Acetazolamide and renal ammoniagenesis

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CHAPMAN, SHARON K., AND MICHAEL S. HOOVER. *Acetazolamide and renal ammoniagenesis.* Am. J. Physiol. 234(3): F235–F237, 1978 or Am. J. Physiol.: Renal Fluid Electrolyte Physiol. 3(3): F235–F237, 1978. —The effect of acetazolamide on ammonia-producing enzyme systems was determined in vitro at concentrations comparable to those which have been shown to abolish ammonium excretion in vivo. No change in the activity of glutaminase or γ-glutamyl transpeptidase could be observed at concentrations up to 0.2 mM acetazolamide, and concentrations up to 1 mM were without effect on D-glutamyltransferase activity. Therefore, the effect of acetazolamide to abolish ammonium excretion cannot be explained by an action of the drug to inhibit ammoniagenesis.

carbonic anhydrase; glutaminase; γ-glutamyl transpeptidase

THE INHIBITION OF RENAL CARBONIC ANHYDRASE by acetazolamide is recognizable by the accompanying increase in bicarbonate output and the decrease in hydrogen and ammonium output (6). The mechanism underlying the fall in ammonium excretion has received considerable attention recently.

In the past it was suggested that a relatively alkaline pH of urine prevents the trapping of protons by ammonia to yield ammonium (12). In this view, acetazolamide decreases ammonia excretion simply by the diversion of renal cellular ammonia from the alkalinized urine to the more acidic renal venous blood (2, 10).

More recently, however, the decline in ammonia excretion has been attributed directly to the inhibition of ammoniagenesis by acetazolamide. Phenix and Welbourne (11) perfused isolated rat kidneys with increasing concentrations of the substrate glutamine. In control rats, there was a rise in ammonia production with a progressive rise in substrate concentration, whereas the rate of increase was reduced in rats pretreated with 20 mg/kg acetazolamide. The apparent K_m was increased from 18 mM in the controls to 80 mM in pretreated animals. Their results were interpreted to indicate that acetazolamide was a competitive inhibitor of glutamyltransferase, an enzyme which they consider to be the major ammonia producer in the nonacidotic rat kidney (17).

The circulating precursor of (excreted) ammonia is glutamine (16). Ammonia can be liberated from glutamine by either glutaminase (1), D-glutamyltransferase (18, 19), or γ-glutamyl transpeptidase (8).

In the present study, we sought a more direct measure of the inhibition of ammoniagenesis by determining if acetazolamide could inhibit any of these ammonia-producing pathways in vitro at concentrations comparable to those which have been shown to reduce ammonia excretion in vivo.

METHODS

The following reactions were studied in vitro.

Reaction 1: Glutaminase

L-glutamine ⟶ L-glutamate ⟶ 2-oxoglutarate

Reaction 2: Glutamyltransferase

L-glutamine + hydroxylamine
→ glutamylhydroxamate + NH_4^+

Reaction 3: Glutamyl transpeptidase

γ-glutamyl-*p*-nitroanilide + glycylglycine
→ *p*-nitroanilide + glutamylglycylglycine

The glutaminase activity (*reaction 1*) was assayed spectrophotometrically by coupling the glutaminase reaction to the subsequent glutamic dehydrogenase and following the time course of the reduction of NAD^+ at 340 nm. A Gilford model 2400 spectrophotometer was used. The assay mixture contained 47.5 mM Tris sulfate, pH 8.5, 5 mM L-glutamine, 1 mM NAD^+, 5.9 U L-glutamic dehydrogenase (Sigma, type I), and 0.04 U of glutaminase (Sigma, grade VI).

The glutamyltransferase activity (*reaction 2*) was assayed by the method described by Thorndike and Reif-Lehrer (14). The enzyme source for the assay was the supernatant fraction of kidney homogenates prepared from male Holtzman rats weighing 200–300 g. The rats were stunned by a blow on the head, decapitated, exsanguinated, and quickly nephrectomized. The 20% homogenates were prepared in ice-cold 0.14 M NaCl solutions in a Potter-Elvehjem homogenizer with a Teflon pestle. Homogenates were centrifuged for 15 min at 3,000 × *g* in a refrigerated Sorvall model RC2-B centrifuge. The assay mixture contained 5 mM L-glutamine, 40 mM hydroxylamine, 42 mM sodium arsenate, 25 mM sodium citrate, 0.5 mM $MnCl_2$, 75 μM ADP, and an aliquot of the rat kidney supernatant fraction equivalent to 10 mg of kidney tissue.

The glutamyl transpeptidase activity was assayed by the method of Rosalki and Tarlow (13) (*reaction 3*). The

enzyme source for the assay was the 3,000 × *g* supernatant fraction from 10% kidney homogenates prepared in Tris glycylglycine buffer, pH 8.5, as described above.

RESULTS

The catalytic rate for the glutaminase assay, expressed as nanomoles of NADH + H^+ formed per minute, mean SE of six determinations, was 3.02 ± 0.2. Acetazolamide was added in concentrations up to 0.2 mM with no significant alteration in reaction rate (3.01 ± 0.4 nmol/min).

At substrate concentrations of 2.5–10 mM, the catalytic rates for glutamyl transpeptidase varied from 73.8 to 132.0 μmol *p*-nitroanilide formed/min per g kidney. The reaction rates in five parallel studies which contained acetazolamide in concentrations up to 0.2 mM were identical to those which contained substrate alone.

Since previous studies (11) suggested that glutamyltransferase is competitively inhibited by acetazolamide in vivo with an increase in the apparent K_m from 18 to 80 mM, it was our concern to study this interaction in vitro at a lower (and more physiological) substrate concentration. To do this, a concentration of 5 mM glutamine was chosen, and the linearity of the enzyme assay was established in the presence and absence of acetazolamide (Fig. 1). Concentrations of acetazolamide up to 1 mM were without effect on the amount of glutamylhydroxamate formed or on the linearity of the reaction. From these results, a 10-min incubation was selected and the effects of 1 mM acetazolamide were studied over a range of substrate concentrations (Fig. 2). The initial rate of the reaction was directly proportional to substrate concentration and no change in glutamyltransferase activity was observed with 1 mM acetazolamide.

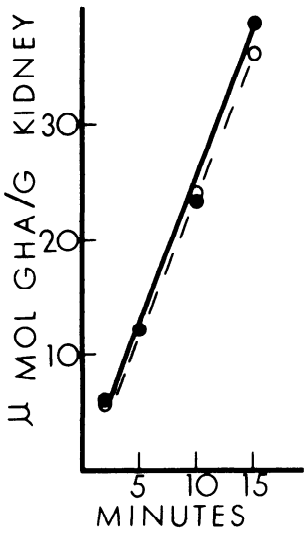

FIG. 1. Effect of acetazolamide on rate and linearity of glutamyltransferase. Glutamylhydroxamic acid (GHA) formation was measured as described in METHODS. Substrate concentration was 5 mM and the reaction was stopped at times indicated by immersion of tubes in ice and addition of 15% TCA. Solid line is control enzyme activity; broken line is enzyme activity in presence of 0.5, 0.75, or 1.0 mM acetazolamide. Data represent means for 6–8 replicate assays performed in presence or absence of acetazolamide.

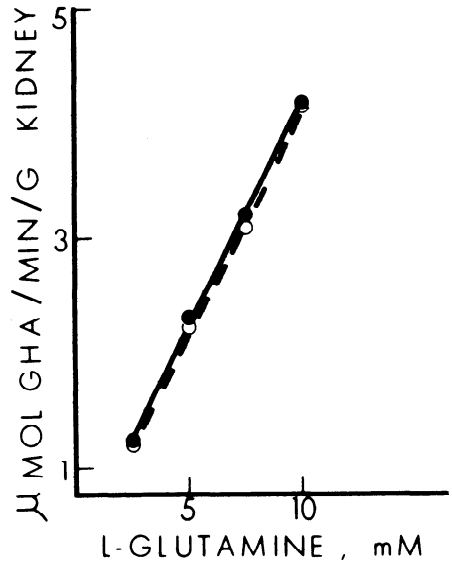

FIG. 2. Effect of acetazolamide on glutamyltransferase activity as a function of substrate concentration. Assay was carried out as described in METHODS; incubation time was 10 min. Solid line is control enzyme activity; broken line is enzyme activity in presence of 1 mM acetazolamide. Data represent means for 6 replicate assays performed in presence or absence of acetazolamide.

DISCUSSION

The concentration of drug found in the dog renal cortex 45 min after the administration of 5 mg/kg acetazolamide was 58 μmol/kg; the medullary drug concentration was within 2 times the level found in the cortex (5). Maximal effects of acetazolamide to abolish ammonium excretion are observed at this dose (7). Therefore, the concentration of acetazolamide available to inhibit ammoniagenesis in the dog at a time when ammonia excretion was abolished in vivo would be in the range 0.06 – 0.12 mM. Inhibition of carbonic anhydrase at 99.99% has been reported with 0.1 mM acetazolamide (7), but our in vitro data indicate that acetazolamide will not inhibit any of the major ammonia-producing enzyme systems at these concentrations.

Phenix and Welbourne (11) have suggested that acetazolamide (20 mg/kg) reduces ammonia excretion by a competitive inhibition of glutamyltransferase. In their studies with perfused kidneys and kidney homogenates, the ammonia produced from D- rather than L-glutamine was used as an index to differentiate glutamyltransferase activity from other ammonia-producing enzymes (e.g., glutaminase); the ammonia production was always less in the acetazolamide-pretreated group.

It has long been considered (8) that glutamyltransferase can utilize D-glutamine as a substrate, whereas glutaminase hydrolyzes only the L isomer. However, as reported in a recent paper by Herzfeld (3), a more purified glutamyltransferase preparation was isolated, and the substrate and inhibitor specificity were reevaluated. The transferase and synthetase activities, which were believed to reside at different sites on the same protein molecule, were separated by differential extraction. Glutamyl synthetase catalyzes the formation of L-glutamine from L-glutamate by the following reaction

$$\text{L-glutamate} + NH_4^+ + ATP \xrightarrow{Mg^{++}} \text{L-glutamine} + ADP + P_i$$

In the Herzfeld study (3), D- or L-glutamate served equally as substrates for the synthetase reaction, whereas the glutamyltransferase activity was virtually absent when L-glutamine was replaced by the D isomer. These results question the validity of the methodology used in studies (11) showing acetazolamide inhibition of glutamyltransferase. Measuring ammonia production from the substrate D-glutamine may not be a true index of glutamyltransferase activity.

In the present study, glutamylhydroxamate formation was used as an index of glutamyltransferase activity (see *reaction 2*), and both the substrate (L-glutamine) and inhibitor (acetazolamide) were added to the incubation mixture. This method has been employed in previous studies showing the inhibition of glutamyltransferase by DL-methionine-DL-sulfoximine (MSO) (17); there is a stoichiometric production of ammonia and glutamylhydroxamate under these assay conditions. D-Glutamine does not serve as a good substrate in this system. No inhibition of glutamyltransferase activity could be demonstrated at concentrations up to 1 mM acetazolamide; the concentration of acetazolamide in rat kidney tissue after a dose of 20 mg/kg is 0.2–0.4 mM (15).

Although other investigators have shown that glutaminase activity is inhibited by acetazolamide in vitro, the inhibition occurred only at very high concentrations (i.e., 10–15 mM) (4). Our results with lower concentrations are consistent with those of Phenix and Welbourne (11). These investigators found no inhibition of glutaminase in kidney homogenates prepared from acetazolamide-treated (20 mg/kg) rats.

It is still puzzling why the diversion of ammonia from urine cannot be quantified, consistently, in the renal vein; there is evidence for (2, 10) and against (11) an increase in renal venous release of ammonia after acetazolamide administration. We conclude from our experiments, however, that the fall in ammonium excretion following the acute administration of acetazolamide cannot be explained by a direct effect of the drug to inhibit ammonia-producing enzyme systems.

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