AMONG THE MOST WIDELY prescribed drugs in clinical practice, thiazide diuretics are mainstays in the therapy of hypertension and in the management of edema in patients with well-preserved renal function. Thiazides are also currently used in the management of hypercalcemia and diabetes insipidus (DI).

Three possible mechanisms of NaCl transport in distal segments have been considered: 1) Na\(^+\)-Cl\(^-\) cotransport, 2) Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransport, and 3) Na\(^+\)-H\(^+\) and Cl\(^-\)/HCO\(_3\)\(^-\) parallel antiport. At present, it is generally believed that the major site of action of thiazides is located in the distal convoluted tubule, as demonstrated in in vivo microperfusion and microperfusion studies (5–7). Constanzo and Windhager (6), Velazquez et al. (33, 34), and Ellis et al. (12) demonstrated that thiazides act on the electroneutral Na\(^+\)-dependent Cl\(^-\) transport mechanism to decrease Cl\(^-\) and Na\(^+\) reabsorption. Using metolazone, a thiazide-type diuretic, Tran et al. (31) proposed that in the Na\(^+\)-Cl\(^-\) cotransport, the binding site for thiazide is on the luminal side of the transporter protein, sharing the same site with Cl\(^-\).

When thiazides occupy this site, they may be able to block the ion translocating process.

The administration of thiazide diuretics to patients with DI results in a paradoxical antidiuresis (8,10,28). In a study on Brattleboro rats with hereditary hypothalamic DI, Walter et al. (35) showed that the antidiuresis that follows acute administration of hydrochlorothiazide (HCTZ) is entirely secondary to the natriuresis and consequent sodium depletion induced by the drug. Shirley et al. (27) showed that the mechanism of sustained antidiuresis during chronic HCTZ administration in DI differs from that of the acute response. They demonstrated that the proximal tubule was able to increase the fractional fluid reabsorption, reducing the fluid delivery to the distal nephron segments, but they proposed that these changes in proximal tubular function only partially account for the decrease in urinary volume, and for this reason they assumed that there is a direct or an indirect action of thiazide on other segments.

Bachmann et al. (2) concluded that the predominant site of thiazide-sensitive Na\(^+\)-Cl\(^-\) cotransporter mRNA expression in rabbit distal nephron is the distal convoluted tubule and that the sites of mRNA expression of electroneutral Na\(^+\) and Cl\(^-\) transport are similar in rabbits, rats, and mice. Congenital nephrogenic DI (NDI) is characterized by renal tubular resistance to antidiuretic hormone (ADH), resulting in the excretion of an increased volume of diluted urine, a decrease in total body water, and a rise in plasma osmolality.

No definitive therapy for NDI currently exists. Previous reports have indicated that HCTZ in combination with a prostaglandin synthesis inhibitor is an effective therapy (20). In view of the adverse effects of hypokalemia induced by HCTZ, or the renal complications induced by prostaglandin inhibitors, combined treatment with HCTZ and amiloride, the potassium-sparing diuretic, has been used (1,16).

In an attempt to elucidate the hypothesis presented above and considering that no data are available about the effect of HCTZ on water transport in the final segments, the purpose of the present study was to directly analyze the effect of HCTZ on water permeability in the in vitro microperfused rat inner medullary collecting duct (IMCD). Our results showed that HCTZ...
enhanced water permeability in the absence of ADH, providing an improvement in water absorption in the last segment of the nephron when used for DI therapy.

**METHODS**

The isolated IMCD was perfused by previously described techniques (4, 23).

Male Wistar and Brattleboro rats weighing 120–125 g were studied. All animals were maintained on a standard diet and had free access to tap water. Tubules were isolated from a small slice that was immersed in a dish of chilled Ringer-HCO₃ buffer, oxygenated, and kept at pH 7.4 by bubbling the solution with 5% CO₂-95% O₂. The IMCD was dissected without the use of collagenase or other enzymatic agents. After isolation, the segment was transferred to a temperature-regulated chamber (37°C) mounted on the stage of an inverted microscope. The bathing solution used was 298 ± 4 mosmol/kgH₂O Ringer-HCO₃ of the following composition (in mM): 115.0 NaCl, 25.0 NaHCO₃, 10.0 sodium acetate, 5.0 KCl, 1.0 CaCl₂, 1.2 MgSO₄, 1.2 NaH₂PO₄, and 5.5 D-glucose. For the hypotonic solution (70.0 mosmol/kgH₂O), NaCl (115.0 mM) was withdrawn. F&D&C green dye was added to the perfusate as a visual marker.

Net water absorption (Jₚ) was measured with [¹⁴C]inulin dialyzed immediately before the experiments. The dialyzed isotope was added to the perfusion solution at a final concentration of 25–100 cpm/ml. Jₚ was calculated as Vₒ – Vᵢ/L, where Vₒ is the perfusion rate, Vᵢ the collecting rate, and L the length of the tubule studied (tubule length and inner diameter can be measured to within 0.05 mm with a precalibrated micrometer in one eyepiece of the inverted microscope used to observe the perfusion). Vᵢ was directly measured on the basis of collection time, whereas Vₒ was calculated by the rate of appearance of the impermeant marker [¹³C]inulin in the collection pipette according to the equation Vᵢ = Vₒ (Iₒ/Iᵢ), where Iₒ is the counts per minute of the collected fluid, and Iᵢ is the counts per minute of the perfusate. Timed tubular samples were collected for analysis under mineral oil by aspiration into a calibrated pipette. All measurements were made 60–90 min after perfusion of a given nephron segment was started. The inner diameter (Iₐ) of the perfused segments ranged from 25 to 35 μm and the tubular length (L) from 1.5 to 2.0 mm. The area (A) was calculated as Iₐ·π·L, and expressed as ×10⁻⁴ cm².

Osmotic water permeability (Pₒ) was determined by measuring net fluid movement in response to an imposed gradient. Net fluid reabsorption was induced by perfusion with hypertonic perfusion solution, 80–90 mosmol/kgH₂O (40 mM NaCl), and 300 mosmol/kgH₂O bathing solution. The osmolality of the perfusate and bath fluid was measured, and Pₒ (μm/s) was calculated in each experiment by the following equation (9)

\[ Pₒ = \frac{R}{L₉} \frac{Vₒ}{Vᵢ} \]

where L₉ is the hydraulic conductivity and is determined by the equation

\[ L₉ = \frac{1}{RTAC₉} \]

\[ (Cᵢ - Vᵢ - Vₛ) + Cᵥn \left[ \frac{Lₐ}{C₉} - \frac{Lₐ}{Cᵥ} \right] \]

where Cᵢ and Cᵥ are the osmolalities of the bath and the initial perfusion fluid, respectively; R is the gas constant; T is the absolute temperature; Vₛ is the partial molar volume of water; and A the luminal surface area. This equation rests on the assumption that the reflection coefficient of NaCl is 1.0.

Diffusional permeability (Pᵦ) was determined from the unidirectional flux of tritiated water (³H₂O) added to the perfusion solution. The Pᵦ (×10⁻⁶ cm²/s) value was calculated using the following expression (26)

\[ Pᵦ = \frac{Vᵢ}{A} \left[ \frac{Cᵢ}{Cₒ} \right] \]

where Cᵢ and Cₒ are the activities of ³H₂O (cpm/ml) in the initial perfusion fluid and collected fluid, respectively; and Vᵢ and A are as defined above.

The baths were checked for osmolality and pH with an osmometer (Advanced Instruments) and a pH meter (Iris 7; Tecnon, São Paulo, Brazil), respectively. The bath fluid was changed every 10 min to reduce the effect of evaporation and consequently the increase in osmotic gradient. The bath osmolality did not change significantly during the 10-min period (from 295 ± 1 mosmol/kgH₂O at 0 min to 297 ± 2 mosmol/kgH₂O at 10 min).

Timed fluid collections were made with a constant-volume constriction pipette that was rinsed four times with 0.5 ml of water; 5 ml of scintillation liquid was then pipetted into the vial (Aquasol universal cocktail; New England Nuclear, Boston, MA). The isotopic concentration was determined with a liquid scintillator spectrometer (Tri-Carb 1600TR; Packard, Downers Grove, IL).

The isotopic materials used were from Amersham International and New England Nuclear. HCTZ was kindly supplied by the Pharmacy Division of the University Hospital, Faculty of Medicine of São Paulo, University of São Paulo. All other drugs were purchased from Sigma Chemical (St. Louis, MO).

Results are expressed as means ± SE. The data for each period are the mean of three to four collections. Statistical analysis was performed using the Student’s t-test and ANOVA.

**RESULTS**

The dose added to the perfusate (10⁻⁶ M) was chosen because it was the lowest dose with reproducible results, although others had used higher doses (10⁻⁴ M) in micropuncture studies (30, 35). Unless otherwise indicated, the concentration in the perfused fluid was always 10⁻⁶ M. HCTZ added to the bath fluid had no effect. In a pilot study, it was demonstrated that HCTZ was able to enhance water transport only when applied to the luminal side. In this investigation, a dose-dependent study was performed by adding 10⁻⁴, 10⁻⁵, and 10⁻⁶ M of HCTZ to the bath fluid, and the results were as follows: control, 5.79 ± 1.41; 10⁻⁶ M, 5.65 ± 0.99; 10⁻⁵ M, 6.08 ± 0.71; and 10⁻⁶ M, 7.06 ± 0.42.

The effect of HCTZ on water transport is presented in Table 1. In this group of IMCDs, we measured the effect of 6 M HCTZ on the Pₒ of normal rats in the absence of ADH. HCTZ increased Jₚ from 1.90 ± 0.57 to 3.11 ± 0.73 nl·min⁻¹·mm⁻¹·mm H₂O (P < 0.05), which represents an increase in Pₒ from 6.36 ± 0.56 to 19.08 ± 1.70 μm/s (P < 0.01). The reversibility of the action of HCTZ was observed during the recovery period, when Pₒ decreased to 5.93 ± 1.41 μm/s (P < 0.01) after HCTZ withdrawal.

In a second group of IMCDs (Table 1), we examined the effect of HCTZ (10⁻⁶ M) on Pᵦ. HCTZ increased Pᵦ from 38.01 ± 4.52 to 52.26 ± 4.38 × 10⁻⁵ cm²/s (P < 0.01) with a return to initial values (37.73 ± 6.22 × 10⁻⁵ cm²/s) during the recovery period in the absence of HCTZ.
We tested the effect of HCTZ in Brattleboro rats to avoid possible effects of endogenous ADH. HCTZ increased P, from 3.53 ± 1.41 to 11.16 ± 1.13 µm/s (P < 0.01), and P returned to initial values in the absence of HCTZ (4.24 ± 0.99 µm/s) (P < 0.01) (Table 1).

In the fourth group we examined the effect of prostaglandin (PGE2). PGE2 (10⁻⁵ M) was tested to understand the practice of the use of indomethacin, a PG inhibitor, as an adjuvant of HCTZ in DI therapy. The results presented in Table 2 show that PGE2 added to the bath fluid inhibited the HCTZ-stimulated P, from 29.65 ± 5.67 (P < 0.02) to 10.46 ± 1.84 (P < 0.01), with a recovery to 16.77 ± 4.07 µm/s.

In a final group we decided to investigate whether the effect of the HCTZ would be due to its action on Na⁺-Cl⁻ cotransport. We performed an experiment using 10⁻⁴ M ouabain, added to the bath. The results presented in Table 2 showed that ouabain did not block the action of HCTZ (control, 7.35 ± 3.25; HCTZ, 23.17 ± 7.49; HCTZ + ouabain, 25.15 ± 5.37; and HCTZ, 21.34 ± 4.24 µm/s).

**DISCUSSION**

Despite the widespread use of the thiazides in clinical practice for many years, their sites of action and their effects on collecting duct function have not been fully assessed. At present, it is generally believed that the major site of action of thiazides is the distal convoluted tubule. This notion is based on data derived from both osmolar clearance and micropuncture studies. The observation that thiazides inhibit free water clearance without affecting free water reabsorption (11, 25, 29) supports the view that these drugs inhibit solute transport in the cortical diluting segments. More direct evidence with regard to the site of action of thiazides has been provided by micropuncture studies (6, 12, 17, 30, 31, 33).

The present in vitro microperfusion study was designed to determine whether HCTZ has any effect on water transport in the rat IMCD. The results showed that HCTZ, in the absence of ADH (Table 1, Fig. 1A), was able to enhance water transport measured both in terms of osmotic and diffusional permeability. The data from the diffusional permeability, showing an increase in water conductance in absence of an osmotic gradient, provide acceptable evidence that the effective epithelial permeability was changed.

However, results were obtained only when the diuretic was applied to the lumen of the tubule and not just added to the bath fluid. It is well known that HCTZ, which is a sulfonamide compound, is an organic acid and consequently is secreted into the proximal tubule by the organic acid secretory pathway, reaching the tubular lumen of the distal segment where its principal target, the Na⁺-Cl⁻ cotransport, is located. Since the vasopressin receptor is located on the basolateral side of the IMCD cell and since HCTZ works only in the lumen, our data lead us to hypothesize that thiazide did not act on the vasopressin receptor to enhance water transport, but required entering the cells through the luminal membrane, reaching intracellular structures, and then performing its action. Although our data are convincing, this hypothesis requires further investigation.

To assure the absence of endogenous ADH, all experimental measurements were performed ~60 min after the rats were killed, with the sampling procedure beginning after an equilibration period of 30–45 min. Another alternative to rule out possible effects of endogenous ADH was to use rats with DI (Brattleboro strain), which cannot elaborate ADH (32). The data from these experiments confirmed what was demonstrated for normal rats, i.e., HCTZ enhanced water absorption also in Brattleboro rats (Fig. 1A).

**Table 1. Effect of 10⁻⁶ M luminal HCTZ on P⃗⃗ in Brattleboro and normal rats and P⃗⃗ in normal rats**

<table>
<thead>
<tr>
<th>P, µm/s</th>
<th>n</th>
<th>Area, ×10⁻⁴ cm²</th>
<th>V, nl/min Exp</th>
<th>HCTZ</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brattleboro</td>
<td>5</td>
<td>11.76 ± 0.3</td>
<td>26.2 ± 1.7</td>
<td>3.53 ± 1.4×</td>
<td>28.9 ± 1.5</td>
</tr>
<tr>
<td>Normal</td>
<td>5</td>
<td>20.6 ± 1.4</td>
<td>22.0 ± 2.3</td>
<td>6.36 ± 0.56×</td>
<td>25.0 ± 3.0</td>
</tr>
<tr>
<td>P, ×10⁻⁵ cm²</td>
<td>6</td>
<td>21.9 ± 0.8</td>
<td>39.1 ± 1.5</td>
<td>38.01 ± 4.52×</td>
<td>39.2 ± 1.1</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of IMCDs. P<sub>f</sub>, osmotic water permeability; P<sub>dw</sub>, water diffusional permeability. Net water absorption values for control, hydrochlorothiazide (HCTZ)-treated, and recovery in IMCDs from Brattleboro and normal rats were 0.28 ± 0.53, 2.04 ± 0.12, and 1.39 ± 0.64, and 1.90 ± 0.57, 3.11 ± 0.73, and 1.42 ± 0.10 nl·min⁻¹·mm⁻², respectively. V<sub>j</sub>, perfusion rate (nl/min); Exp, experimental periods; *P < 0.01 compared with the preceding group.

**Table 2. Effect of prostaglandin and ouabain on P⃗⃗ in presence of HCTZ**

<table>
<thead>
<tr>
<th>P, µm/s</th>
<th>n</th>
<th>Control</th>
<th>HCTZ</th>
<th>HCTZ + Inhibitor</th>
<th>HCTZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGE&lt;sub&gt;2&lt;/sub&gt; (10⁻⁵ M)</td>
<td>6</td>
<td>16.93 ± 2.64</td>
<td>29.65 ± 5.67†</td>
<td>10.46 ± 1.84*</td>
<td>16.77 ± 4.07*</td>
</tr>
<tr>
<td>Ouabain (10⁻⁴ M)</td>
<td>6</td>
<td>7.35 ± 3.25</td>
<td>23.17 ± 7.49</td>
<td>25.15 ± 5.37</td>
<td>21.34 ± 4.24</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of IMCDs. Mean V<sub>j</sub>, 30.9 ± 0.08 nl/min; mean area 15.7 ± 1.0 × 10⁻⁴ cm². *P < 0.01 and †P < 0.02 compared with preceding period.
According to studies reported by Earley and Orloff (10) and Skadhauge (28), the administration of thiazide diuretics to patients with DI results in a paradoxical antidiuresis. This antidiuresis is not well understood, but Walter et al. (35) tried to explain this phenomenon using micropuncture techniques applied to Brattleboro rats. They demonstrated that acute HCTZ administration produced a marked antidiuresis due to a rise in fractional fluid reabsorption by proximal tubules, diminishing distal delivery and restricting urinary flow. In a long-term HCTZ treatment, Shirley et al. (27) proposed that “the changes in proximal tubular function during chronic thiazide treatment only partially account for the reduction in urine volume; it seems probable that the raised papillary osmolality, by enhancing water reabsorption at sites beyond the proximal tubule, makes a greater contribution to antidiuresis.” On this basis, our data confirm a direct action of this diuretic on the last segment of the nephron from both normal ADH-depleted rats and Brattleboro rats.

PGE₂ was directly used to try to understand why indomethacin is used as a coadjuvant in DI therapy. Indeed, PGE₂ is known to be a blocker of vasopressin-adenylate cyclase stimulation when it participates in the loop of the “negative feedback” in the vasopressin cascade (22). Our results showed that PGE₂ decreased the effect of HCTZ when added after HCTZ, i.e., PGE₂ produced a rapid blockade in the HCTZ-stimulated Pᵢ. Thus these results inform us that when indomethacin was administered with HCTZ, it can enhance the action of this diuretic, providing evidence as to why this association is effective in DI treatment.

During the PGE₂ period (Table 2), in the absence of ADH and HCTZ, PGE₂ drastically reduced Pᵢ, in contrast to the data reported by Nadler et al. (21) and Hébert et al. (15). The present experiments and other in vitro studies from our laboratory (18, 19) showed that HCTZ (1–2 mg·kg⁻¹·day⁻¹) and indomethacin (0.75 to 1.5 mg/kg) used with a low osmolality and low-sodium diet substantially reduced water excretion (1, 3).

Although the effect of thiazide has been described in the distal convoluted tubule, we cannot rule out the possibility that HCTZ enhances water transport by its effect on Na⁺–Cl⁻ cotransport in IMCD. To study this possibility, it would be necessary to inhibit this cotransport, but it is well known that all substances that block this cotransport are thiazides and thiazide-like diuretics and that these drugs act by the same mechanism, i.e., using the same chemical radical operating at the same binding sites of the Na⁺–Cl⁻ cotransporter (14).

On this basis, it would not be correct to use a thiazide diuretic to study this. The only way to try to overcome this problem was to use a strategy to indirectly reduce the action of this Na⁺–Cl⁻ cotransporter by blocking Na⁺–K⁺–ATPase with ouabain. With the reduction of the Na⁺ gradient between the intracellular and the luminal fluid, the Na⁺–Cl⁻ symport would then decrease. Rocha and Kudo (24) showed that ouabain (10⁻⁴ M) reduced the Na⁺ lumen-to-bath flux in IMCD, demonstrating that the entry of Na⁺ through luminal membranes is directly or indirectly coupled to Na⁺–K⁺–ATPase. Our data showed that the effect of HCTZ was not abolished in the presence of this glycoside, leading us to suppose that the HCTZ effect on water absorption is not linked to the performance of the NaCl cotransporter. A possible effect produced by the carbonic anhydrase-inhibiting capability of HCTZ was not considered, because this capability is very low (14).

Finally, the present results provide some additional information about the effect of HCTZ on IMCD. Although this effect was shown in an in vitro study, we believe that it could be extended to human beings and, when evaluated in general, explain the capacity of HCTZ to enhance water absorption in a patient with DI. The data showing the blocker effect of PGE₂, together with the absence of an effect on the basolateral side, may shed some light on a possible mechanism of action of HCTZ, i.e., the above-mentioned idea, that HCTZ must enter the cell to produce its effect and a possible action may be to stimulate structures of the vasopressin cascade inside the cell, finishing with the enhancement of the water permeability. This hypothesis requires confirmation, through studies designed to clarify the mechanism by which the thiazide acts.

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Address for reprint requests and other correspondence: A. J. Magaldi, Faculdade de Medicina da USP, Av. Dr. Arnaldo, 455, 01246-903, São Paulo, SP, Brazil (E-mail: biomag@usp.br).

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