Copper-Sodium Linkage during Intestinal Absorption: Inhibition by Amiloride (43208)

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Abstract. **The possible association between copper and sodium small intestinal absorption in the rat was investigated in the presence or absence of the electrolyte transport inhibitors amiloride, acetazolamide, and furosemide, at pharmacologic concentrations, using an *in situ* perfusion procedure. Amiloride (1 m*M*) produced a significant decrease in copper, net water, and sodium absorption, in solutions with sodium. Copper tissue retention was not altered, but was much higher in the absence of sodium. Acetazolamide and furosemide (1 m*M*), in separate experiments, had no effect on copper removal from the lumen, but generally reduced sodium and water transport. The presence or absence of sodium in the perfusate influenced rates of copper uptake. These data are compatible with a more effective passage of copper across the enterocyte basolateral membrane in the presence of sodium than in its absence.** [P.S.E.B.M. 1991, Vol 196]

Copper intestinal absorption and nutritional status can be altered by several components of the diet. A large amount of information has been reported on the effect of carbohydrates, particularly fructose, although metabolic effects can now be considered species-specific (1–5). In addition, amino acids and other protein breakdown products can also modulate copper utilization and absorption in humans (6), rodents (7, 8), and birds (9). Other constituents of the diet have not been as extensively studied. The role of sodium on copper metabolism has been investigated in relation to kidney function and structure, but only to a limited extent with regard to the absorptive process. Excess dietary sodium superimposed to copper deficiency produces kidney lesions in rats (10), with consequent renal failure and structural damage (11).

We have previously shown an association between copper and sodium in the luminal phase of the absorption of the metal (12), which was unrelated to unidirectional water fluxes and, hence, to the bulk transport of copper. Presumably the presence or absence of sodium affected the mediated transport of copper. The goal of this study was to further investigate a possible linkage between sodium (and chloride) intestinal epithelial transport and copper absorption using pharmacologic agents that affect ion exchanges across membranes. Specifically, we investigated amiloride, an inhibitor of sodium channel transport; furosemide, which inhibits an electroneutral sodium-potassium-chloride cotransporter; and acetazolamide, an inhibitor of carbonic anhydrase, which also affects membrane sodium/proton transfer.

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Materials and Methods

Changes in the jejunal absorption of copper were monitored by an *in situ* perfusion procedure extensively used in our laboratory (8, 12). In brief, male rats weighing 125–150 g (Crl:[WI]BR; Charles River Breeding Laboratories, Kingston, NY) were anesthetized with urethane (1.3 g/kg ip) after an overnight fast. A 20- to 30-cm length of the upper jejunum was cannulated, rinsed with warm saline, and perfused with a peristaltic pump (model 1203; Harvard Instruments, Boston, MA) at a rate of 11–12 ml/hr with the solutions described below. Copper was present at 31.5 μM (2 mg/liter) in isotonic solutions of either NaCl (140 mM) or glycerol (280 mM). Phenol red (20 mg/liter) was added as the nonabsorbable marker. The solutions were buffered at pH 6.8 with 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-KOH, an agent with negligible affinity for divalent cations. Tracer amounts of ^3H_2O (5 μCi/liter; ICN Radiochemicals, Irvine, CA) were also included in the solutions to allow for the calculation of unidirectional mucosa-to-serosa water fluxes. The putative inhibitors were tested at 1 mM concentration each, in separate experiments. Eight 15-min effluent

fractions were collected after allowing for 1 hr of equilibration. This resulted in an absorptive steady state for the solutes in which the fractional collections did not differ by more than 15% of the mean. Every 15-min sample was separately analyzed, but a single value was averaged for each rat. At the end of the perfusions, the cannulated segment was excised, measured under a 3-g tension, weighed, slit open, and rinsed in ice-cold isotonic NaCl. The tissue was placed in a test tube and treated with 1 ml of 15 *N* HNO_3 until it completely disintegrated. This solution was then made up to 10 ml, centrifuged, and analyzed for copper content. During every perfusion run, up to 12 rats were handled at a time, and control or drug treatments were randomly assigned. The experiments were conducted in two series. Due to the poor water solubility of amiloride, this substance had to be initially dissolved in dimethyl sulfoxide and the stock further diluted into NaCl or glycerol solutions. The controls contained a similar amount of dimethyl sulfoxide (1.0 ml/500 ml of final volume). Acetazolamide and furosemide were directly dissolved in the perfusing solutions. Therefore, the presentation of the data is divided in two subsections. Each animal was perfused with a single solution. Ten to 14 rats were used for each of the variables tested, as indicated in tables and figures. Copper was assayed by atomic absorption spectroscopy (Varian Spectra AA10; Varian Instruments, Palo Alto, CA) and ^3H_2O was determined in a beta scintillator counter (Beckman LS3800; Beckman Instruments, Fullerton, CA). Net copper absorption values were computed taking into consideration net water absorption, obtained from phenol red assays (13), and unidirectional water fluxes were derived from isotope dilution. Recirculation of the labeled water was shown in earlier studies to be negligible within the fraction collection period. All remaining reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Copper certified standards (Fisher Chemical Co., Fair Lawn, NJ) were used to calibrate the atomic absorption spectrophotometer and as stocks for the perfused solutions. The results were assessed by either a nonpaired *t* test, or by analysis of variance and subsequent Dunnett's and Tukey's test for contrasting against a reference solution or among treatments (14).

Results

Effects of Amiloride. The intestinal absorption of copper was reduced by luminal 1 m*M* amiloride during perfusions in the presence ([+Na]) or absence ([−Na]) of sodium (Fig. 1). The decline in copper absorption was more marked in [−Na] conditions. In the [+Na] experiments, the reduction of copper absorption paralleled a decrease of net water and sodium absorption (Fig. 2). However, amiloride did not alter net water absorption in the absence of sodium [mean ± SE: 1.53 ± 0.10 with amiloride and 1.66 ± 0.13 μl/min × cm

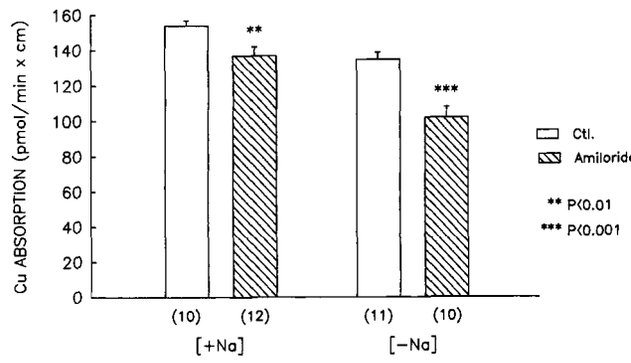

Figure 1. Copper absorption in the presence or absence of 1 m*M* amiloride and during perfusions containing isotonic sodium chloride [+Na] or glycerol [−Na]. See text for other details of the technique. Ctl, control.

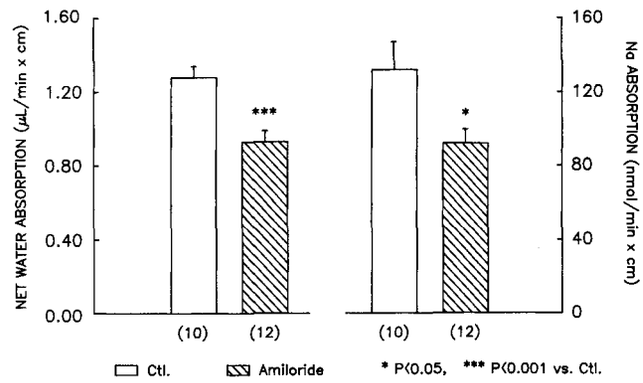

Figure 2. Net water (left panel) and sodium (right panel) absorption during [+Na] perfusions in the presence or absence of 1 m*M* amiloride. Ctl, control.

for controls (t = 0.79, NS)]. The differences in the mucosa-to-serosa water influx were negligible, either in [+Na] or in [−Na] conditions (Table I, first series). Copper retention by the perfused tissue was unaffected by any of the solutions perfused (Table II, first series). However, the differences in copper retention were very significantly higher following perfusions in the [−Na] status than after [+Na] conditions.

Effects of Acetazolamide and Furosemide. The rate of copper removal from the intestinal lumen was unaltered by either 1 m*M* acetazolamide or furosemide, with or without sodium in the perfusing solutions (Fig. 3). However, a two-way analysis of variance indicated that the presence or absence of sodium was a significant factor in the results ($P < 0.05$) and produced an alteration of the response.

As in the case of amiloride, there were no differences in the amount of copper retention by the perfused intestinal tissue (Table II, second series). However, the accumulation of copper in experiments conducted in the absence of sodium resulted in more than a doubling of the trace element retention by the perfused segment. Acetazolamide significantly reduced net water absorption, both in [+Na] and in [−Na] conditions, and

Table I. Mucosa-to-Serosa Water Influx in Perfusions with either Isotonic NaCl [+Na] or Glycerol [−Na]

	First series (μl/min × cm)		Second series (μl/min × cm)		
	Amiloride	Control	Acetazolamide	Furosemide	Control
[+Na]	4.81 ± 0.12[a] (12)[b]	4.99 ± 0.06 (10)	4.75 ± 0.18 (13)	5.24 ± 0.24 (11)	4.81 ± 0.16 (11)
[−Na]	4.78 ± 0.11 (10)	4.70 ± 0.19 (11)	4.50 ± 0.10 (14)	4.71 ± 0.24 (10)	4.70 ± 0.11 (12)

[a] All data are mean ± SE. No significant differences in either series of experiments.
[b] Numbers in parentheses, number of rats.

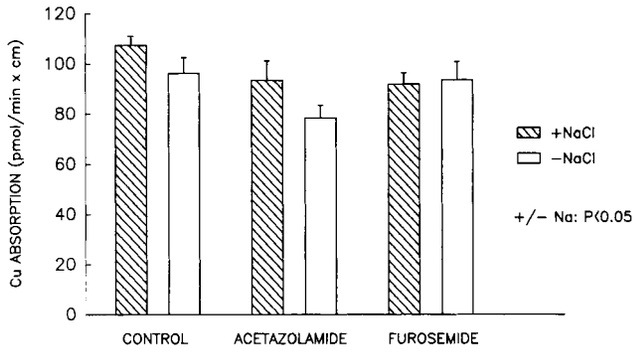

Figure 3. Copper absorption in the presence of either 1 m*M* acetazolamide, furosemide, or no additive, and in solutions containing either sodium chloride [+Na] or glycerol [−Na] as osmotic solutes.

sharply decreased sodium absorption, during the perfusions carried out [+Na] (Fig. 4). The decline of net water absorption during furosemide perfusion in [+Na] experiments was not significant. In contrast, furosemide induced an increase of net water absorption in [−Na] perfusions. As in the case of acetazolamide, sodium absorption, in the presence of furosemide, was diminished. The changes in the mucosa-to-serosa water transport due to the presence of these drugs in the medium were not significant (Table I, second series).

Discussion

The results presented here support earlier findings linking sodium with the intestinal transport of copper (12). In the present study, 1 m*M* luminal amiloride led to a reduction of copper absorption in the presence or the absence of sodium (Fig. 1), as well as sodium and water absorption in perfusions with the electrolyte [+Na]. The effect on copper was proportionally more marked in perfusions made with glycerol as the osmotic agent. It should be noted that the alteration of luminal copper removal occurred even under circumstances of net sodium secretion, when no sodium was added to the medium [−Na]. Measurements of unidirectional water influx are consistent with a greater serosa-to-mucosa outflow in the presence of amiloride and sodium, since the data of mucosa-to-serosa inflow (Table I), which are directly obtained, showed no differences due to the presence of amiloride.

Amiloride and chemically similar derivatives are organic bases originally demonstrated to inhibit the sodium channel in urinary epithelia, but act on other tissues as well. These drugs also inhibit the sodium/proton antiporter and the sodium/calcium exchanger (15–17). It has been previously reported that amiloride reduced water absorption only in rats made sodium-deficient by dietary restriction for over 10 days (18). This situation may physiologically resemble the perfusions conducted in [−Na] conditions. A linkage between sodium, mineralocorticoids, and sensitivity to amiloride has been demonstrated in the gut, since elevations of aldosterone induced by a sodium-deficient diet result in an enhancement of the sodium transport system, which can be inhibited by amiloride (19, 20). Such changes are not observable in the normally fed

Table II. Copper Retention by Perfused Intestinal Tissue in the Presence [+Na] or the Absence [−Na] of Sodium

	First series (pmol/min × g)		Second series (pmol/min × g)		
	Amiloride	Control	Acetazolamide	Furosemide	Control
[+Na]	419 ± 40[a,b] (12)[c]	373 ± 48[b] (10)	541 ± 50[b] (13)	408 ± 31[b] (11)	454 ± 40[b] (11)
[−Na]	953 ± 47 (10)	929 ± 48 (11)	1175 ± 51 (14)	906 ± 102 (10)	1066 ± 127 (12)

[a] All data are mean ± SE.
[b] $P < 0.001$ [+Na] versus [−Na].
[c] Numbers in parentheses, number of rats.

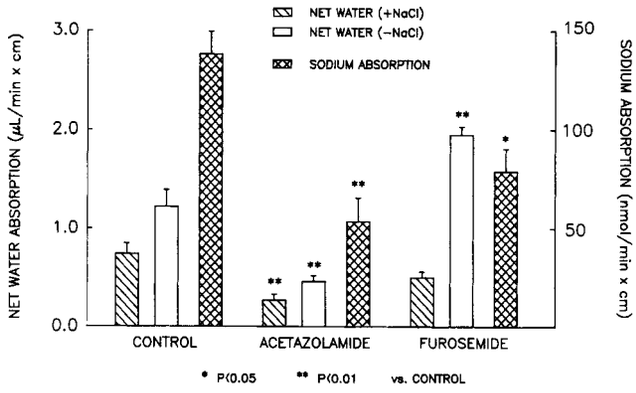

Figure 4. Net water and sodium absorption during perfusions in the presence or absence of sodium chloride. The data on sodium absorption correspond to the [+Na] perfusions. *Significance of differences against controls.

animal. Hence, the rapid effect achieved in the experiments described here confirms a considerable sensitivity of the rat jejunum to that diuretic, as well as the parallelism between copper and sodium transport, and the possible association of copper entry through the sodium apical channel, or its sharing a translocation route via the sodium/proton antiporter.

In vitro techniques, namely Ussing chamber and everted sacs, have been the main tools applied to demonstrate inhibition of sodium and chloride transport by furosemide and amiloride (21). Furosemide acts on the electroneutral sodium-potassium-chloride cotransporter, and it has mostly been studied in the thick ascending loop of Henle (17). This may explain why, in the mammalian small intestine, there is only a reduction of net sodium absorption, whereas water entry into the enterocyte is not altered, and is even enhanced in [−Na] conditions. Hence, there is no significant effect on copper absorption, suggesting that the sodium-potassium-chloride cotransporter does not play a significant role in copper passage across the jejunal apical membrane. The underlying mechanism appears to be related to a decrease of membrane conductance.

Carbonic anhydrase is responsive to acid-base balance in many epithelial tissues. However, the activity of this enzyme in rat jejunum and ileum is low when compared with the stomach and the colon (22). Acetazolamide and other carbonic anhydrase inhibitors directly inhibit luminal sodium/proton exchanges by reducing intracellular proton load and delaying the splitting of the carbonic acid formed from bicarbonate and extruded protons into water and carbon dioxide (17). In our experimental setup, this action was translated into a sharp reduction of net water absorption in both [−Na] and [+Na] conditions. This is understandable, since sodium ions are not directly affected and the extent of the sodium/proton exchange is quantitatively smaller than neutral sodium-potassium-chloride transport. Nevertheless, in [−Na] perfusions there was a tendency toward a diminished rate of copper extraction from the luminal fluid.

In all cases, retention of copper by the small intestinal mucosa following perfusions with sodium ([+Na]) was much smaller than without sodium ([−Na]). These results are compatible with a more effective transport of copper across the basolateral membrane in the presence of sodium. Future studies using inhibitors of basolateral sodium transport, such as ouabain or spironolactone, which respectively inhibit (Na-K)ATPase and an aldosterone-regulated mechanism and ethacrynic acid, which interferes with the electroneutral sodium-potassium-chloride transporter both in the basolateral and in the apical membranes (17), may provide additional clarification on the copper-sodium linkage presented in this and our preceding report (12).

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