Thyroid hormone modulates ClC-2 chloride channel gene expression in rat renal proximal tubules

D Santos Ornellas, R Grozovsky, R C Goldenberg, D P Carvalho, P Fong¹, W B Cuggino¹ and M Morales

Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, RJ, Brazil, CEP 21949-900

¹Department of Physiology of the Johns Hopkins University School of Medicine, Baltimore, Maryland, 21205, USA

(Requests for offprints should be addressed to M M Morales, Instituto de Biofísica Carlos Chagas Filho-UFRJ, CCS- Bloco G, 21949-900 Rio de Janeiro, RJ, Brasil; Email: mmorales@biof.ufrj.br)

Abstract

Thyroid hormones has its main role in controlling metabolism, but it can also modulate extracellular fluid volume (ECFV) through its action on the expression and activity of Na⁺ transporters. Otherwise, chloride is the main anion in the ECFV and the influence of thyroid hormones in the regulation of chloride transporters is not yet understood. In this work, we studied the effect of thyroid hormones in the expression of ClC-2, a cell volume-, pH- and voltage-sensitive Cl⁻ channel, in rat kidney. To analyze the modulation of ClC-2 gene expression by thyroid hormones, we used hypothyroid (Hypo) rats with or without thyroxine (T₄) replacement and hyperthyroid (Hyper) rats as our experimental models. Total RNA was isolated and the expression of ClC-2 mRNA was evaluated by a ribonuclease protection assay, and/or semi-quantitative

RT-PCR. Renal ClC-2 expression decreased in Hypo rats and increased in Hyper rats. In addition, semi-quantitative RT-PCR of different nephron segments showed that these changes were due exclusively to the modulation of ClC-2 mRNA expression by thyroid hormone in convoluted and straight proximal tubules. To investigate whether thyroid hormones action was direct or indirect, renal proximal tubule primary culture cells were prepared and subjected to different T₄ concentrations. ClC-2 mRNA expression was increased by T₄ in a dose-dependent fashion, as analyzed by RT-PCR. Western blotting demonstrated that ClC-2 protein expression followed the same profile of mRNA expression.

Journal of Endocrinology (2003) **178**, 503–511

Introduction

Chloride is the most abundant anion in the glomerular ultrafiltrate and its reabsorption along the nephron can occur through either paracellular or transcellular pathways. Several different proteins expressed in the renal tubule cells, including chloride channels, are responsible for the transcellular Cl⁻ transport (Aronson & Giebisch 1998). In this report we examine ClC-2 regulation by thyroid hormones as well as the participation of ClC-2 in chloride transport along the rat nephron.

ClC-2 belongs to the ClC family of voltage-gated chloride channels. Several members of the ClC family are involved in human hereditary diseases (Waldegger & Jentsch 2000). In both rats and humans, ClC-2 is normally expressed in many cells, including those constituting the renal epithelium. Membrane hyperpolarization, changes in cellular volume, extracellular pH and intracellular chloride concentration (Stephen & Klip 1995, Fritsch & Edelman 1997, Jordt & Jentsch 1998, Furukawa *et al.* 1999) all regulate the gating of ClC-2. Murray *et al.* (1995),

suggested an involvement of ClC-2 in the fetal development of the rat lung. The high levels of mRNA found in the kidney indicate that this channel may be important for Cl⁻ reabsorption along the nephron (Ornellas *et al.* 2002).

It is known that hormones that are involved in extracellular fluid volume (ECFV) regulation can modulate sodium chloride transport along the nephron. Two well-understood examples of hormones that regulate ECFV are atrial natriuretic factor (ANF) and aldosterone. ANF increases urinary sodium excretion by inhibiting sodium transporters in nephron segments, especially those of the inner medullary collecting ducts (Light *et al.* 1999). Aldosterone, another hormone important in regulation of ECFV, acts to increase sodium chloride reabsorption, primarily in the collecting ducts (Masilamani *et al.* 1999). Besides the classic hormones, other hormones can modulate renal function. For example, the thyroid hormones, triiodothyronine (T₃) and T₄ (thyroxine), affect both renal morphology and function. They are required for kidney growth and development, and thyroid deficiency results in decreased renal plasma flow and glomerular

filtration rate, as well as in impaired urinary concentration and dilution (Katz *et al.* 1985). In hyperthyroidism, decreased distal tubule delivery of chloride has been observed in the kidney (Shirotta *et al.* 1992). The fractional excretion of sodium and chloride in the hypothyroid (Hypo) patients was significantly higher than that in the controls patients (Liu *et al.* 1990). Hypo patients have also a decrease in glomerular filtration rate and renal plasma flow that are completely reversed by T_4 administration (Capasso *et al.* 1999); on the other hand, hyperthyroid (Hyper) subjects exhibit a significant increase in both parameters (Capasso *et al.* 1999). In addition, thyroid hormones can modulate the Na^+/K^+ ATPase (Stephen & Klip 1995) and activity of the Na^+/H^+ exchanger, mostly in the proximal tubule (Morales *et al.* 1997). Furthermore, thyroid hormones alter not only sodium transport suggesting their importance in renal transport dynamism. In Hypo rats, for example, increased sodium and bicarbonate excretion rates were observed in urine compared with control animals. This change is probably related to the modulation of Na^+/H^+ exchanger by thyroid hormone, mainly in proximal tubules (Kumar & Prasad 2002). It was also suggested the thyroid hormones activate calcium reabsorption in proximal tubules via the Na^+/Ca^{2+} exchanger through a cAMP-dependent pathway (Kumar & Prasad 2002).

In a recent publication, we showed the expression of CIC-2 mRNA in all nephron segments except cortical and outer medullary collecting ducts and its modulation by aldosterone in cortical and medullary thick ascending limbs of Henle's loop (HL), indicating a possible role of this channel in the chloride transport in the kidney (Ornellas *et al.* 2002). It was also demonstrated that CIC-2 mRNA expression is decreased in the absence of arginine vasopressin in Brattleboro rats. Here, based on the clear effect of thyroid hormones in renal transport, we evaluate the action of this hormone on CIC-2 expression in rat kidney, and in so doing, provide insight into the role of these channels in Cl^- transport along the nephron.

Materials and Methods

Animal preparation

The Ethics Committee from The Biophysics Institute, Federal University of Rio de Janeiro, previously approved all procedures and protocols using animals mentioned in this manuscript. Male adult Wistar rats (weight of 200–250 g, from Laboratório de Fisiologia Endócrina, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro) were kept in a room with a controlled lighting (12 h light:12 h darkness, lights on at 0800 h, off at 2000 h) and temperature (23–27 °C). The control group had free access to food and tap water until the time of the experiment. Hypothyroidism was induced in the second

group by the addition of 0.03% methimazole (Sigma Chemical Co., St Louis, MO, USA), an inhibitor of the synthesis of T_3 and T_4 (Ortiga-Carvalho *et al.* 1997), to the drinking water for 21 days (Hypo rats). Both groups received s.c. injections of saline during the last 10 days before killing. The third group was induced to hypothyroidism as described for the Hypo group, but also received daily s.c. injections of T_4 (Sigma) at 1 µg/100 g body weight (BW) during the 10 days before killing (Hypo+ T_4 rats). Hyperthyroidism was induced by s.c. injections of T_4 (10 µg/100 g BW) to normal animals for 10 days prior to killing (Hyper rats). All rats had unlimited access to standard rodent diet. The animals were killed by decapitation 18 h after the last injections and blood samples were then collected for hormonal evaluation (Ortiga-Carvalho *et al.* 1997).

Serum T_4 quantification

Serum T_4 was measured in whole plasma by a specific and highly sensitive RIA using specific antiserum (Kit Coat A Count; Diagnostic Products Corporation, Los Angeles, CA, USA), using a standard curve made with rat serum free of hormones.

Primary cell culture of proximal tubules

Proximal tubules were dissected from normal rats (weighing 250–300 g) by a modification of the method described by Toutain *et al.* (1991). Rats were anesthetized with pentobarbitone sodium (5 mg/100 g BW) for all procedures involving surgery. The abdominal aorta of each animal was accessed and the left kidneys were perfused with salt solution (1.4 mM NaCl, 0.5 mM KCl, 0.1 mM $Na_2PO_4 \cdot 8H_2O$, 0.3 mM sodium acetate, 0.12 mM $NaSO_4$, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 5 mM HEPES, 5.5 mM glucose). The left kidneys were dissected into fine transverse slices and incubated at 37 °C for 30 min with salt solution containing 0.125 mg/ml collagenase B (Sigma). The tissue was then transferred to Dulbecco's modified Eagle's medium and nutrient mixture F-12 Ham (DMEM/F-12, 1:1) (Sigma) and the proximal tubule micro-dissection was performed under stereoscopy. After dissection, approximately 80 segments of proximal tubules, each 2 mm long, were placed in DMEM/F-12 supplemented with 5 µg/ml insulin and 5 µg/ml transferrin (both from Gibco BRL, Grand Island, NY, USA), 10^{-7} M hydrocortisone, 5 ng/ml sodium selenite, 25 IU/ml penicillin and 25 µg/ml streptomycin (all from Sigma), as described by Toutain *et al.* (1991). The proximal tubules were attached to the bottoms of rat-tail collagen-coated, 20 mm plastic culture plates using fine needles and cultured in a humidified, 37 °C, 5% CO_2 incubator. Tubules were cultured for 20–25 days or until confluent (Toutain *et al.* 1991).

At confluence, T_4 was added to achieve 10^{-9} , 10^{-8} and 10^{-7} M final concentrations. Control cells had no T_4 added to the medium. Twenty-four hours after T_4 exposure, total RNA was isolated and the RT-PCR was performed.

Isolation of total RNA

Total RNA was extracted from whole kidney and also from proximal tubule cell primary cultures with Trizol reagent (Gibco BRL) using a modification of the single-step RNA isolation method developed by Chomczynski & Sacchi (1998).

Isolation of mRNA from rat nephronal segments (3 mm each) was performed as previously described (Morales *et al.* 2000).

Ribonuclease protection assay (RPA)

The *ClC-2* probe, corresponding to nucleotides 345–960 of the *ClC-2* rat sequence, was described before (Morales *et al.* 2001). The pTRI-rat- β -actin or pTRI-rat-GAPDH (125 and 315 bp respectively) (Ambion, Austin, TX, USA) was used as template to make probes for internal controls. Following the Maxscript Kit protocol (Ambion), all templates were transcribed *in vitro* in the presence of [α - ^{32} P]UTP, 3000 Ci/mmol (Amersham, Arlington Heights, IL, USA) with RNA polymerase T7 to generate radiolabeled antisense RNA probes. Prior to use in the RPA (RPAII kit; Ambion), all probes were evaluated with 10 μ g yeast transfer RNA in the presence and absence of RNase A and T_1 , according to the manufacturer's instructions. The undigested *ClC-2*, β -actin and GAPDH probes were 116, 63 and 91 nucleotides larger than the digested probes respectively. Total RNA (30 μ g) from rat renal tissue was mixed with each radiolabeled antisense probe (1×10^5 c.p.m./sample) along with β -actin or GAPDH internal control probes and hybridized at 45 °C for 19 h. Both RNase A and T_1 (RPAII kit; Ambion) were used to treat the samples at 37 °C for 30 min. The protected fragments were separated electrophoretically on 9 M urea gels containing 50 g/l polyacrylamide. The samples were transferred to chromatography papers and exposed to X-ray films with an intensifying screen at –90 °C prior to exposure. The density of the bands corresponding to the expected sizes of the probes was analyzed with the aid of Sigma Gel v1.1 software (Jandel Scientific, San Rafael, CA, USA).

RT-PCR

To prepare first-strand deoxyribonucleic acid (cDNA), total RNA samples isolated from whole kidney, dissected renal segments and primary proximal tubule cell cultures were firstly primed with oligodeoxythymidilic primer and

then reverse transcribed with SuperScript (Gibco BRL) at 37 °C for 60 min.

PCR amplification of the synthesized cDNA utilized the following reaction conditions: 0.2 μ M of the primers, 0.2 μ M of each deoxynucleotide triphosphate and 50 mM KCl, 10 mM Tris-HCl (pH 9.3) and 1.5 mM $MgCl_2$ plus 2.5 U of Amplitaq (Perkin Elmer, Norwalk, CT, USA). Primers for *ClC-2* (sense 5'-CTATGCCATCGCGTC TG-3' and antisense 5'-GAAGTCGAGTCGGAACCG-3') corresponding to nucleotides 345–362 and 943–960 of rat *ClC-2* cDNA sequence (Thiemann *et al.* 1992) respectively, were synthesized. PCR cycling parameters were: 35 cycles of denaturation (94 °C, 1 min), annealing (55 °C, 1 min) and extension (72 °C, 1 min).

To detect *ClC-2* mRNA expression along the rat nephron, procedures for dissection of nephron segments (3 mm each) and reverse transcription of mRNA were followed as described previously (Katz *et al.* 1985). These experiments were performed at least seven to nine times for each segment dissected from different rats.

Semi-quantitative RT-PCR was used to compare the expression of *ClC-2* in each of the following nine groups: (i) glomeruli, (ii) proximal convoluted tubule (PCT), (iii) proximal straight tubule (PST), (iv) cortical and (v) medullary thick ascending limbs (cTAL and mTAL respectively), (vi) thin limbs of HL, and the (vii) cortical, (viii) outer and (ix) inner medullary collecting tubules (CCD, OMCD and IMCD respectively) dissected from the following groups of rats: control, Hypo, Hypo+ T_4 and Hyper. PCR conditions were the same as those described above, with the addition of rat β -actin primers (sense 5'-TATGCCAACACAGTGCTGTCTGG-3' and antisense 5'-AAGAAAGCAAGACAGTGATTGTG-3', corresponding to nucleotides 2762–2794 and 2965–2997 of the rat β -actin gene and amplifying a predicted internal control product of 226 bp) to the same RT-PCR reaction tubes. The PCR products were separated using 1.5% agarose gel electrophoresis. *ClC-2* and β -actin bands from the same sample were densitometrically analyzed and normalized by dividing the *ClC-2* values by the corresponding β -actin values.

The same technique was also used to compare the expression of *ClC-2* in both control and T_4 -treated primary proximal tubule cell cultures. T_4 (Sigma) was added at different concentrations (10^{-9} , 10^{-8} and 10^{-7} M) in the test cultures.

The semi-quantitative method of RT-PCR was validated in preliminary experiments as described before (Ornellas *et al.* 2002). The PCR conditions were the same as described in previous work (Ornellas *et al.* 2002).

Western blot analysis

Expression of *ClC-2* protein in whole rat kidney from control, Hypo, Hypo+ T_4 and Hyper groups was assessed

and compared by immunoblotting using an antibody directed toward a synthetic peptide corresponding to amino acids 888–906 of the rat *ClC-2* (Sigma). Kidneys from all rat groups were homogenized and the protein was obtained as described before (Park *et al.* 2001) and the membrane proteins (100 µg/lane) were subjected to 70 g/l SDS-PAGE and transferred to 0.45 µm Immobilon-NC nitrocellulose membranes (Sigma). We used the rabbit anti-*ClC-2* antibody (1:300 dilution) diluted in Tris-buffered saline-Tween solution (136.8 mM NaCl, 74.5 mM KCl, 24.8 mM Tris base, pH 7.4, 0.5% Tween 20) containing 30 g/l non-fat milk, at room temperature for 1 h. Sequential incubation was performed with a secondary antibody goat anti-rabbit IgG (H+L) for alkaline phosphatase (1:1000). The *ClC-2* protein bands were detected using an alkaline solution (100 mM NaCl, 5 mM MgCl₂, 100 mM Tris, pH 9.5), 75 mg/ml nitro-blue tetrazolium chloride (NBT) and 50 mg/ml 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt (BCIP) (all from Life Technologies, Rockville, IL, USA) for 5 min. As an internal control, the same experiment was performed in the absence of rabbit anti-*ClC-2*. In this condition, no bands were detected, suggesting that there was not any non-specific binding.

Alkaline phosphatase assay

To validate that primary cultures indeed were derived from proximal tubules, alkaline phosphatase activity was compared with that measured in rat kidney cortex homogenate as described before (Fine & Sakhrani 1997). After confluence, cells in the primary cell culture were lysed with a hypertonic solution (250 mM sucrose, 1 mM EDTA). Alkaline phosphatase was assayed in a reaction containing 7.5 mM *p*-nitrophenyl phosphate, 5 mM magnesium chloride, 0.1% Triton X-100 and 5 mM borate (pH 10.5) (Edelman 1975). The reaction was stopped by the addition of 100 µl 1000 mM NaOH and read at 425 nm (visible light) in a spectrophotometer (Pharmacia Biotech Ultrospec 1000; Cambridge, UK).

Statistics

Results are presented as means ± S.E.M. One-way ANOVA followed by Newman-Keuls multiple comparison test was used to compare the changes in the mRNA expression of *ClC-2*. Differences were assumed to be significant when $P \leq 0.05$.

Results

Quantification of serum T_4

For the control group, the mean level of T_4 was 44 ± 3.7 nM, whereas the Hypo group presented

undetected levels of serum T_4 ($n=10$). The Hypo+ T_4 group showed normal serum T_4 (35 ± 6.3 nM) ($n=10$). Treatment of normal animals with supraphysiological doses of T_4 for 10 days (Hyper) resulted in a significant increase in serum thyroid hormone levels to 119 ± 10 nM, about 2.7-fold ($n=10$ and $P < 0.05$).

Quantification of *ClC-2* mRNA expression in different groups

Here, we used an RPA to test and quantify the influence of thyroid hormone on *ClC-2* mRNA expression in the kidney. A 615 bp fragment corresponding to *ClC-2* was detected in RNA isolated from total rat kidney from the control animals as well as three experimental groups: Hypo, Hypo+ T_4 and Hyper. The resultant bands were then subjected to densitometric analysis ($n=9$). GAPDH signal measurements, which were hybridized in the same reaction, were then used as an internal control, against which the *ClC-2* measurements were normalized. Figure 1 expresses the mean densitometric values ratio between *ClC-2* and GAPDH in all the four groups studied. Compared with the control group, renal *ClC-2* mRNA expression in Hypo rats decreased 40% ($P < 0.05$), whereas it increased significantly by 63% in Hyper rats ($P < 0.05$). T_4 administration to Hypo rats (Hypo+ T_4) restored the expression of renal *ClC-2* mRNA to levels comparable with those observed for control rats.

Quantification of *ClC-2* mRNA along the nephron by RT-PCR

In previous experiments, we detected the expression of *ClC-2* mRNA in all nephron segments, except in the CCD and the OMCD (Ortiga-Carvalho *et al.* 1997). Indeed, in the present study, RT-PCR revealed bands of the expected size, 615 bp, in most nephron segments. Figure 2 summarizes the ratios between the mean densitometric values for *ClC-2* and β -actin for different dissected nephron segments taken from the four experimental groups. Relative to controls, this ratio increased by 47% in PCTs of Hyper rats. Hypo animals showed a decrease in PCT *ClC-2* mRNA expression of 49% ($n=6$, $P < 0.05$; Fig. 2). In PSTs, mRNA expression of *ClC-2* mRNA increased by 56% in Hyper animals and decreased by 49.3% in Hypo rats related to control ($n=6$, $P < 0.05$; Fig. 2). In both segments, PST and PCT, the expression of mRNA of *ClC-2* returned to the control values in Hypo+ T_4 rats. None of the following segments showed variation in *ClC-2* mRNA expression between groups: glomeruli ($n=5$, Fig. 2), HL ($n=5$, Fig. 2), mTAL ($n=5$, Fig. 2), cTAL ($n=5$, Fig. 2) and IMCD ($n=5$, Fig. 2). Consistent with our previous observations, *ClC-2* mRNA expression was detected in neither CCD ($n=4$, Fig. 2) nor OMCD ($n=4$, Fig. 2).

Figure 1 *ClC-2* mRNA detection by RPA assay of transcripts performed on total RNA extracted from kidneys of rats under different experimental conditions. (A) *ClC-2* (not digested probe): *ClC-2* probe not digested with RNase. *ClC-2* (Digested probe): *ClC-2* probe digest with RNase. *GAPDH* (not digested probe): *GAPDH* probe not digest with RNase. *GAPDH* (Digested probe): *GAPDH* probe digest with RNase. The protected fragments of 615 and 317 bp correspond to *ClC-2* and *GAPDH* respectively. Control, (Hypo): hypothyroid rats, (Hypo+T₄): hypothyroid rats treated with physiological doses of T₄ (1 µg/100 g BW/10 days), (Hyper): normal rats treated with high doses of T₄ (10 µg/100 g BW/10 days). In the lower panel is shown a less exposed autoradiographic film containing the *GAPDH* bands, from which the densitometric values were used to normalize the correspondent *ClC-2* values. (B) Values obtained for the ratio between the densitometric analyses of the specific activity of the *ClC-2*/*GAPDH* bands obtained from four RPA experiments performed from nine different rats in each group. The results are expressed as means ± S.E.M and values are relative to control. *Statistically different from control group. #Statistically different from Hypo+T₄ group.

ClC-2 mRNA expression modulation by thyroid hormone in proximal tubule primary culture cells

Means of densitometric values ($n=3$) showed an increase in *ClC-2* mRNA expression after T₄ administration in a dose-dependent manner. Control cell mRNA expression was taken as 100%. T₄ added to cell culture medium, at concentrations of 10^{-9} , 10^{-8} and 10^{-7} M, significantly increased rat renal primary culture proximal tubule cell *ClC-2* mRNA respectively, by 37, 79 and 100% of control levels ($n=3$, $P<0.05$) (Fig. 3).

Western blot analysis of *ClC-2* protein expression in rat kidney

The mRNA levels do not necessarily correlate directly with the amount of protein expression. To examine *ClC-2* expression in the four different experimental groups, we performed Western blot analysis using 100 µg protein for each sample group ($n=5$). A fragment of 90 kDa, corresponding to the predicted molecular mass of *ClC-2* protein, was detected in all experimental groups (Murray *et al.* 1995). Comparisons of renal *ClC-2* protein expression between groups were related to control. Renal *ClC-2* protein expression decreased significantly by 62% in the Hypo group whereas it significantly increased by 49.3% in the Hyper group ($P<0.05$). In the Hypo+T₄ group, *ClC-2* expression was restored to control levels (Fig. 4).

Discussion

ClC-2 was originally isolated from a rat heart and brain cDNA library. Its wide expression in diverse organs and epithelia has pointed to function as a housekeeping gene (Waldegger & Jentsch 2000). We note, however, that *ClC-2* is also highly expressed in the kidney (Murray *et al.* 1995) and that its role in this organ is not completely understood. Our group has previously shown that *ClC-2* is distributed in all nephron segments except in cortical and outer medullary collecting ducts (Ornellas *et al.* 2002) supporting its possible importance in the kidney. The renal *ClC-2* is modulated by aldosterone (Ornellas *et al.* 2002). Aldosterone plays an important role in ECFV regulation (Thiemann *et al.* 1992, Nasemann & Horster 1998), and its modulation of *ClC-2* is pronounced in the thick ascending limbs of HL, suggesting a possible physiological role for *ClC-2* in ECFV regulation. Because chloride is the main sodium co-ion, it is reasonable to postulate that hormones involved in ECFV regulation via an action on renal sodium transporters may also modulate renal chloride transporters. Besides the classic hormones, such as aldosterone, that are known to be involved in the regulation of NaCl transport by the kidney (Masilamani *et al.* 1999),

Figure 2 Relative expression of CIC-2 mRNA in different nephron segments obtained by amplification of CIC-2 and β -actin by semi-quantitative RT-PCR. RT (–): negative control using RNA from each segment in the absence of reverse transcriptase; M.W: 100 bp ladder (Gibco BRL). Different nephron segments were stereoscopically dissected and assayed by semi-quantitative RT-PCR. The respective graphics represents the means $\pm$ S.E.M. for the ratio between the densitometric values of CIC-2 and β -actin bands obtained in RT-PCR experiments. * $P \leq 0.05$ at minimum vs each other in each case. Glomeruli ($n=5$), PCT ($n=7$), PST ($n=7$), cTAL ($n=5$), mTAL ($n=5$), HL ($n=5$), OMCD ($n=4$), CCD ($n=4$), IMCD ($n=5$). Control, hypothyroid (Hypo) rats, (Hypo+T4) hypothyroid rats treated with physiological doses of T_4 (1 μ g/100 g BW/10 days) and normal rats treated with high doses of T_4 (10 μ g/100 g BW/10 days) (Hyper).

other hormones, such as thyroid hormone, are also capable of regulating the ECFV via modulation of nephron ion and fluid transport. Thyroid hormones has been shown to increase fluid reabsorption in rat proximal tubules (Capasso *et al.* 1999). Importantly, thyroid hormone modulates the

expression of Na^+/K^+ ATPase mRNA and protein, and hence regulates the activity of this critical component of renal sodium transport (McDonough *et al.* 1988, Azuma *et al.* 1993, Barlet *et al.* 1995). In proximal tubule, thyroid hormone acts on the Na^+/H^+ exchanger to change

Figure 3 Effect of different concentrations of T_4 on mRNA expression of *ClC-2* in proximal tubule primary cultured cells using semi-quantitative RT-PCR. *Statistically different from control ($n=3$, $P<0.05$). #Statistically different from control and 10^{-9} M groups ($n=3$, $P<0.05$). In all experiments the *ClC-2* expression was expressed as the ratio between *ClC-2* (615 bp) and the corresponding β -actin (125 bp) band intensity; both originated in the same reaction tube. The graphics represent the mean of the three independent experiments. Insert: M.W., molecular weight (lane 1), RT (-) (lane 2), Control, experimental control (lanes 3–5), cells treated with 10^{-9} M (lanes 6–8), 10^{-8} M (lanes 9–11) and 10^{-7} M (lanes 12–14) T_4 .

intratubular acidification dynamics (Morales *et al.* 1997). Alteration of renal function in hyperthyroidism decreases distal tubule delivery of chloride (Shirota *et al.* 1992). Thus, the notion that thyroid hormones acts by regulating the expression of different sodium and chloride transporters in the kidney is plausible in the light of its involvement in increasing renal fluid reabsorption (Edelman 1975).

We report here our observation that thyroid hormones modulates *ClC-2* in the rat kidney. The modulation was detectable in total rat kidney, and our RPA results showed a decrease of *ClC-2* mRNA expression in Hypo rats compared with control levels. Hyper rats that received supra-physiological doses of T_4 , had their mRNA expression increased by 1.5-fold relative to control levels. Hypo rats with hormone replacement at physiological doses showed expression of *ClC-2* mRNA at levels comparable with those measured in control rats. The same pattern of expression was observed for *ClC-2* protein in rat kidney between the studied groups. More detailed analysis, using RT-PCR on isolated nephron segments as well as cultured proximal tubule cells, showed that the results of RPA were attributable to the modulation of *ClC-2*

Figure 4 Expression of *ClC-2* protein in total membranes of renal tissue of rats subjected to different experimental conditions. The results are expressed as means $\pm$ S.E.M. Values are for the densitometric analysis of the *ClC-2* protein bands obtained in the immunoblotting experiments ($n=5$; $P<0.05$). *Statistically different from control. Insert: a representative immunoblot for *ClC-2* in membrane extracts prepared from rat kidneys: control, hypothyroid rats (Hypo), hypothyroid rats treated with physiological doses of T_4 ($1 \mu\text{g}/100 \text{ g BW}/10 \text{ days}$) (Hypo+ T_4) and normal rats treated with high doses of T_4 ($10 \mu\text{g}/100 \text{ g BW}/10 \text{ days}$) (Hyper). One hundred micrograms of proteins were loaded into each lane. The blot was probed with the anti-*ClC-2* antibody (1:1000) and the signals were obtained after incubation with NBT and 50 mg/ml BCIP.

message only in rat PCT and PST respectively. The specific thyroid hormones action in proximal tubules is consistent with the fact that this nephron segment has the greatest density of thyroid hormone receptors and is thus more responsive to the action of thyroid hormones (Barlet *et al.* 1995). This characteristic of the proximal tubule may be due to its high metabolic activity, an attribute that is necessary for its role in transporting ions and hence 70% of the total filtered fluid (Capasso *et al.* 1999).

We noted a statistically significant dose-dependent increase in *ClC-2* mRNA expression in T_4 -treated rat proximal tubule primary cell cultures. This modulation indicates a direct action of T_4 on *ClC-2* mRNA expression in PST and PCT epithelial cells in rat kidney, segments where *ClC-2* mRNA occurred in high abundance. However, our findings do not rule out an indirect action of thyroid hormone on *ClC-2* mRNA expression in proximal tubules. In other words, thyroid hormones could be inducing the release of other hormones that in turn affect *ClC-2* mRNA expression. For example, in Hypo rats, the reduction of plasma renin activity consequently decreases plasma concentrations of angiotensin I and II, as

well as aldosterone (Asmah *et al.* 1998, Park *et al.* 2001). Therefore, the renin–angiotensin system could be modulating the expression of many genes, such as CIC-2, under the influence of thyroid hormone (Stephen & Klip 1995).

That CIC-2 protein expression in rat kidney follows the same pattern as found for CIC-2 mRNA confirms the physiological importance of CIC-2 in this organ. Our findings also support previous work showing that the lack or decrease of plasma thyroid hormones leads to a diminished expression (Stephen & Klip 1995) and activity of Na⁺ and Cl[−] transporters (Stephen & Klip 1995), as well as the prediction that hormonal replacement with T₃ or T₄ leads to normalization or even an increase in fluid reabsorption, depending on the dose, in the proximal tubule of rat nephrons. Taken together, our findings lead us to suggest the involvement of CIC-2 in the chloride transport modulated by thyroid hormones in rat renal proximal tubules.

Acknowledgements

The present work was supported by grants from NIH (HL47122 and DK 32753), Fundação José Bonifácio (FUBJ), Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ), Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

References

- Aronson PS & Giebisch G 1998 Mechanisms of chloride transport in the proximal tubule. *American Journal of Physiology. Renal Physiology* **283** F189–F192.
- Asmah BJ, Wan Nazaimoon WM, Tan T & Khalid BAK 1998 Plasma renin and aldosterone in thyroid diseases. *Hormone and Metabolic Research* **29** 590–593.
- Azuma KK, Hensley CB, Tang MJ & McDonough A 1993 Thyroid hormone specifically regulates skeletal muscle Na⁺–K⁺ATPase α 2- and β 2-isoforms. *American Journal of Physiology. Cell Physiology* **275** C790–C798.
- Barlet C, Ben Abdelkhalik M & Doucet A 1995 Sites of thyroid hormone action on Na⁺–K⁺ATPase along the rabbit nephron. *Pflügers Archiv* **405** 52–58.
- Capasso G, De Tommaso G, Pica A, Anastasio P, Capasso J, Kinne R & De Santo NG 1999 Effects of thyroid hormones on heart and kidney functions. *Mineral and Electrolyte Metabolism* **25** 57–74.
- Chomczynski P & Sacchi N 1998 Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Annals of Biochemistry* **172** 157–159.
- Edelman IS 1975 Thyroidal regulation of renal energy metabolism and (Na–K)-activated adenosine triphosphatase activity. *Medical Clinics of North America* **59** 605–614.
- Fine LG & Sakhrani LM 1997 Proximal tubular cells in primary culture. *Mineral and Electrolyte Metabolism* **12** 51–58.
- Fritsch J & Edelman A 1997 Modulation of the hyperpolarization-activated Cl[−] current in human intestinal T94 epithelial cells by phosphorylation. *Journal of Physiology* **490** 115–129.
- Furukawa T, Ogura T, Katayama Y and Hiraoka M 1999 Characteristics of rabbit CIC-2 current expressed in *Xenopus* oocytes and its contribution to volume regulation. *American Journal of Physiology. Cell Physiology* **284** C500–C512.
- Jordt SE & Jentsch TJ 1998 Molecular dissection of gating in the CIC-2 chloride channel. *EMBO Journal* **16** 1592–1592.
- Katz AI, Emmanouel DS & Lindheimer MD 1985 Thyroid hormone and the kidney. *Nephron* **15** 223–249.
- Kumar V & Prasad R 2002 Molecular basis of renal handling of calcium in response to thyroid hormone status of rat. *Biochimica et Biophysica Acta* **24** 331–343.
- Light DB, Schwiebert EM, Karlson KH & Stanton BA 1999 Atrial natriuretic peptide inhibits a cation channel in renal inner medullary collecting duct cells. *Science* **243** 393–395.
- Liu XM, Bai Y & Guo ZS 1990 Study on urinary function and metabolism of water and electrolytes in primary hypothyroidism. *Zhonghua Neike Zazhi* **29** 299–302.
- Masilamani S, Kim GH, Mitchell C, Wade JB & Knepper MA 1999 Aldosterone-mediated regulation of ENaC α , β , and γ subunit proteins in rat kidney. *Journal of Clinical Investigation* **104** R19–R23.
- McDonough AA, Brown TA, Horowitz B, Chiu R, Schlotterbeck, Bowen J & Schmitt 1988 Thyroid hormone coordinately regulates Na⁺–K⁺ATPase α - and β -subunit mRNA levels in kidney. *American Journal of Physiology. Cell Physiology* **254** C323–C329.
- Morales MM, Brucoli HCP, Malnic G & Lopes AG 1997 Role of thyroid hormones in renal tubule acidification. *Molecular and Cellular Biochemistry* **154** 18–21.
- Morales MM, Capella MA, Sanches MV, Lopes AG & Guggino WB 2000 Modulation of the mdr-1b gene in the kidney of rats subjected to dehydration or a high-salt diet. *Pflügers Archiv* **43** 357–372.
- Morales MM, Nascimento DS, Capella MA, Lopes AG & Guggino WB 2001 Arginine vasopressin regulates CFTR and CIC-2 mRNA expression in rat kidney cortex and medulla. *Pflügers Archiv* **443** 202–211.
- Murray CB, Morales MM, Flotte TR, McGrath-Morrow AS & Guggino WB 1995 CIC-2: a developmentally dependent chloride channel expressed in the fetal lung and downregulated after birth. *American Journal of Respiratory Cell and Molecular Biology* **12** 597–604.
- Nasemann J & Horster MF 1998 Aldosterone induces chloride transport in the cortical collecting tubule. *Journal of Developmental Physiology* **9** 528–537.
- Ornellas DS, Nascimento DS, Christoph DH, Guggino WB & Morales MM 2002 Aldosterone and high NaCl diet modulate CIC-2 chloride channel gene expression in rat kidney. *Pflügers Archiv* **444** 193–201.
- Ortiga-Carvalho TM, Polak J, MacCann S & Pazos-Moura CC 1997 Effect of thyroid hormones on pituitary neuromedin B and possible interaction between thyroid hormones and neuromedin B on thyrotropin secretion. *Regulatory Peptides* **78** 48–53.
- Park CW, Shin YS, Ahn SJ, Kim SY, Choi EJ, Chang YS & Bang BK 2001 Thyroxine treatment induces upregulation of renin–angiotensin–aldosterone system due to decreasing effective plasma volume in patients with primary myxoedema. *Nephrology, Dialysis, Transplantation* **7** 1899–1907.
- Shirota T, Shinoda T, Yamada T & Aizawa T 1992 Alteration of renal function in hyperthyroidism: increased tubular secretion of creatinine and decreased distal tubule delivery of chloride. *Metabolism* **41** 402–405.
- Stephen EH & Klip A 1995 Hormonal regulation of the Na⁺–K⁺ATPase: mechanisms underlying rapid and sustained changes in pump activity. *American Journal of Physiology. Cell Physiology* **279** C295–C311.

Thiemann A, Gründer S, Pusch M & Jentsch TJ 1992 A chloride channel widely expressed in epithelial and non-epithelial cells. *Nature* **357** 58–70.

Toutain H, Vauclin-Jacques N, Fillastre JP & Morin JP 1991 Biochemical, functional, and morphological characterization of a primary culture of rabbit proximal tubule cells. *Experimental Cell Research* **194** 9–19.

Waldegger S & Jentsch TJ 2000 From tonus to tonicity: physiology of CLC chloride channels. *Journal of the American Society of Nephrology* **11** 1331–1339.

Received in final form 5 June 2003

Accepted 5 June 2003