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

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Key Words
Thyroid hormones • Δ337T • TR-β receptor • Thyroid hormone resistance syndrome • CIC-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 CIC-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 CIC-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⁻). CIC-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 CIC-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 CIC-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 CIC-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) CIC-1, the major skeletal muscle chloride channel leads to myotonia [1, 2]; (b) CIC-K, a kidney-specific chloride channel, leads to either nephrogenic diabetes insipidus or Bartter syndrome [2, 3]; and (c) CIC-5 leads to Dent
disease [2, 4]. CIC-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. CIC-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.

CIC-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 CIC-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 CIC-2 expression, mainly in proximal tubules.

Thyroid hormones, triiodothyronine (T₃) and thyroxine (T₄) are important in renal physiology and their actions are mediated by high affinity to nuclear thyroid hormone receptors (TRs) that bind T₃. There are several T₃-binding TR isoforms (β₁, β₂, β₃, Δβ₁, 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 T₃ 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 T₃, T₄ and thyroid-stimulating hormone (TSH), hyperplastic thyroid gland, increased basal heart frequency and inner ear defects, and is an 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 CIC-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 CIC-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-triiodothyronine and thyroxine (T₃ and T₄, 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-T4 group of animals were Hypo mice subjected to daily subcutaneous injections of thyroxine (1 μg /100 g BW; Sigma) for 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 (A337T) 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 (A337T) 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 T₄ was measured by radioimmunoassay using specific antibodies (Kit Coat A Count, Diagnostic Products Coro-
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) 1 (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 each primer, 50 mmol/l of KCl, 10 mmol/l of Tris–Cl (pH 8.3) and 2.5 mmol/l of MgCl₂, 2.5 U of Amplitaq (Perkin Elmer, New Jersey, USA). Primers for CIC-2 (sense 5’-CTATGC CAT CGC GTG TG-3’ and antisense 5’-GAA GTC GAG TCG -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 CIC-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 CIC-2 in each of the following groups: (1) control, (2) hypothyroid (Hypo), (3) hypothyroid replaced with thyroxine (Hypo + T₄), (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 TGA GCC C-3’ and antisense 5’-GCT CTG GGA TGA CCT TGC C-3’), GenBank accession no. XM_906931 to the same RT-PCR reaction tubes. CIC-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 CIC-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 CIC-2 (Sigma-RBI, USA). This antibody specifically recognizes CIC-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-µM Immobilon-NC nitrocellulose membranes (Sigma). We used rabbit anti-CIC-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-CIC-2; no bands were detected under these conditions.

**DNA transfection and CIC-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 Ca²⁺- and Mg²⁺-free solution, reseded in 6-well plates, reaching 70% confluence on the day of transfection. The cells were transiently co-transfected with 2.0 µg of CIC-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 µl of Lipofectamine 2000 reagent (Invitrogen) diluted in 250 µl D-MEM without serum and antiboiiotic 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 10⁻³ M, 10⁻⁴ 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 MgSO₄, 4 mM EGTA, Triton X (25%) and 1 mM DTT) for 20 min at room temperature. Luciferase activity was quantified using 200 µl of cell lysates, 100 µl of β-mercaptoethanol (Molecular Probes Inc., Eugene, OR, USA) and 100 µl of luciferase assay buffer (25 mM gly-gly, 15 mM MgSO₄, 4 mM EGTA, 15 mM KH₂PO₄, 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 NaH₂PO₄, 40 mM NaNH₂PO₄ and 2 mg/ml of ONPG) and 810 µl of buffer β-gal (60 mM Na₂HPO₄, 729

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230 mM NaH$_2$PO$_4$, 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 $p \leq 0.05$.

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).

CIC-2 chloride channel expression

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

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<td>FECI (%)</td>
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<td>0.83±0.04</td>
<td>1.41±0.08*</td>
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<tr>
<td>Urine flux (µl/g BW per 24 h)</td>
<td>88.12±1.1</td>
<td>86.25±2.4</td>
<td>85.87±2.57</td>
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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 CIC-2 mRNA and protein by thyroid hormones. (A) Expression of CIC-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 CIC-2 and GAPDH bands obtained in RT-PCR experiments. (B) Expression of CIC-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 CIC-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 Na$_2$HPO$_4$, 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 $p<0.05$. 

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).

CIC-2 chloride channel expression

To test and quantify the influence of thyroid hormones on CIC-2 mRNA expression in mice kidney, we
used semi-quantitative RT-PCR. We observed 615 bp bands, corresponding to CIC-2 in all analyzed groups. Densitometric analysis of the bands corresponding to CIC-2 and normalized by GAPDH mRNA showed that hypothyroid animals presented a decrease in CIC-2 renal expression compared with control levels (75% of control) \((n=6, p<0.05, \text{Fig. 1A})\). Treatment of hypothyroid mice with a physiological dose of thyroxine \((1 \mu g/100 \text{ g of BW/day})\) for 15 days normalized the amount of CIC-2 mRNA renal expression to control levels. Treatment of normal mice with a supraphysiological dose of T4 \((10 \mu g/100 \text{ g of BW per day})\) for 15 days induced an increase in CIC-2 renal mRNA expression compared with control animals \((35\% \text{ of control})\) \((n=5; p<0.05, \text{Fig. 1A})\). As shown in Fig. 1B, CIC-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 CIC-2 protein expression compared with control animals \((n=5; p<0.05, \text{Fig. 1B})\). CIC-2 protein levels were normalized with the physi-
ological dose of T4 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 homoyzgous (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 T3 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}, 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M T3, 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-
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