High Cholesterol Feeding May Induce Tubular Dysfunction Resulting in Hypomagnesemia

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Key Words

Hypomagnesemia · Hypercholesterolemia · Magnesium · TRPM6 · Renal hemodynamic · Renal transporter · Renal dysfunction

Abstract

Background/Aims: Hypomagnesemia may induce hypercholesterolemia, but the contrary has not been described yet. Thus, magnesium homeostasis was evaluated in rats fed a cholesterol-enriched diet for 8 days. This study has a relevant clinical application if hypomagnesemia, due to hypercholesterolemia, is confirmed in patients with long-term hypercholesterolemia. **Methods:** Both hypercholesterolemic (HC) and normocholesterolemic rats (NC) were divided into sets of experiments to measure hemodynamic parameters, physiological data, maximum capacity to dilute urine (C_{H_2O}), variations (Δ) in $[Ca^{2+}]_i$ and the expression of transporter proteins. **Results:** HC developed hypomagnesemia and showed high magnesuria in the absence of hemodynamic abnormalities. However, the urinary sodium excretion and C_{H_2O} in HC was similar to NC. On the other hand, the responses to angiotensin II by measuring $\Delta [Ca^{2+}]_i$ were higher in the thick ascending limb of Henle's loop (TAL) of HC than NC. Moreover, high expression of the cotransporter NKCC2 was found

in renal outer medulla fractions of HC. Taken together, the hypothesis of impairment in TAL was excluded. Actually, the expression of the epithelial Mg^{2+} channel in renal cortical membrane fractions was reduced in HC. **Conclusion:** Impairment in distal convoluted tubule induced by hypercholesterolemia explains high magnesuria and hypomagnesemia observed in HC.

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Introduction

Hypercholesterolemia is one of the major risk factors in the development of cardiovascular diseases including hypertension and ischemic damage in distinct organs. Currently, atherosclerosis is considered an inflammatory disease in which cytokines, proteases and vasoactive molecules are released from the endothelium due to oxidative stress generated by LDL cholesterol accumulated in the intima [1, 2]. Consequently, an impairment of endothelium-dependent vasodilatation occurs and nonvascular tissues may also be targeted by the inflammatory effector mechanism [3]. Hence, hypercholesterolemia may induce vascular and nonvascular injuries in the organs.

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Previous studies conducted by our laboratory demonstrated that hypercholesterolemia is associated with poor prognosis in acute renal failure [4], aggravates radiocontrast nephrotoxicity [5] and may impair renal autoregulation [6]. In these studies, the vascular factor was probably the major mechanism involved in the pathophysiology of these findings. In another study [7], maternal hypercholesterolemia was evaluated and pregnancy abnormalities were observed in the absence of hypertension. Moreover, disturbances in the development of the offspring including reduction in creatinine clearance associated with high urinary excretion of sodium, potassium and water in adulthood were also observed. Again, these observed effects were in the absence of hypertension. Thus, a nonvascular mechanism may also play a role in the development of the abnormalities induced by maternal hypercholesterolemia.

Magnesium deficiency may alter metabolism of elastin, proliferation of collagen, calcification, lipid metabolism and platelet aggregation [8]. Magnesium is the second most important intracellular cation and it functions as a cofactor for many cellular processes which include the regulation of several ion channels and the activity of Na^+, K^+ -ATPase [8, 9]. Hence, magnesium depletion may induce failure in the renal and cardiovascular systems and may induce hypercholesterolemia in humans [9–13]. It has been reported that magnesium intake is inversely associated with the incidence of type 2 diabetes [14] and the prevalence of the metabolic syndrome in older adults [15]. However, studies demonstrating that hypercholesterolemia may induce hypomagnesemia have not been carried out to date.

The aim of this study was to evaluate if hypercholesterolemia can induce abnormalities in electrolyte homeostasis due to changes in renal tubular function. Thus, a short period of high cholesterol feeding was chosen to induce hypercholesterolemia in rats. This protocol elicits cholesterol plasma without changes in blood pressure (BP), glomerular filtration rate (GFR) and renal blood flow (RBF) [6]. Although the short period of high cholesterol feeding may seem to be an acute effect of hypercholesterolemia in renal rat function, the results will have clinical relevance if the hypothesis is confirmed, especially if hypomagnesemia occurs in consequence of a renal tubular dysfunction. Thus, studies conducted in humans with long-term hypercholesterolemia will be required to evaluate magnesium homeostasis. It is possible that chronic hypercholesterolemia is in fact the cause of hypomagnesemia that is often observed in critically ill patients and which has been associated with increased morbidity and mortality in hospitalized patients [12, 13].

Methods

Experimental Groups

Male Wistar rats, weighing ~200 g, were randomly housed in individual cages. They were fed a standard diet or a standard diet supplemented with 4% (w/w) cholesterol and 1% (w/w) cholic acid for 8 days [4–7]. The animals were anesthetized with sodium thionembutal (50 mg/kg b.w., i.p.) to perform all surgical preparations or to harvest the kidneys in the case of in vitro experiments.

This study was conducted in accordance with the guidelines of the Ethics Committee of the Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo.

Renal Blood Flow

RBF was measured under anesthesia, as previously described [6]. Briefly, catheters were placed in the trachea, external jugular vein for fluid infusion and in femoral artery to measure BP. A midline incision in abdomen was made and the left renal pedicle was carefully microdissected to place an ultrasonic flow probe (T106 Transonic Systems, Bethesda, Md., USA). RBF was recorded every 10 s during 10 min for each experiment and the renal vascular resistance (RVR) was calculated as the ratio of BP and RBF.

Metabolic Cage Studies

Rats fed either a standard diet or a cholesterol-enriched diet were placed in individual metabolic cages to collect urine under mineral oil for 24 h. To avoid contamination in urine samples the diet was restricted, but they were allowed to drink water ad libitum. At the end of this period, the animals were anesthetized to collect blood samples via a PE-200 inserted into the abdominal aorta.

Urine and plasma osmolality was determined by the freezing-point method (The Advanced Osmometer; 3D3, Mass., USA). Sodium and potassium were measured in urine and plasma by flame photometry (CELM-FC280, Brazil). The enzymatic colorimetric method (Labtest; Minas Gerais, Brazil) was used to quantify levels of magnesium, calcium, phosphorus, chloride and creatinine in urine and plasma. In the serum, total cholesterol and aldosterone were also measured using enzymatic colorimetric method and serum by radioimmunoassay, respectively (Coat-A-Count; DPC, Los Angeles, Calif., USA).

Free Water Clearance

Free water clearance ($\text{C}_{\text{H}_2\text{O}}$) was measured in another set of animals [16]. Briefly, a tracheotomy was performed and two polyethylene (PE-60) catheters were inserted in two external jugular veins for infusion of inulin and fluids. A hypotonic saline solution (150 mosm) was infused at a rate of 0.03 ml/min for 15 min immediately after a jugular vein had been cannulated. Thereafter, a more hypotonic saline solution (135 mosm) was infused at different rates (0.03, 0.06 and 0.08 ml/min) according to the diuresis observed [16]. Subsequently, one PE-60 was inserted in a carotid artery to collect blood samples and to measure BP and one PE-240 was placed in the bladder to collect urine samples. When the surgical procedure was completed, a priming dose of inulin (100 mg/kg b.w.) was administered by a jugular vein, followed by a constant inulin infusion (10 mg/kg b.w.) at a rate of 0.04 ml/min. Blood samples were collected at the beginning and at the end of the experiment and inulin was assayed by the anthrone method in all

blood and urine samples [17]. C_{H_2O} and the distal delivery of sodium chloride (DD_{NaCl}) were calculated using the following formulas:

$$C_{H_2O} = \frac{\text{Urine volume (ml/min)} - \text{Osmolar clearance (ml/min)}}{\text{Inulin clearance (ml/min)}} \times 100$$

$$DD_{NaCl} = \frac{\text{Sodium clearance (ml/min)} + C_{H_2O} \text{ (ml/min)}}{\text{Inulin clearance (ml/min)}} \times 100$$

Na⁺,K⁺-ATPase Assay

In another series of anesthetized animals, the kidneys were harvested to dissect the cortex and the outer medullas at 4°C. The tissue fragments were homogenized with a Teflon pestle in a 20/1 (v/w) cold solution (4°C) containing 0.25 M sucrose, 6 mM EDTA, 20 mM imidazole, 2.4 mM sodium deoxycholate (pH 6.8), and filtered through a double layer of surgical gauze. The Na⁺,K⁺-ATPase activity was measured by the inorganic phosphate (P_i) released from ATP labeled with γ[³²P]. The Na⁺,K⁺-ATPase activity was defined as the difference of ³²P liberated in total and basal ATPase assays, corrected for the spontaneous nonenzymatic breakdown of ATP and reported as μM P_i/mg protein · h⁻¹, as described by Assis et al. [7].

Isolation and Calcium Signaling in Isolated Nephron Segments

After anesthesia, the medullary segments of the thick ascending limb of Henle's loop (mTAL) and collecting duct (OMCD) were isolated from collagenase-treated kidney [18, 19]. The tubules were microdissected in standard solution (composition in mM: 127 NaCl, 5 KCl, 0.8 MgSO₄, 0.33 Na₂HPO₄, 0.44 KH₂PO₄, 2 CaCl₂, 1-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes), pH 7.4, and 1 mg/ml bovine serum albumin) at 4°C under a stereomicroscope (Hund GmbH, Germany). The tubules were identified by morphological and topographical criteria. Each isolated tubule was transferred to a thin glass microscope coverslip in 1 μl standard solution containing 1% agarose (type IX; Sigma-Aldrich) that was fixed by cooling the slide for 5 min on ice. The tubules embedded in jellied agarose were then loaded with 10 μM acetoxymethyl ester of fura 2 (fura 2-AM; Molecular Probes, USA) for 45 min at room temperature in darkness.

The glass slip with the sample was glued to the bottom of a superfusion chamber and fixed on the stage of an inverted fluorescent microscope (Nikon, Japan) equipped with Fluor ×40 objective to measure intracellular calcium concentration ([Ca²⁺]_i) by using a Photocan microfluorimeter (Photon Technology International, USA). The sample was continuously superfused with the standard solution (0.6 ml/min, 37°C) carrying or not carrying angiotensin II (Ang II) for 5 min. The sample was alternately excited at 340 and 380 nm (4 s/cycle), and the fluorescence emitted at 510 nm from an area defined by an adjustable window (about 25 × 30 mm) was measured. Background was routinely recorded for both wavelengths at the end of each experiment and subtracted from all measurements.

[Ca²⁺]_i was calculated using the equation of Grynkiewicz et al. [20]:

$$[Ca^{2+}]_i = K_d \times \lambda (R - R_{min}) / (R_{max} - R)$$

where K_d is the dissociation constant for the fura 2-Ca²⁺ complex, R is the ratio of fluorescence emitted for each wavelength (340/380), R_{max} is the maximal ratio emitted in the presence of saturating free calcium, R_{min} is the minimal ratio measured in the absence of free calcium, and λ is the ratio of fluorescence obtained at 380 nm in the absence and presence of saturating free calcium. The values of R_{min}, R_{max} and λ were periodically determined by external calibration using a buffer kit (Molecular Probes, USA) that mimicked the intracellular medium [19].

Specific Antibodies to Aquaporin 2 (AQP2), Na,K-2Cl Cotransporter (NKCC2), Renal Outer Medullary Potassium Channel (ROMK) and Transient Receptor Potential Channel Melastatin Subtype 6 (TRPM6)

The peptide-derived polyclonal antibody specific to TRPM6 was kindly supplied by Dr. Vladimir Chubanov (Institute for Pharmacology and Toxicology, Philipps University Marburg, Marburg, Germany). The peptide-derived polyclonal antibodies specific to AQP2, ROMK and actin were obtained from Santa Cruz Biotechnology (Santa Cruz, Calif., USA) and the peptide-derived polyclonal antibody specific to NKCC2 was from Millipore (USA).

Preparation of Membrane Fractions

Using a Teflon pestle glass homogenizer (Schmidt & Co., Frankfurt am Main, Germany), cortex and medulla samples were homogenized in an ice-cold isolation solution (200 mM mannitol, 80 mM Hepes and 41 mM KOH, pH 7.5) containing protease inhibitor cocktail (Sigma, St. Louis, Mo., USA). The homogenates were centrifuged at low speed (2,000 g) for 15 min at 4°C to remove nuclei and cell debris. The supernatants were then spun at 100,000 g for 1 h at 4°C (70 Ti rotor; Beckman Coulter, Inc., Fullerton, Calif., USA), producing pellets containing membrane fractions enriched with plasma membranes and intracellular vesicles. The pellets were suspended in the same isolation solution.

Electrophoresis and Immunoblotting

Samples of membrane fractions were run on polyacrylamide minigels, 12% (for AQP2 and ROMK) or 8% (for NKCC2 and TRPM6). After transfer by electroelution to nitrocellulose membranes (PolyScreen, PVDF Transfer; NEN Life Science, Boston, Mass., USA), blots were blocked with 5% milk and 0.1% Tween 20 in phosphate-buffered saline (NaCl 8.7 g/l, dibasic phosphate 7.2 mM and monobasic phosphate 2.8 mM) for 1 h. Blots were then incubated with anti-AQP2 antibody (1:2,000), NKCC2 antibody (1:1,000), ROMK (1:500) or TRPM6 antibody (1:500). The labeling was visualized with horseradish peroxidase-conjugated secondary antibody (anti-rabbit IgG, diluted 1:2,000, or anti-goat IgG, diluted 1:5,000; Sigma) using an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Piscataway, N.J., USA).

Quantitation of Renal Protein Levels

The enhanced chemiluminescence films presenting bands within the linear range were scanned using the ImageJ (National Institute of Mental Health, Bethesda, Md., USA). These bands were normalized through evaluation of densitometric actin protein abundance. Bands corresponding to protein expression of AQP2, NKCC2, ROMK, and TRPM6 were expressed as percentages of control.

Table 1. Body weight and hemodynamic measurements in HC and NC rats

	Body weight g	Blood pressure mm Hg	Renal blood flow ml/min/ 100 g b.w.	Renal vascular resistance mm Hg/ ml/min
NC (n = 8)	229.2 ± 3.8	106.2 ± 5.3	3.80 ± 0.18	12.3 ± 0.7
HC (n = 9)	233.8 ± 6.5	108.0 ± 3.2	3.92 ± 0.22	12.3 ± 1.0

Data are expressed as mean ± SEM. There were no differences between groups.

Table 2. Characteristics of HC and NC rats during metabolic cage studies

	NC	HC
Number of rats	8	8
Final body weight, g	233 ± 4	214 ± 5
Feed intake, g/day	24.9 ± 1.8	24.1 ± 0.9
Water intake, ml/day	21.4 ± 3.5	35.2 ± 1.5**
Serum concentrations		
Na, mM	142 ± 1	142 ± 1
K, mM	4.84 ± 0.18	4.41 ± 0.26
Mg, mM	0.82 ± 0.03	0.70 ± 0.02*
Ca, mM	2.38 ± 0.04	2.41 ± 0.14
P, mM	2.74 ± 0.13	3.11 ± 0.29
Cl, mM	114 ± 1	110 ± 3
Creatinine, mg/dl	0.64 ± 0.05	0.66 ± 0.04
Osmolality, mosm/kg H ₂ O	286 ± 3	290 ± 3
Total cholesterol, mg/dl	88 ± 7	240 ± 30**
Aldosterone, ng/dl	13.9 ± 3.0	39.8 ± 7.5**
Excretory rates		
Urine output, ml/day	19.0 ± 3.2	26.9 ± 0.8*
Na excretion, μmol/day	518 ± 130	460 ± 91
K excretion, μmol/day	1,212 ± 137	708 ± 96*
Mg excretion, μmol/day	5.0 ± 0.7	13.7 ± 2.1*
Ca excretion, μmol/day	6.4 ± 1.2	23.4 ± 5.3*
P excretion, μmol/day	420 ± 72	400 ± 46
Cl excretion, μmol/day	1,295 ± 281	1,343 ± 292
Creatinine clearance, ml/min/ 100 g b.w.	0.54 ± 0.07	0.60 ± 0.12
Osmolar clearance/creatinine clearance, %	2.07 ± 0.25	2.00 ± 0.29
Free water reabsorption/creatinine clearance, %	1.01 ± 0.26	0.15 ± 0.16*
Free water reabsorption/osmolar clearance, %	42.7 ± 9.0	8.62 ± 7.06**

Data are expressed as mean ± SEM.

* p < 0.05 vs. NC; ** p < 0.01 vs. NC.

Statistical Analysis

Data are expressed as means ± SE. Unpaired Student's t test was used for comparisons between groups and paired Student's t test was used for dual comparisons on the same nephron segment between baseline and Ang II treatment. The Mann-Whitney test was used for densitometric analysis. Values of p < 0.05 were considered significant.

Results

As expected, high cholesterol feeding for 8 days did not change BP, RBF or RVR (table 1). In addition, the metabolic cage studies confirmed that high cholesterol feeding for 8 days was enough to induce hypercholesterolemia without changes in GFR that was determined by creatinine clearance (table 2).

With regard to electrolyte homeostasis, all of them were in a normal range in the serum of hypercholesterolemic rats (HC) with the exception of magnesium (table 2). It was observed that HC developed hypomagnesemia with a marked increase in urinary excretion of this cation (table 2). Moreover, a marked increase in urinary excretion of calcium was also observed in HC (fig. 1), and HC had a lower capacity to excrete potassium than normocholesterolemic rats (NC) despite a significant increase in serum aldosterone (fig. 2).

Concerning renal handling of water, the urine output and the water intake were significantly higher in HC than NC. Urine osmolality and the calculus of free water reabsorption corrected for either creatinine clearance or osmolar clearance were significantly lower in HC than in NC (fig. 3).

Free Water Clearance

In another series of animals, the renal capacity to excrete water was evaluated. The free water clearance reached the maximum in both HC and NC with similar distal delivery of sodium chloride (fig. 4). Both HC and NC reduced the urinary osmolality to the same low range and it took them the same time to reach this maximal effect. However, differences in diluting capacity were observed between HC and NC when the free water clearance was correlated with the distal delivery of sodium chloride. As can be seen in figure 5, the free water clearance was low when the distal delivery of sodium chloride was also low. On the other hand, when the distal delivery of sodium chloride was elicited, the magnitude of the free water clearance in HC was proportionally lower than in NC ($F_0 = 34.96 > F_{\alpha} = 4.085$, $p < 0.0001$).

Fig. 1. Urinary excretion of magnesium (**a**) and calcium (**b**) in HC, n = 8, and NC rats, n = 8. Data are means $\pm$ SEM. $p < 0.01$ as compared with NC.

Fig. 2. Capacity to excrete potassium in HC, n = 8, and NC rats, n = 8. **a** Urinary excretion of potassium (U_KV). **b** Urinary potassium/sodium ratio (U_K/U_{Na}). **c** Trans-tubular potassium gradient (TTKG). **d** Serum aldosterone. Data are means $\pm$ SEM. $p < 0.01$ and $p < 0.02$ as compared with NC.

Na⁺,K⁺-ATPase Activity

As illustrated in figure 6, the Na⁺,K⁺-ATPase activity in cortex and outer medulla of HC did not differ from that measured in NC.

Calcium Signaling in mTAL and OMCD

[Ca²⁺]_i responses to 10⁻⁷ M Ang II were tested in isolated medullary nephron segments. Either mTAL or OMCD microdissected from NC and HC kidneys responded to Ang II (table 3). However, mTAL of HC kidneys showed higher sensitivity to Ang II than mTAL of NC kidneys (fig. 7). In contrast, the magnitude of response to Ang II in OMCD of HC did not differ from NC.

Immunoblot Using Membrane Fractions Prepared from Renal Cortex Samples to TRPM6 and Renal Medulla Samples to AQP2, NKCC2 and ROMK

As demonstrated in table 4 and illustrated in figure 8, TRPM6 protein expression was significantly reduced in the renal cortical membrane fractions of HC than in NC. In addition, NKCC2 and ROMK protein expressions were significantly higher in renal outer medulla fractions in HC than in NC. With regard to AQP2, its protein expression in renal outer medulla fraction of HC did not differ from that measured in NC.

Fig. 3. Capacity to reabsorb free water in HC, n = 8, and NC rats, n = 8. **a** Urine volume. **b** Water intake. **c** Urine osmolality (U_{osmolar}). **d** Free water reabsorption corrected for creatinine clearance ($T_{\text{cH}_2\text{O}}/C_{\text{creatinine}}$). **e** Free water reabsorption corrected for osmolar clearance ($T_{\text{cH}_2\text{O}}/C_{\text{osmolar}}$). Data are means $\pm$ SEM. $p < 0.01$ and $p < 0.05$ as compared with NC.

Fig. 4. Capacity to eliminate free water in HC, n = 6, and NC rats, n = 7. **a** The maximal capacity to dilute urine determined by free water excretion corrected for inulin clearance ($C_{\text{H}_2\text{O}}/C_{\text{inulin}}$). **b** The urinary osmolality (U_{osmolar}) reached at the maximal capacity to dilute urine. **c** Time elapsed to reach the maximal capacity to dilute urine. **d** Distal delivery of sodium chloride corrected for inulin clearance ($\text{DD}_{\text{NaCl}}/C_{\text{inulin}}$). Data are means $\pm$ SEM.

Fig. 5. Magnitude of the free water clearance corrected for inulin clearance (C_{H_2O}/C_{inulin}) plotted against the distal delivery of sodium chloride corrected for inulin clearance (DD_{NaCl}/C_{inulin}) in HC, $n = 6$, and NC rats, $n = 7$.

Fig. 6. Na^+,K^+ -ATPase activity measured in the cortex and the outer medulla in HC, $n = 6$, and NC rats, $n = 6$. Data are means $\pm$ SEM.

Fig. 7. In vitro studies using cytosolic $[Ca^{2+}]_i$ responses to 10^{-7} M Ang II measured in a medullary segment of the mTAL (a) and an outer medullary collecting duct (OMCD) (b) microdissected from HC and NC rats. Data are mean $\pm$ SEM. $p < 0.05$ as compared with NC.

Table 3. $[Ca^{2+}]_i$ responses to 10^{-7} M Ang II in medullary nephron segments microdissected from kidneys of HC and NC rats

	mTAL				OMCD			
	n	basal	Ang II	$\Delta[Ca^{2+}]_i$	n	basal	Ang II	$\Delta[Ca^{2+}]_i$
NC	7	66 ± 6	$109 \pm 11^*$	42 ± 8	7	88 ± 8	$130 \pm 16^*$	42 ± 9
HC	6	70 ± 7	$135 \pm 8^*$	$65 \pm 12^{**}$	7	101 ± 9	147 ± 11	47 ± 5

Data are expressed as mean $\pm$ SEM.

$\Delta[Ca^{2+}]_i$ = Variation in intracellular calcium concentration.

* $p < 0.01$ vs. basal; ** $p < 0.05$ vs. mTAL of NC and OMCD of NC and HC.

Discussion

This study showed that high cholesterol feeding in rats induced hypomagnesemia with high magnesium urinary losses due to a reduction in the expression of epithelial

Mg^{2+} channel in the renal cortex. Additionally, increases in urinary excretion of Ca^{2+} and a reduction in the capacity to excrete K^+ were also observed in HC. All these tubular dysfunctions occurred in the absence of a vascular mechanism.

Fig. 8. Immunoblotting of membrane fractions prepared from kidney cortex and medulla samples: AQP2 (a), NKCC2 (b), ROMK (c), and TRPM6 (d).

In agreement with our previous study, BP, GFR, RBF and RVR values remained within the normal range in the rats feeding a high cholesterol diet for 8 days [6]. Thus, the results will be analyzed considering the characteristics of each nephron segment transport in order to verify which nephron segment could be targeted by hypercholesterolemia.

The possibility that changes in the proximal tubule function occurred was not considered because the phosphorus filtered is reabsorbed in the proximal tubule and the urinary excretion of phosphorus was similar in HC and NC. Moreover, the distal delivery of sodium chloride measured in free water clearance experiments did not differ between HC and NC.

The chances of impairment in TAL would be expected because HC showed high magnesium and calcium urinary losses and less capacity to reabsorb and eliminate free water. However, urinary sodium excretion in HC was similar to NC.

Table 4. Quantitation of immunoblotting of membrane fractions prepared from kidney cortex and medulla samples; densitometric analysis of all samples from kidneys of HC and NC rats

	AQP2	NKCC2	ROMK	TRPM6
NC (n = 8)	100.5 ± 3.0	102.4 ± 3.0	106 ± 4.4	101.4 ± 1.5
HC (n = 8)	98.0 ± 5.5	138.1 ± 4.4**	128 ± 8.1*	66.6 ± 3.1**

Data are expressed as mean ± SEM.

* p < 0.05 vs. NC; ** p < 0.001 vs. NC.

It is well known that a NKCC2 is expressed in the apical side in TAL resulting in transcellular reabsorption of sodium chloride, which is the initial step for the counter-current mechanism. For this reason, TAL is the decisive nephron segment to form concentrated or diluted urine [21–23]. In consequence of chloride reabsorption, a lumen-positive voltage is generated, allowing passive paracellular transport of Ca^{2+} and Mg^{2+} [11, 21]. The protein kinase A, 20-hydroxyeicosatetraenoic acid and nitric oxide regulate the NKCC2 by inhibiting its action [24, 25].

Considering that increments in oxidized lipoproteins increase the arachidonic acid metabolites and activate phospholipase A₂ [2], an inhibition in the NKCC2 could be expected.

Actually, an increment in the expression of NKCC2 was observed in renal medulla samples of HC that justifies the similar urinary sodium excretion in HC compared to NC. NKCC2 expression may be elicited due to a decrease in renal nitric oxide production caused by hypercholesterolemia. Studies by our laboratory in which rats were also fed a high-cholesterol diet showed benefits in renal function with L-arginine supplementation, suggesting decreases in nitric oxide production [4, 5]. In a recent study, Kopkan et al. [26] demonstrated that infusions of cholesterol into the renal artery in rats induced antinatriuresis and antidiuresis. They showed that these effects were mediated by a decrease in nitric oxide production which stimulated the NKCC2.

In vitro tests measuring variations in $[\text{Ca}^{2+}]_i$ demonstrated that HC have also shown high sensitivity to Ang II in mTAL. This finding may be associated with the increases in the expression of NKCC2 in renal medulla samples of HC.

Taken together, the hypothesis that high urinary losses of divalent cation were due to impairment in TAL was not supported.

The distal nephron segment was also evaluated and the expression of TRPM6 was measured. This divalent cation-permeable channel is located at the luminal side of the distal convoluted tubule and is responsible for the influx of Mg^{2+} across the luminal membrane [11]. A reduced expression of TRPM6 in renal cortical of HC was observed, which explains high losses of Mg^{2+} in urine and the development of hypomagnesemia. Expression of other transporter proteins, such as TRV5 that is responsible for calcium reabsorption in distal convoluted tubule, may be also reduced. In addition, the free water clearance results also suggest impairment in distal convoluted tubule. Both HC and NC reached the maximum capacity to dilute urine. However, when the delivery of NaCl elicited, the free water clearance in HC was proportionally lower than in NC. The urinary diluting mechanism requires a generation of hypotonic fluid to reach the collecting duct. Thus, reduction in the ionic transport capacity in distal convoluted tubule may prevent a less hypotonic fluid from reaching the collecting duct in conditions of maximum free water diuresis.

With regard to the collecting duct, the $[Ca^{2+}]_i$ responses to Ang II and the expression of AQP2 did not differ between HC and NC, suggesting that high-cholesterol feeding did not induce abnormalities in this nephron segment. However, a reduction in the capacity to excrete K^+ was observed in HC.

It is well established that collecting duct cells are responsible for K^+ secretion which is coupled with Na^+ reabsorption [27]. The Na^+,K^+ -ATPase present in basal membrane releases the energy, allowing sodium reabsorption through the amiloride-sensitive epithelial Na^+ channel (ENaC). A lumen-negative voltage is generated, favoring the secretion of K^+ through ROMK channels. Aldosterone plays an important role by increasing the activity of Na^+,K^+ -ATPase and ENaC function which favors K^+ secretion [27].

In this study, a significant increment in serum aldosterone was observed in HC. The possibility of the activation of the renin-angiotensin system was thought, but it was not justified. The plasma chloride levels and the urinary excretion of chloride in HC were within the normal range, suggesting euvoolemia in these animals. Moreover, Andrade et al. [5], using the same protocol to induce hypercholesterolemia, demonstrated that changes in volume status do not occur in HC. Thus, a possible explanation for high aldosterone serum levels may be attributed to the need to maintain potassium homeostasis. In chronic renal failure, high levels of aldosterone are present even in the presence of expanded extracellular volume [28, 29]. In recent reviews, Arroyo et al. [30] and Hoorn et al. [31] described the aldo-

sterone paradox in which aldosterone plays a role in regulating K^+ in collecting duct cells in the absence of Ang II action. In this situation, aldosterone is required to maintain K^+ homeostasis and hypovolemia is not present. The mechanism is not totally known, but interactions of aldosterone and several serine/threonine kinases, especially WNK4, with ion transport proteins have been suggested, like activation of ROMK and ENaC channels.

In this study, the expression of ROMK was increased in HC, but the capacity to excrete K^+ was reduced and the Na^+,K^+ -ATPase activity did not increase in HC in the presence of high aldosterone serum levels.

The Na^+,K^+ -ATPase activity in HC was twofold higher in homogenates from the outer medulla than from the cortex, which is considered within the normal range [7, 32, 33]. It is possible that Mg^{2+} deficiency could be responsible for the absence of increments in the Na^+,K^+ -ATPase activity in HC. We may hypothesize that a difficulty to maintain K^+ homeostasis may take a place, which resulted in an increment in aldosterone secretion that elicited the expression of ROMK by a stimulation of WNK4.

In conclusion, hypercholesterolemia induced tubular dysfunctions in the absence of hemodynamic abnormalities like high magnesuria due to a reduction in the expression of TRPM6 in the renal cortex, allowing the development of hypomagnesemia. Considering that hypomagnesemia is a risk factor to increase mortality rate, the control of the plasma Mg^{2+} concentration in HC patients is justified and required.

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Disclosure Statement

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