to determine them independently (Hortelano *et al.* 1990). PIG activity is high in proximal straight tubules localized at the brush border (Curthoys and Lowry 1973, Dass and Kurtz 1990, Schoolwerth and Gesek 1990), but also at the basolateral membrane (Spater *et al.* 1982). Moreover, it is also present in the microvascular compartment (Dass *et al.* 1981). Another principle is based upon the influence of inorganic phosphate on enzyme activities. PDG is inactive if inorganic P is absent in the incubation media while PIG is P-independent.

Both principles were combined in the past. However, the recently developed methodology by Lardner and O'Donovan (1994) exploited just the enzyme activity principle with adequate separation of PIG and PDG activities. This methodology was also employed in our experiments.

PDG participation in ammoniagenesis

Under basal conditions, the activity of PDG is twice as high as that of PIG and is markedly increased by acidification. This is in accordance with previous studies, performed on total (PIG + PDG) activity. The elevation is caused mainly by the PDG localized in proximal tubule cells (Curthoys and Lowry 1973, Challa *et al.* 1993, Spustová *et al.* 1997). Surprisingly, PDG is inhibited by hippurate, during acidification and probably also under basal conditions. Insulin stimulates PDG, but it increases PDG inhibition by hippurate both under basal and acidotic conditions.

The increased activity of PDG is caused by lower Km and not by any change in velocity (V_{max}) (Hortelano *et al.* 1990). However, increased ammonia production is not limited just to PDG activation. PDG mRNA expression already increases 4 h after the acidification and it rises further by 10 h and diminishes to almost normal values at 30 h (Schoolwerth *et al.* 1994) simultaneously with the increased mRNA expression of Phosphoenolpyruvate carboxykinase and glutamate dehydrogenase. Moreover, even the expression of cDNA encoding kidney mitochondrial glutaminase is significant (Shapiro *et al.* 1991).

PIG participation in ammoniagenesis

Basal PIG activity is lower than that of PDG. PIG activity is significantly stimulated by metabolic acidosis and insulin, and even more by hippurate. The effects are additive. In this respect, PIG stimulation by hippurate basically differs from that of PDG. However, on the contrary to PDG, no data are available about the mechanism of stimulation, especially the PIG mRNA expression. There is only some indirect evidence for the increase of PIG activity by the decreased K_m (Welbourne and Dass 1982, Silbernagl 1986).

The relevance of PDG and PIG in kidney ammoniagenesis

There are no doubts about the dominance of PDG in ammoniagenesis, while the relevance of PIG in ammoniagenesis is a matter of controversy. Generally, it is suggested to be low (Tannen 1978, Silbernagl 1985, May *et al.* 1987, Simon *et al.* 1990), while others stress the PIG relevance, though not dominance in ammoniagenesis (Dass and Kurtz 1990, Nonoguchi *et al.* 1990, Mályusz *et al.* 1994). Detailed studies have shown that control rats produced 38–48 % ammonia

by the PIG pathway under basal conditions, about 37 % in metabolic acidosis (Welbourne and Dass 1988, Dass and Martin 1990) and about 44 % (Dass and Martin 1990) in renal insufficiency. The stimulation by acidosis and insulin reflects both the PDG and PIG activity stimulation. On the other hand, hippurate points to the significance of PIG participation. More studies on additional modulators are needed to evaluate the relevance of PIG. Another important point appears to concern the PIG mRNA expression. These experiments are now in progress in our laboratory.

In conclusion, both PDG and PIG participate in ammoniagenesis. The dominant stimulation of PDG by insulin and acidosis is unambiguous; the inhibition by hippurate is surprising. On the other hand, PIG is stimulated by insulin. acidosis and hippurate; their effects are additive and the modulation by modulators both at the translation and transcription level remains to be elucidated.

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