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Thyroid Hormone Beta Receptor Mutation Causes Renal Dysfunction and Impairment of ClC-2 Chloride Channel Expression in Mouse Kidney

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Key Words

Thyroid hormones • Δ337T • TR-β receptor • Thyroid hormone resistance syndrome • ClC-2 • Ion channel • Kidney

Abstract

Background/Aims: Mutations in the thyroid hormone receptor β (TR-β) gene result in resistance to thyroid hormone (RTH). Mutation Δ337T in the TR-β gene has been shown to have the characteristics of RTH syndrome in mice. The aim of this work was to study the possible involvement of TR-β receptor in thyroid modulation of ClC-2 in mouse kidney. Methods: Expression of mouse (Δ337T and normal C57BL/6) renal RNA and protein expression were studied by reverse transcriptase-polymerase chain reaction and Western blot, respectively, in mice with hyper- or hypothyroidism. Renal function was studied by analysis of urinary electrolyte excretion. Studies of the ClC-2 promoter region were performed in immortalized renal proximal tubule (IRPT) cells. Results: In RTH syndrome mice (Δ337T), renal dysfunction was found to be associated with changes in the fractional excretion of sodium (FE_{Na}) and chloride (FE_{Cl}). ClC-2 chloride channel mRNA and protein expression were found to be decreased by 40% in heterozygous and homozygous mutant mouse kidneys and high levels of plasma thyroid hormone were detected in both groups. Hypothyroidism induced by methimazole decreased the renal expression of ClC-2 in normal mice but not in Δ337T mutant mice. In *in vitro* studies performed on IRPT cells subjected to thyroid hormone treatment, the promoter region of the ClC-2 chloride channel was stimulated in a dose-dependent manner. Conclusions: This work emphasizes the importance of thyroid hormone in electrolyte handling along the nephron and suggests its participation in renal ClC-2 gene transcription via the TR-β receptor pathway.

Introduction

Several isoforms of the CLC family of voltage-gated chloride channels are involved in several physiological functions in human tissues. Mutations in some of these isoforms cause human hereditary diseases. Mutation in: (a) ClC-1, the major skeletal muscle chloride channel leads to myotonia [1, 2]; (b) ClC-K, a kidney-specific chloride channel, leads to either nephrogenic diabetes insipidus or Bartter syndrome [2, 3]; and (c) ClC-5 leads to Dent

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disease [2, 4].ClC-2 is a member of the CLC family and is broadly expressed in a variety of tissues [5], however, its function has not been fully elucidated. ClC-2 was found to be important in the fetal stage of lung development [6] and disruption of this chloride channel in knockout mice was found to lead to a degeneration of the seminiferous tubule of the testis and the retina [7]. These findings strongly suggest its participation in secretory processes in epithelia.

ClC-2 is abundantly expressed in the kidney and the channel is activated by different stimuli commonly found in the renal environment, such as: (a) membrane hyperpolarization [8]; (b) increase in cellular volume [9]; (c) a decrease in extracellular pH [10]; and (d) increase in intracellular chloride concentrations [10].

Our group have shown that ClC-2 is modulated by vasopressin and aldosterone in rat kidney indicating the importance of this channel in the transport of chloride modulated by hormones classically involved in the regulation of extracellular fluid volume [11, 12]. We also found that other hormones, such as estrogen, can lead to significant increase in renal ClC-2 expression, mainly in proximal tubules.

Thyroid hormones, triiodothyronine (T_3) and thyroxine (T_4) are important in renal physiology and their actions are mediated by high affinity to nuclear thyroid hormone receptors (TRs) that bind T_3. There are several T_3-binding TR isoforms (β1, β2, β3, Δβ3, α1) and isoforms that do not bind T3 (α2, Δα1 and Δα2) derived from α- and β-TR genes by alternative splicing of the primary transcripts or derived from alternative promoters [13,14]. TR mRNA expression depends on development and cellular differentiation [15]. TR-α1, TR-α2 and TR-β1 mRNAs are expressed in most tissues, including the kidney. TR-β1 is most abundantly expressed in liver, kidney and brain; TR-β2 is expressed mainly in the pituitary gland and other sites in the brain [15]. Rat TR-β3 is expressed mainly in the liver, kidneys and lungs. Mutations in the TR-β gene generate mutant receptors with impaired binding of T_3 causing the human syndrome of resistance to thyroid hormone (RTH) [16, 17]. Transgenic mice generated by mutation in TR-β present characteristics similar to human RTH, such as high levels of T_3, T_4 and thyroid-stimulating hormone (TSH), hyperplasic thyroid gland, increased basal heart frequency and inner ear defects, and is a excellent model to study the action of thyroid hormones [17–19].

It is well known that thyroid hormones are important for renal morphology and function [20] and they are essential for kidney growth and development. Thyroid deficiencies can result in decreased renal plasma flow and glomerular filtration rate (GFR), as well as impaired urinary concentration and dilution [20]. A decrease in distal tubule delivery of chloride was found in patients with hyperthyroid Graves disease [21]. Previous studies from our group have shown that ClC-2 chloride channel gene expression is modulated by thyroid hormone [22] but the mechanisms involved in this phenomenon remain unclear. The main goal of the present work was to study the possible involvement of TR-β receptor in thyroid modulation of ClC-2 in the kidney and possible involvement of this channel promoter region in this process.

Materials and Methods

Animal treatment

Male adult 129/C57BL/6 strain mice weighing 20–25 g (from Laboratory of Molecular Endocrinology, IBCCf, UFRJ) were used. Animals were kept in a room with controlled lighting (12-h light/12-h dark, lights on at 7:00 a.m., lights off at 7:00 p.m.) and temperature (23-26°C) with free access to food and tap water. All animal experiments were performed according to the NIH Guidelines for the Care and Use of Laboratory Animals and protocols were approved by our institutional animal ethics committee. Hypothyroidism was induced in 129/C57BL/6 mice (Hypo group) by the addition of 0.1% methimazole (MMI; Sigma Chemical Co., St. Louis, MO, USA), an inhibitor of the synthesis and release of 3,5,3′-L-triidothyronine and thyroxine (T_3 and T_4, respectively) [23], to the drinking water for 45 days. The Hypo+T4 group of animals were Hypo mice subjected to daily subcutaneous injections of thyroxine (1 μg /100 g body weight (BW); Sigma) for 15 days before sacrifice. The Hypo group received subcutaneous injections of saline for the last 15 days before sacrifice.

Hyperthyroidism was induced by daily subcutaneous injection of thyroxine (10 μg /100 g BW; Sigma) in normal animals for 15 days (Hyper group).

All animals were killed by decapitation 18 h after the last injections and blood samples were then collected for hormonal evaluation [23]. TR-β (Δ337T) mutated mice, homozygous (Homo group) or heterozygous (Hetero group), were provided by Molecular Endocrinology Laboratory, Federal University of Rio de Janeiro. These mutated animals were generated by Dr Fredric E. Wondisford's group, Department of Medicine, Johns Hopkins University, Baltimore, MD, USA. This mutation avoids the TR binding to T3. TR-β (Δ337T) mutated homozygous and heterozygous mice were subjected to treatment with MMI as described above and the groups were identified as the Homo MMI group and the Hetero MMI group, respectively.

Radioimmunoassay

Plasma T_4 was measured by radioimmunoassay using specific antibodies (Kit Coat A Count, Diagnostic Products Cor-

poration, Los Angeles, USA), according to the manufacturer's suggested protocol.

Isolation of total RNA

Total RNA was extracted from kidney or cell cultures using Trizol® reagent (Invitrogen, BRL, Grand Island, NY, USA), following the manufacturer's suggested protocol. Purified total RNA was treated with 1 U/μl of ribonuclease (RNase)-free deoxyribonuclease (DNase) I (GIBCO BRL, Grand Island, NY, USA) for 30 min to eliminate any genomic DNA contamination.

Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR analysis was carried out on 500 ng of total RNA using reverse transcriptase SuperScript™ (GIBCO BRL, Grand Island, NY, USA).

To prepare first-strand deoxyribonucleic acid (cDNA), total RNA samples isolated from whole kidney were first primed with oligo-deoxythymidylic (oligo dT) primer and then reverse-transcribed with SuperScript (Gibco BRL, Grand Island, NY, USA) at 37°C for 60 min. PCR amplification of the synthesized cDNA was carried out under the following reaction conditions: 0.2 mmol/l of the primers, 0.2 mmol/l of each deoxynucleotide triphosphate (dNTP) and 50 mmol/l of KCl, 10 mmol/l of Tris–Cl (pH 8.3) and 1.5 mmol/l of $MgCl_2$ plus 2.5 U of Amplitaq (Perkin Elmer, New Jersey, USA). Primers for ClC-2 (sense 5′-CTA TGC CAT CGC GTC TG-3′ and antisense 5′-GAA GTC GAG TCG GAA CCG-3′) GenBank accession no. X64139 [5], 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). Primers for rat ClC-2 were used after searching for homology in the BLAST program (GenBank accession no. BC137625.1). Preliminary experiments validated the semi-quantitative RT-PCR methods used as described previously [22, 24].

Semi-quantitative RT-PCR was used to compare the expression of ClC-2 in each of the following groups: (1) control, (2) hypothyroid (Hypo), (3) hypothyroid replaced with thyroxine (Hypo + T_4), (4) hyperthyroid (Hyper), (5) heterozygous, (6) (Hetero), (7) homozygous (Homo), (8) control treated with MMI, (9) heterozygous treated with MMI (Hetero MMI), (10) homozygous treated with MMI (Homo MMI). PCR conditions were the same as those described earlier, with the addition of mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene primers (sense 5′-CAA CCA ACT TGC TTA GCC C-3′ and antisense 5′-GCT CTG GGA TGA CCT TGC-3′, corresponding to nucleotides and amplifying a predicted internal control product of 206 bp, GenBank accession no. XM_906931) to the same RT-PCR reaction tubes. ClC-2 and GAPDH bands amplified in the same sample were analyzed by densitometry and normalized by dividing the target gene values by the corresponding GAPDH values. Sigma Gel v1.1 (Jandel Scientific, Corte Madera, CA, USA) software was used for densitometric analysis of the bands.

Western blot analysis

Expression of ClC-2 protein in whole mouse kidney from different groups of animals was assessed and compared by immunoblotting using an antibody directed towards a synthetic peptide corresponding to amino acids 808–906 of the rat ClC-2 (Sigma-RBI, USA). This antibody specifically recognizes ClC-2 protein [5]. Kidneys from all mouse groups were homogenized and protein was obtained as described previously [25]. Membrane proteins (200 μg/lane) were subjected to 70 g/l sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred to 0.45-mm Immobilon-NC nitrocellulose membranes (Sigma). We used rabbit anti-ClC-2 antibody (1:300 dilution) diluted in Tris-buffered saline (TBS, pH 7.4) solution plus 0.5% Tween 20 (TBS-T solution) containing 30 g/l non-fat milk (TBS-T+milk) at room temperature for 1 h. The specificity of this antibody has been reported previously [5]. Subsequent to incubation with a 1:1000 dilution of the alkaline phosphatase-labeled secondary antibody goat anti-rabbit IgG (H+L), detection was performed by exposure to an alkaline phosphatase substrate containing 75 mg/ml of nitroblue tetrazolium chloride (NBT) and 50 mg/ml of 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt (BCIP) (all from Life Technologies, Rockville, MD, USA) for 5 min. To rule out the possibility of non-specific secondary antibody binding, the same experiment was performed in the absence of rabbit-anti-ClC-2; no bands were detected under these conditions.

DNA transfection and ClC-2 promoter region

Immortalized renal proximal tubule (IRPT) cells were grown to confluence and the day before transfection, cells were detached by exposure to 0.05% trypsin in Ca^{2+}- and Mg^{2+}-free solution, reseeded in 6-well plates, reaching 70% confluence on the day of transfection. The cells were transiently co-transfected with 2.0 μg of ClC-2 rat promoter construct plasmid DNA [26] (kindly provided by Dr P. Zeitlin, Department of Pediatrics, Johns Hopkins University, Baltimore, MD, USA) [27] for the luciferase reporter assay and 2.0 μg of pSV-galactosidase plasmid diluted in 250 μl D-MEM without serum or antibiotic, using 6 ml of Lipofectamine 2000 reagent (Invitrogen) diluted in 250 μl D-MEM without serum and antibiotic added to the cells in a final volume of 500 μl per well. Cells were incubated at 37°C for 3 h. The medium was then removed and 2 μl of supplemented D-MEM were added per well. The cells were cultured for 24 h. After this period, the cells were washed and treated with triiodothyronine 10^{-7} M, 10^{-6} M, 10^{-5} M for 24 h at 37°C. Cells were washed twice with 3 ml of phosphate buffered saline (PBS) and then lysed in 250 μl (per well) with lysis buffer (25 mM gly-gly, 15 mM $MgSO_4$, 4 mM EGTA, Triton X (25%) and 1 mM DTT) for 20 min at room temperature. Luciferase activity was quantified using 200 ml of cell lysates, 100 ml of D-luciferin (Molecular Probes Inc., Eugene, OR, USA) and 100 μl of luciferase assay buffer (25 mM gly-gly, 15 mM $MgSO_4$, 4 mM EGTA, 15 mM KH_2PO_4, 6 mM ATP and 3 mM DTT) per sample. Light emission was detected using a TD-20/20 luminometer (Turner Designs) for 30 s at room temperature. β-Galactosidase activity utilized for the normalization of the results, was quantified using 30 μl of cell lysates, 160 μl of 2-nitrophenyl-β-D-galactopyranoside (ONPG) reagent (60 mM Na_2HPO_4, 40 mM NaH_2PO_4 and 2 mg/ml of ONPG) and 810 μl of buffer β-gal (60 mM Na_2HPO_4,

	Control	Hetero	Homo
GFR (µl/min per g KW)	755±107.82	820±68.8	710±85.83
FE_{Na} (%)	0.79±0.01	0.63±0.08	1.22±0.08*
FE_K (%)	11.04±1.71	10.27±1.25	10.83±0.89
FE_{Cl} (%)	0.83±0.04	0.83±0.04	1.41±0.08*
Urine flux (µl/g BW per 24 h)	88.12±1.1	86.25±2.4	85.87±2.57

Table 1. Comparison of renal functional characteristics of TR-β mutated mice. Normal 129/C57BL/6 (Control); homozygous (Homo) and heterozygous (Hetero) for thyroid hormone receptor β; KW, kilogram weight; BW, body weight; FE, fractional excretion. *Significantly different from control; $p<0.05$.

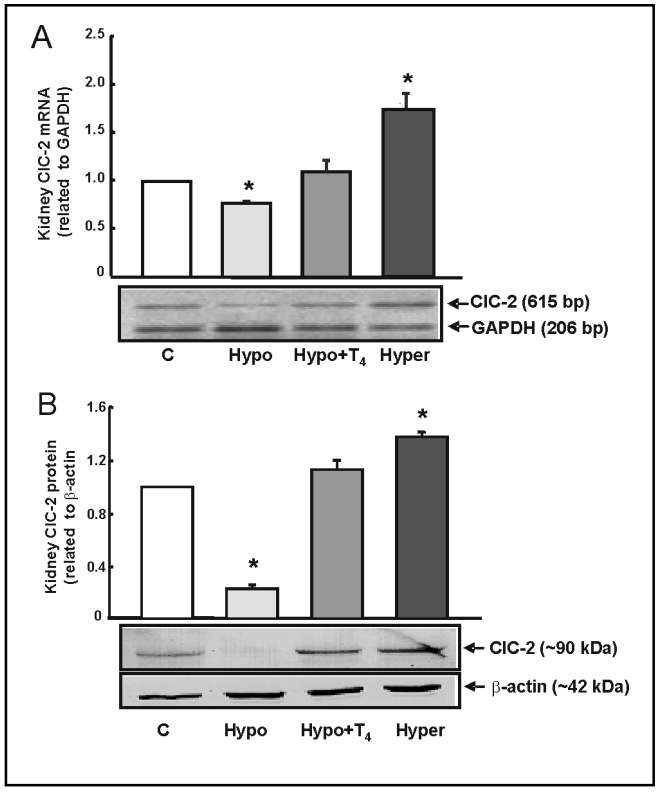

Fig. 1. Modulation of ClC-2 mRNA and protein by thyroid hormones. (A) Expression of ClC-2 mRNA in the kidney of control, hypo- and hyperthyroid mice according to semi-quantitative RT-PCR analysis. Graphs represent the mean±SEM for the ratio between the densitometric values of ClC-2 and GAPDH bands obtained in RT-PCR experiments. (B) Expression of ClC-2 protein in the kidney of control, hypo- and hyperthyroid mice according to Western blot analysis. Graphs represent the mean±SEM for the ratio between the densitometric values of ClC-2 and β-actin bands obtained in Western blot experiments. Hypothyroid mice were treated with saline (Hypo) or 1 µg thyroxine/100 g BW (Hypo+T_4) for 15 days. The Hyper group were normal mice receiving T_4 (10 µg/100 g BW) for 15 days. *$p<0.05$. Statistically different from control ($n=5$).

40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgCl_2$ and 50 mM β-mercaptoethanol) at 420 nm.

Statistical analysis

ANOVA followed by the Newman–Keuls multiple comparison test was used to compare differences between groups. Results are presented as means±SE. Differences were assumed to be significant when $p\leq0.05$.

Results

T_4 plasma levels

The T_4 plasma level for the control group was 4.29±0.41 µg/dl ($n=6$). Hypothyroid animals showed nearly undetectable levels of T_4 (0.01±0.00, $n=5$, $p<0.05$). Treatment of hypothyroid mice with a physiological dose of thyroxine (1 µg/100 g BW per day; 15 days) normalized T_4 levels. Animals treated with ten-fold higher doses of T_4 (Hyper group) developed hyperthyroidism (6.43±0.95 µg/dl, $n=6$, $p<0.05$). In heterozygous TR-β mutated mice, the plasma level of T_4 was 4.9±0.41 µg/dl ($n=6$, $p<0.05$) and for homozygous animals the level was >20 µg/dl ($n=6$, $p<0.05$).

Renal function

TR-β mutated mice showed no changes in GFR, urinary flow and fractional excretion of potassium between mutated (heterozygous and homozygous) and control groups. The heterozygous mice showed no changes in the parameters analyzed, showing renal function similar to that of normal animals. However, the fractional excretion of Na^+ and Cl^- was increased compared with control mice homozygous for the mutation of receptor β (1.215±0.083 and 1.413±0.079), respectively ($n=10$, $p<0.05$) (Table 1).

ClC-2 chloride channel expression

To test and quantify the influence of thyroid hormones on ClC-2 mRNA expression in mice kidney, we

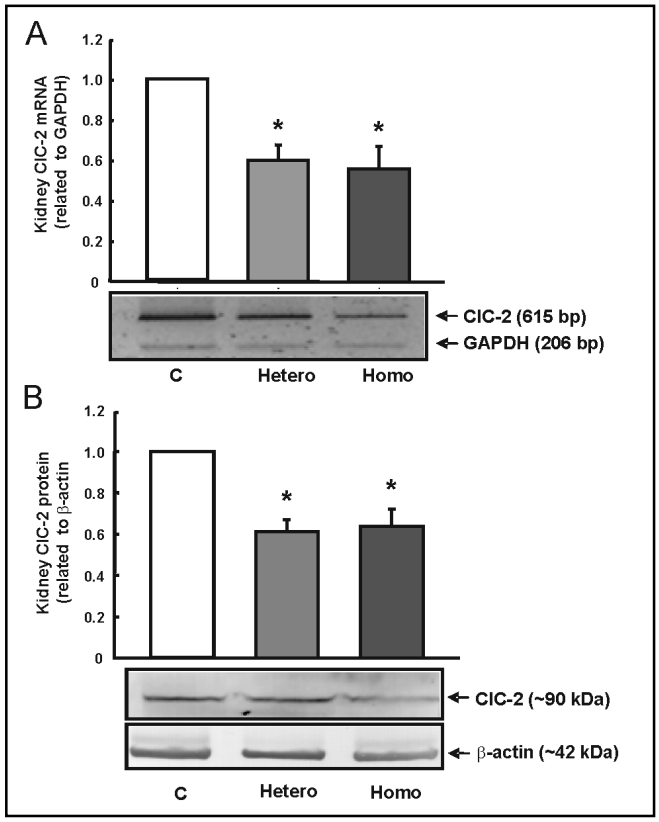

Fig. 2. Modulation of ClC-2 mRNA and protein in the kidney of TR-β mutated mice. (A) Expression of ClC-2 mRNA in the kidney of control, heterozygous and homozygous mice according to semi-quantitative RT-PCR analysis. Graphs represent the mean±SEM for the ratio between the densitometric values of ClC-2 and GAPDH bands obtained in RT-PCR experiments. (B) Expression of ClC-2 protein in the kidney of control, Hetero and Homo mice according to Western blot analysis. Graphs represent the mean±SEM for the ratio between the densitometric values of ClC-2 and β-actin bands obtained in Western blot experiments. (1) Control (C), (2) heterozygous (Hetero), (3) homozygous (Homo). *p<0.05. Statistically different from control (n=5).

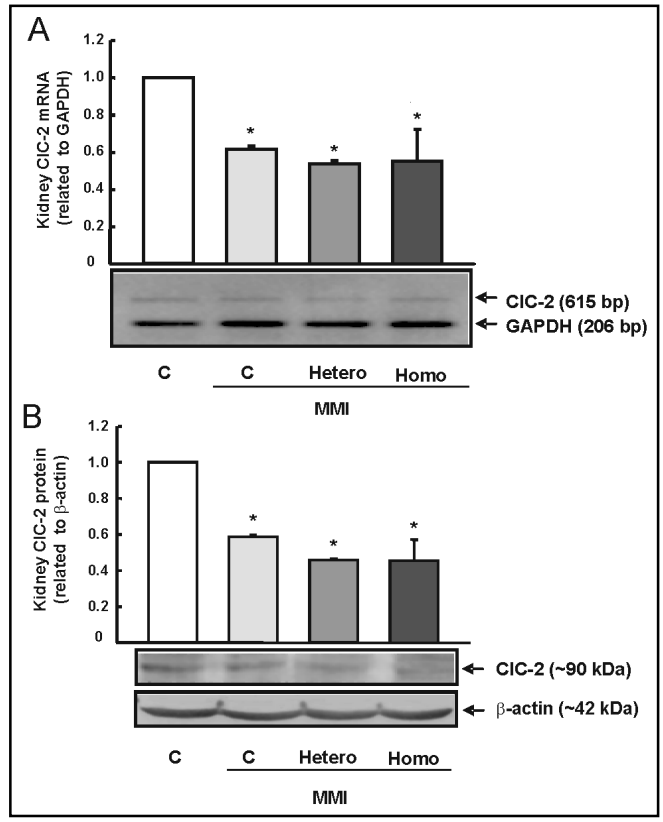

Fig. 3. Modulation of ClC-2 mRNA and protein in kidney of TR-β mutated mice (heterozygous and homozygous) treated with methimazole compared with the control group. (A) Expression of ClC-2 mRNA in the kidney of control, Hetero and Homo mice according to semi-quantitative RT-PCR analysis. Graphs represent the mean±SEM for the ratio between the densitometric values of ClC-2 and GAPDH bands obtained in RT-PCR experiments. (B) Expression of ClC-2 protein in kidney of control, Hetero and Homo mice according to Western blot analysis. Graphs represent the mean±SEM for the ratio between the densitometric values of ClC-2 and β-actin bands obtained in Western blot experiments. Control (C) was used to compare the expression of ClC-2 in each of the following groups: control (C) treated with methimazole (MMI); mutated mice (heterozygous (Hetero) and homozygous (Homo)) both treated with methimazole. *p<0.05. Statistically different from control.

used semi-quantitative RT-PCR. We observed 615 bp bands, corresponding to ClC-2 in all analyzed groups. Densitometric analysis of the bands corresponding to ClC-2 and normalized by GAPDH mRNA showed that hypothyroid animals presented a decrease in ClC-2 renal expression compared with control levels (75% of control) (n=6, p<0.05, Fig. 1A). Treatment of hypothyroid mice with a physiological dose of thyroxine (1 µg/100 g of BW/day) for 15 days normalized the amount of ClC-2 mRNA renal expression to control levels. Treatment of normal mice with a supraphysiological dose of T_4 (10 µg/100 g of BW per day) for 15 days induced an increase in ClC-2 renal mRNA expression compared with control animals (35% of control) (n=5; p<0.05, Fig. 1A). As shown in Fig. 1B, ClC-2 protein expression presented a similar pattern of expression modulation compared with that observed at the mRNA level. Lack of thyroid hormone caused a 70% decrease in ClC-2 protein expression compared with control animals (n=5; p<0.05, Fig. 1B). ClC-2 protein levels were normalized with the physi-

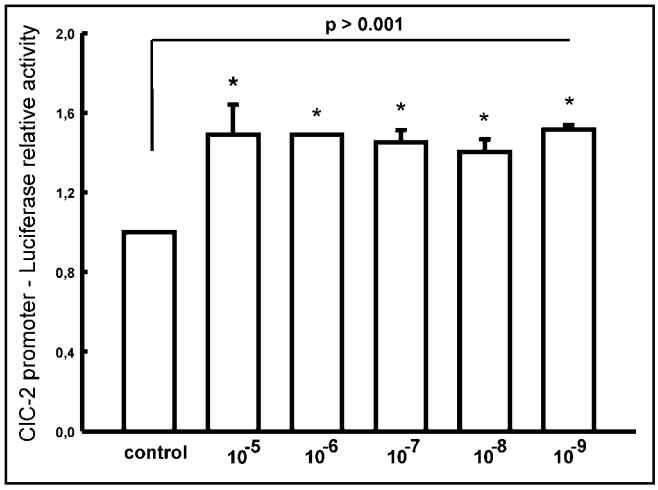

Fig. 4. Modulation of the ClC-2 chloride channel gene promoter region in IRPT cells by T_3. Graph shows luciferase activity in transfected IRPT cells without T_3 (control); or treated with 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} and, 10^{-5} M T_3. Luciferase activity was normalized by co-transfection with pSV-β-galactosidase plasmid (n=5). *p<0.0001 versus control.

ological dose of T_4 and were increased after the supraphysiological dose of thyroxine (22% of control, n=5; p<0.05, Fig. 1B).

In the studies on TR-β mutated mice, it was found that ClC-2 mRNA expression was less expressed in kidneys of the heterozygous (41% of control) and homozygous (45% of control) groups (p<0.001, n=5, Fig. 2A). ClC-2 renal protein expression analyzed by Western blot showed a decrease in the heterozygous (48% of control) and homozygous (46% of control) groups (p<0.001, n=5) compared with the control group (Fig. 2B). In addition to investigating ClC-2 chloride channel expression in TR-β mutated mice (homozygous and heterozygous), we treated the two groups of animals with MMI. We observed a similar decrease in renal ClC-2 mRNA expression in heterozygous (43% of control) and homozygous (40% of control) mice when they were treated with MMI, compared with control levels (p<0.001, n=3) (Fig. 3A). When ClC-2 renal protein expression was studied in MMI-treated heterozygous and homozygous mice, a decrease was observed in both groups (54% and 55% of control levels, respectively; p<0.001, n=4) (Fig. 3B).

ClC-2 promoter region analysis

The luciferase reporter gene assay suggests that T_3 is capable of modulating the ClC-2 gene promoter region. IRPT cells transfected with the plasmid containing the ClC-2 promoter region associated with the luciferase gene presented enzyme activity, indicating a successful transient transfection. Transfected cells treated with 10^{-9} M, 10^{-8} M, 10^{-7} M, 10^{-6} M, 10^{-5} M of T_3 led to an increase in luciferase activity of 51%, 40%, 45%, 49% and 49% (p<0.001, n=6) compared with transfected cells with no hormone treatment (control) (Fig. 4).

Discussion

The regulation of NaCl transport in the kidney is essential for the maintenance of extracellular fluid and blood pressure [27]. Most of these renal transporters are regulated by classic hormones such as aldosterone, atrial natriuretic peptide and vasopressin. Several studies have investigated the role of these hormones in the modulation of sodium (the most abundant ion in extracellular fluid) transport along the nephron. Although chloride is the major anion present in the extracellular compartment, it is not a frequent focus of hormonal regulation studies in the kidney; especially by those not classically involved in extracellular volume regulation, such as thyroid hormones.

Chloride transport along the nephron occurs mainly by the paracellular pathway [28, 29] but an important part is transported transcellularly via chloride channels. Among the many chloride channels, we highlight the CLC family of chloride channels, especially ClC-2, with high expression in the kidney. In addition, ClC-2 has characteristics that are attractive for renal physiology, such as: (a) activation by hyperpolarization of cell membrane; (b) increase in intracellular volume; (c) decrease in extracellular pH; and (d) increase in intracellular chloride concentration [2, 30]. All these characteristics are similar to those found in the interstitial space that surrounds the epithelial cells of the renal tubules (nephron) and they are altered by different hormones that can modulate renal transport such as thyroid hormones.

Thyroid hormones can affect several aspects of renal function participating in hydroelectrolytic equilibrium control. Thyroid hormones can increase fluid reabsorption in mouse proximal tubules [20, 31-32] and modulation of different ion transporters such as Na^+/H^+ was observed in the same segment of nephron [32]. At the molecular level, the expression of mRNA and protein and the activity of renal Na^+/K^+-ATPase are increased by thyroid hormones [20]. In addition, the rate of absorption of chloride in the proximal tubule was found to be diminished in studies of perfusion of proximal tubule in hy-

pothyroid rabbits [29] showing that this modulation is preserved among different species. In a previous publication, our group observed that rat renal ClC-2 mRNA and protein are modulated by thyroid hormones essentially in the proximal tubule in vivo and in vitro [22] although the mechanisms involved in this action were not elucidated. In the present work, we were able to elucidate part of the mechanism of action involved in thyroid hormone modulation of renal expression of the ClC-2 channel. It is well known that thyroid hormone modulates gene expression via thyroid hormone nuclear receptors, TR-α and TR-β, and the complex hormone receptor recognizes consensus sequences (thyroid hormone responsive elements sequence [TREs]) in promoter regions of several genes and can repress or activate gene transcription [18, 22]. Analysis of the ClC-2 promoter region sequence showed three possible sequences with homology that varied between 50 and 70% of TREs. The observation that the ClC-2 promoter region was stimulated by T_3 in the IRPT cells (Fig. 4) strongly suggests the action of this hormone at transcription levels. In addition, hypothyroidism induced in mice led to a reduction in the expression of ClC-2 mRNA (Fig. 1A) and protein (Fig. 1B) expressions, and replacement of thyroid hormone in hypothyroid animals brought the level of ClC-2 renal expression to that of the controls. Hyperthyroid animals had increased renal expression of ClC-2 mRNA (35%) and protein (22%) compared with controls (Fig. 1). The pattern of expression modulation of renal ClC-2 by thyroid hormones was similar to that found for rats [22], corroborating the hypothesis that the physiological role of thyroid hormones in the kidney is conserved among different species. The possible involvement of TR-β in thyroid hormone renal modulation of ClC-2 was investigated using TR-β mutated animals (which present a syndrome of resistance to the thyroid hormone), which led to hyposensitivity to thyroid hormone. This syndrome is characterized mainly by high levels of serum T_3 and T_4 and inappropriate levels of TSH (increased in most cases). We observed that TR-β mutated mice, heterozygous or homozygous, presented decreased expression of ClC-2 renal mRNA (Fig. 2A) and protein (Fig. 2B) compared with wild type animals. This similar pattern of expression of ClC-2 mRNA and protein found for homozygous and heterozygous mice in kidney could be secondary to a a phenomenon called dominant negative regulation by the mutant receptor, in which the mutant thyroid hormone receptor interferes with the function of the normal receptor in the regulation of target genes [33]. The dysfunction of TR-β receptor can also interfere with other chloride transporters such as the chloride channel CFTR [34]. This channel is a modulator of other transporters, including sodium and chloride, and could contribute to the reduction in FE_{Na} and FE_{Cl} observed in our results [35] (Table 1). In addition, many other chloride channels are expressed in the kidney and their expression could also be modulated in the absence of TR-β receptor in mutated mice causing the decrease in renal FE of Cl^-.

The high levels of T_3 and T_4 and TSH found in heterozygous and homozygous animals could lead to misinterpretation of the results because the increased plasma levels of hormones could modulate other proteins and/or factors that may modulate ClC-2 expression in the kidney. The treatment of TR-β mutated animals with MMI minimized this hypothesis because the levels of renal ClC-2 mRNA (Fig. 3A) and protein (Fig. 3B) were not different compared with animals not subjected to MMI treatment (heterozygous and homozygous).

We conclude that TR-β isoform is involved in the mechanism of action of thyroid hormone expression of the renal ClC-2 chloride channel via the ClC-2 promoter region of the gene.

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