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Role of Thyroid Hormones and mir-208 in Myocardial Remodeling in 5/6 Nephrectomized Rats

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Background and Aims. Thyroid hormones exert important effects on heart remodeling through mir-208. The process may have a role in myocardial changes in chronic kidney disease where thyroid abnormalities are common. In this study the effect of T_4 supplementation on left ventricle (LV) remodeling in 5/6 nephrectomized rats (5/6Nx) was analyzed.

Methods. 5/6Nx rats and 5/6Nx under T_4 supplementation (5/6Nx + T_4) were compared with control (C) and thyroidectomized (Tx) rats. After 8 weeks of follow-up, LV was analyzed for α-MHC, β-MHC, TGF-β, and mir-208 expression, hydroxyproline content, and myocardial fibrosis. Serum collagenase activity was also analyzed.

Results. Heart weight increased in 5/6Nx rats compared to C, which was prevented with T_4 supplementation (C, 1.5 ± 0.04; 5/6Nx, 1.8 ± 0.09; 5/6Nx + T_4, 1.6 ± 0.07 g, $p < 0.05$). The same pattern was seen for LV wall thickness, hydroxyproline content, LV fibrosis, and mRNA TGF-β expression (C, 0.47 ± 0.17; 5/6Nx, 10.55 ± 3.4; 5/6Nx + T_4, 3.01 ± 0.52, $p < 0.01$). Tx rats had reduction in heart weight, increased LV wall thickness, and fibrosis. Collagenase activity did not change in any group. mRNA expression of α-, β-MHC, and TGF-β increased in 5/6Nx in comparison to C and 5/6Nx + T_4. Expression of mir-208 decreased in 5/6Nx groups, and levels were restored with T_4 supplementation (4.21 ± 0.28, 3.39 ± 0.29, and 4.26 ± 0.37 RU, respectively, $p < 0.01$).

Conclusions. Decreased plasma level of thyroid hormones or sensitivity at tissue level observed in chronic kidney disease induced by 5/6Nx has an important effect in heart remodeling processes, some of it related or mediated by mir-208 and TGF-β expression in the heart. © 2013 IMSS. Published by Elsevier Inc.

Key Words: Chronic kidney disease, Myocardial remodeling, mir-208, Heart failure, Heart fibrosis, Thyroid hormone.

Introduction

Cardiovascular diseases (CVD) are the leading cause of morbidity and mortality in patients with chronic kidney disease (CKD), particularly in patients in chronic dialysis (1–4). Heart failure is one of the most frequent forms of heart disease in this population; fluid and pressure overload are among the mechanisms underlying this phenomenon. Functional changes are associated with abnormal remodeling with heart enlargement and chamber dilatation, particularly of the left ventricle (LV) where cardiomyocyte hypertrophy and apoptosis, as well as interstitial fibrosis, occur.

Decreased expression of α-myosin heavy chain (α-MHC), overexpression of β-myosin heavy chain (β-MHC), and

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other proteins mainly expressed during fetal life are biochemical manifestations of myocardial remodeling. Myocardial fibrosis is of clinical interest because it contributes to diastolic dysfunction, one of the early alterations found in CKD patients (5). Myocardial fibrosis results from the imbalance between the synthesis and degradation of collagen molecules (6−8). Genetic factors, cytokines, and hormones can modify hypertrophy and fibrosis. Among these, one not-well-understood factor is the reduction in thyroid hormones, which seems to be part of this complex mechanism (9,10).

Low or low-normal plasma levels of triiodothyronine (T_3) and thyroxine (T_4) with normal thyroid stimulating hormone (TSH) is the hormonal pattern commonly seen in CKD patients (9,11). In some studies it has been reported that low levels of T_3 are inversely associated with mortality rates, both in hemodialysis and peritoneal dialysis patients, but the nature of the association is unclear (12−15); heart abnormalities are a possible explanation. Thyroid hormones are linked with the process of hypertrophy as well as fibrosis in the heart in several ways (10). Experimental and clinical studies have shown that thyroid hormones regulate expression of proteins associated with hypertrophy such as α-, and β-MHC and also prevent collagen deposit and/or increase collagen removal (16−20).

In the past few years, a growing number of reports have emerged concerning the post-transcriptional regulation of different proteins in various biological processes. MicroRNAs have a central role in this regulation. One of them, microRNA-208 (mir-208), is selectively expressed in myocardial tissue and is involved in the control of heart remodeling because it regulates the expression of β-MHC and myocardial fibrosis in response to various stimuli (21,22). Thyroid hormones are at the same time one of the most important factors regulating the expression of mir-208 at the pre-transcriptional level. In spite of their potential as regulators of myocardial remodeling, thyroid abnormalities have not been sufficiently studied in terms of myocardial changes in CKD patients or experimental models of uremia.

The aim of the present study was to analyze the effect of thyroxin supplementation on expression of mir-208 as well as of hypertrophy-related proteins and mechanisms of fibrosis in the myocardium of rats with induced CKD.

Materials and Methods

Animals

Male Sprague Dawley rats weighing 250−300 g were studied. Rats were allowed free access to standard chow (5008 Purina chow, Purina SA, Mexico) and tap water and were housed under controlled humidity and temperature with a 12-h light-dark cycle.

Experimental Design and Procedures

Four groups of animals with at least eight rats each were formed. Group C, sham-operated rats, served as controls: Group 5/6Nx, rats with chronic kidney disease induced by 5/6 nephrectomy; Group 5/6Nx + T_4, 5/6Nx rats supplemented with L-thyroxine; Group Tx, thyroidectomized rats.

5/6Nx was performed as previously reported (23). In group 5/6Nx + T_4, thyroxin (T_4) (8 μg/kg/day) (Sigma Chemical Co., St. Louis, MO) was administered intraperitoneally. Hypothyroidism was surgically induced in animals of Tx group. Rats were anesthetized with xylazine-ketamine and the thyroid gland was dissected and excised. Parathyroid glands were dissected and implanted into the sternocleidomastoid muscles.

Rats were followed for 8 weeks after the last surgery. Blood pressure was measured weekly by a non-invasive method in the tail (CODA 2 system model; Kent Scientific Corporation, Torrington, CT). At the end of follow-up, rats were weighed and sacrificed using pentobarbital. Blood samples were taken, plasma was separated and kept frozen at −20°C until biochemical analysis, and the heart was removed and weighed. Left ventricle (LV) samples were prepared and stored in 10% formaldehyde and in physiological solution until assayed.

Methods of Analysis

Serum samples were assayed for creatinine by standard methods in a clinical chemistry analyzer (Syncron CX5, Beckman, Fullerton, CA), and plasma assayed for T_3 and T_4 by ELISA with commercial kits (Milliplex Cat RTHY-30K, Billerica, MA).

Histology

LV fragments fixed in 10% formaldehyde were embedded in paraffin, cut in 4-μm-thick slices and stained using Masson's trichromic method (24). Histological analysis was done using an Olympus BX51 microscope (Olympus American, Melville, NY) at different enlargement degrees and images digitalized and recorded with a VR Evolution half cybernetic digital camera (Madison, WI). Image analysis was done by using a color imaging Image-Pro Plus software v.5.1. Results are expressed as average of pixels for areas of fibrosis (stained blue with Masson trichrome) with the selected color in useful areas that were digitized at 10X recorded in 50 fields.

Immunohistochemistry

Expression of transforming growth factor beta (TGF-β) in LV samples was carried out by conventional technique immunoperoxidase assay (ABC) using an anti-TGF-β antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Immunostained intensity for TGF-β was measured using color analysis capability of imaging software, positivity

in brown immunoperoxidase the indirect technique in fibrosis-free areas and measured at 40X to obtain a measurement in pixels of the positivity in the tissue to the antibody. Analyses were done in a similar manner and equipment as light histology.

Tissue Biochemistry and Molecular Biology Analysis

LV samples were homogenized in PBS solution for biochemical assays. Hydroxyproline was measured in left ventricle as an indicator of fibrosis (25). Collagenase activity was detected by gelatin zymography (26,27). This assay measured collagenase 2 and 9.

Obtaining Left Ventricle RNA

Total RNA was isolated from LV samples homogenized in TRIzol (Invitrogen, Carlsbad, CA) and quantified (NanoDrop, Thermo Scientific, Wilmington, DE) at 260 nm and then used to obtain cDNA.

Synthesis of mir-208 cDNA and RT-PCR was carried out with a qRT-PCR mirVana miRNA detection kit (Ambion, Foster City, CA) according to the manufacturer's protocol. The reaction used SYBER GREEN as fluorophore and U6 as normalizing gene and was incubated at 95°C for 3 min followed by 40 cycles of 95°C for 15 sec and 60°C for 30 sec. All reactions were run in duplicate in a Rotor-Gene thermocycler (Corbertt R6-3000, Concord, NSW).

TGF-β, α and β-myosin Heavy Chain mRNA Measurement

Quantitative PCR were carried out in duplicate (Thermal Cycler ABI Prism 7500, Applied Biosystems, Carlsbad, CA). Sense and anti-sense primers were as follows: 5'AGCTGCAGACAGAGAACGGC3' and 5'GCTTTTTGT CCAGGGCTGCG3' for α-MHC; 5'GCTGGAGCTGATG CACCTGT3' and 5'TCGGCATCTGCCAGGTTGTC3' for β-MHC; 5'TCGGGAAGCAGTGCCAGAAC3' and 5'AG GAGCAGGAAGGGTCGGTT 3' for TNFβ; and 5'ATGGA GAAGGCTGGGGCTCA3' and 5'TTCCAGAGGGGCCAT CCACA3' for glyceraldehyde-3-phosphate dehydrogenase, which served as a normalizing gene. Reactions were run at 95°C for 2 min followed by 40 cycles at 95°C for 30 sec and 52.1°C for 30 sec and 72°C for 32 sec. TGF-β had an annealing temperature of 61°C for 30 sec. Quantification was done with ΔCT.

Statistics

Data are reported as mean ± SEM. Between-group comparisons were done with Student t test; $p < 0.05$ was considered statistically significant.

Results

Rats in all groups had similar characteristics regarding age, body weight, systolic and diastolic blood pressure, and serum creatinine before surgical procedures. Rats from 5/6Nx and 5/6Nx + T_4 had similar characteristics in age, body weight, systolic and diastolic blood pressure, and serum creatinine levels before hormone supplementation. Table 1 shows results after 8 weeks of follow-up; there were no significant differences in body weight among groups. Both systolic and diastolic blood pressure were increased in 5/6Nx and 5/6Nx + T_4 rats and showed a slight decrease in Tx group. Serum creatinine levels rose in both groups of 5/6Nx rats, with and without T_4 supplementation, and had a minor increment in Tx group. T_3 and T_4 levels decreased in CKD groups with and without hormone supplementation as well as in Tx animals when compared to C group.

Table 2 shows macroscopic and histologic data of heart changes at the end of follow-up. Heart/body weight ratio increased in rats of the 5/6Nx when compared to animals in the control group. T_4 supplementation partially prevented this increment. Heart/body weight ratio tended to be lower in Tx rats. The left ventricle wall was thicker in 5/6Nx rats than in the control group or in the T_4 supplemented

Table 1. Body weight, blood pressure, creatinine, and thyroid hormones at the end of eight weeks progression of kidney damage

Group	C	5/6Nx	5/6Nx + T_4	Tx
Body weight (g)	442.8 ± 11.3	414.0 ± 11.5	378.9 ± 12.8	407.5 ± 14.1
SBP (mmHg)	137.3 ± 5.85	210.3 ± 18.25[b]	217.8 ± 7.18[b]	129.5 ± 6.4[d]
DBP (mmHg)	99.3 ± 6.13	169.3 ± 6.29[b]	176.0 ± 24.9[b]	93.5 ± 4.95[d]
Serum Cr (mg/dL)	0.51 ± 0.02	1.36 ± 0.12[b]	1.63 ± 0.30[b]	0.57 ± 0.02[a]
T_3 (ng/mL)	3.87 ± 0.58	1.96 ± 0.56[b]	2.07 ± 0.54[b]	4.65 ± 0.76[c]
T_4 (ng/mL)	244.73 ± 80.20	104.99 ± 10.65[b]	78.64 ± 15.89[b]	190.78 ± 58.48[d]

C, sham-operated rats; 5/6Nx, 5/6 nephrectomized rats; 5/6Nx+T_4, 5/6Nx, rats supplemented with L-thyroxine; Tx, thyroidectomized rats (n = 8); SBP, systolic blood pressure; DBP, diastolic blood pressure; Cr, creatinine.
Each value represents mean ± SEM.
[a] $p < 0.05$ vs. C.
[b] $p < 0.01$ vs C.
[c] $p < 0.05$ vs. 5/6Nx.
[d] $p < 0.01$ vs. 5/6Nx.

Table 2. Cardiac histology and immunohistochemistry in rat heart

	Control	5/6Nx	5/6Nx + T_4	Tx
Heart weight (g)	1.5 ± 0.04	1.8 ± 0.09[b]	1.6 ± 0.07[a]	1.3 ± 0.05[a]
Heart-body weight	3.38 ± 0.33	4.41 ± 0.72[a]	4.36 ± 0.66[a]	3.91 ± 0.78
LV wall thickness (μm)	2912 ± 572	4258 ± 345[b]	3048 ± 651[b,d]	3188 ± 622[b]
Hydroxyproline (mg/g)	11.8 ± 1.1	16.2 ± 1.6[a]	12.1 ± 0.4[c]	10.4 ± 1.0
Fibrosis (Px)	418 ± 74	11544 ± 601[b]	4649 ± 681[b,d]	1990 ± 158[b]
TGF-β (Px)	0.47 ± 0.17	10.55 ± 3.4[b]	3.01 ± 0.52[b,c]	0.85 ± 0.28
MMP (Px)	103 ± 16	105 ± 7	107 ± 4	104 ± 8

C, sham-operated rats; 5/6Nx, 5/6 nephrectomized rats; 5/6Nx+T_4, 5/6Nx, rats supplemented with L-thyroxine; Tx, thyroidectomized rats (n = 8); MMP, metalloproteinase activity; Px, pixels; LV, left ventricular wall thickness.
Each value represents mean ± SEM.
[a]p <0.05 vs C.
[b]p <0.01 vs C.
[c]p <0.05 vs. 5/6Nx.
[d]p <0.01 vs. 5/6Nx.

animals. In the Tx group this variable also increased but to a different extent compared with 5/6Nx rats.

Fibrosis measured by light histology as well as by hydroxyproline content was higher in the 5/6Nx rats compared with controls (Table 2, Figure 1). As in other aforementioned variables, T_4 treatment significantly prevented fibrosis. Tx animals also showed an increase in fibrosis, but to a lesser extent than in 5/6Nx groups. Immunostained areas for TGF-β were greater in the 5/6Nx rats than in either controls or T_4-treated animals (Table 2, Figure 2). TGF-β was increased in Tx rats, but it was below those values seen in 5/6Nx rats. Collagenase activity as measured by zymography was similar in all groups.

Table 3 shows the gene expression of α- and β-MHC, which increased in 5/6Nx rats and slightly decreased in the Tx group when compared to the control group. TGF-β gene expression changed in the same way and follows results observed by immunohistochemistry. Expression of mir-208 decreased in 5/6Nx groups, and levels were restored with T_4 supplementation.

Discussion

Data herein reported support the concept of low thyroid hormone levels as an important factor in the pathophysiology of hypertrophy and fibrosis of the myocardium of rats

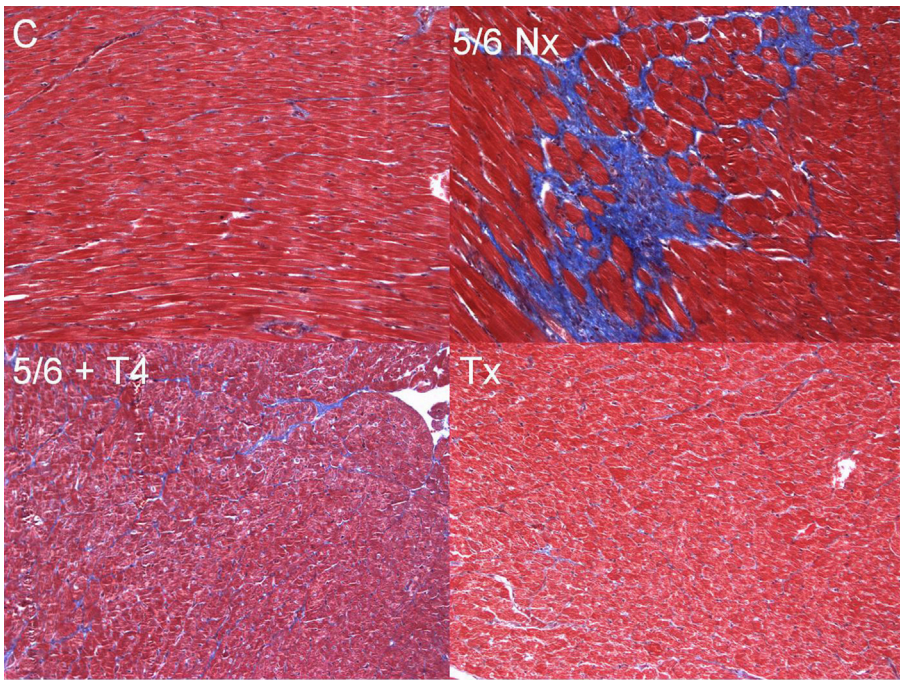

Figure 1. Microphotographs of LV tissue stained with Masson trichrome method (10X). Fibrosis is clearly seen in LV from 5/6Nx group compared to C rats. In T_4 supplemented animals the fibrosis extension much less important.

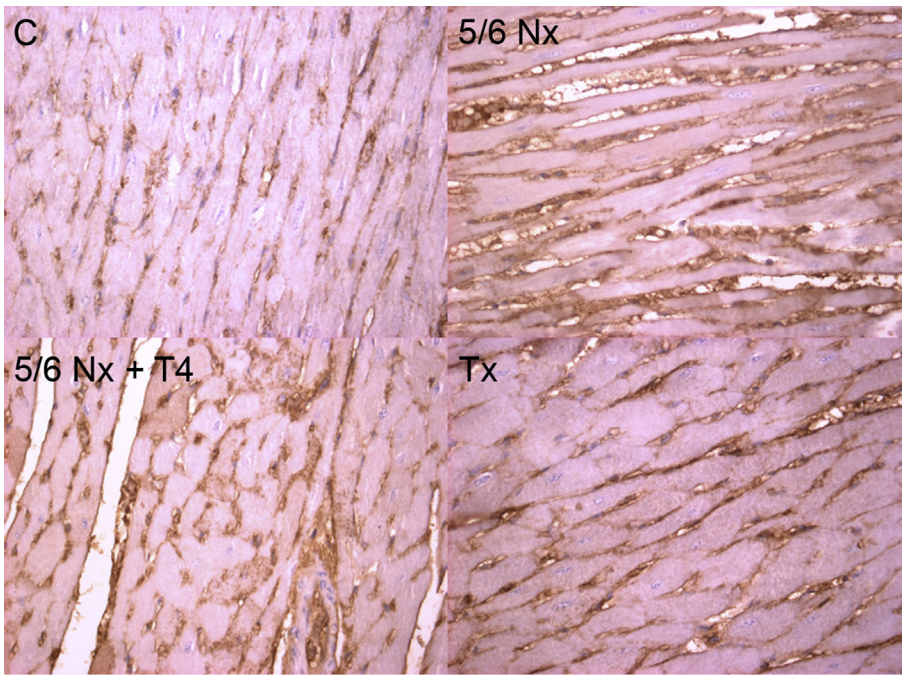

Figure 2. Microphotographs of LV showing TGF-β expression, the indirect immunoperoxidase technique showing positivity in dark brown. Rats from 5/6Nx group express TGF-β with more intensity than controls. T_4 supplementation prevented changes.

with experimentally induced CKD and that mir-208 mediates hormone action.

Appropriate interpretation of these results needs to consider several important aspects. The first is the model: 5/6Nx generates a moderate degree of CKD and not severe (as stage 5 may be in humans) renal failure. The second important aspect refers to the degree of the effect of CKD on thyroid hormone levels. In humans, changes in thyroid hormones are associated with the severity of the primary disease and comprise a broad spectrum of variations, which can range from low-normal or slightly low levels in free T_3 to severe drops in free and total T_3 and T_4 with elevated TSH (28). In this context, the model we analyzed was limited to moderate CKD and moderate changes in thyroid hormones. The third aspect to be considered in the interpretation of the results concerns other previously reported models to study the effects of thyroid hormones on the heart. These models have involved drastic changes in thyroid hormones, which are achieved through total thyroidectomy or the use of high doses of propylthiouracil (PTU) in the case of hypothyroidism or the administration of excessive doses of T_3 or T_4 for the study of hyperthyroidism. In the present study, the Tx group was used only as a qualitative reference because the effects of 5/6 Nx on thyroid function are not equivalent in magnitude to total Tx. Furthermore, it should be stressed that the effect of 5/6Nx on heart is the result of the interaction of many factors and is not limited to the decrease in thyroid hormone levels.

Reductions in thyroid hormones were found in rats with 5/6Nx, which were partially restored by T_4 supplementation. Changes similar in magnitude were found in other studies with the same model of CKD. Decrements may

Table 3. Analysis of the expression of mRNAs and mir-208 in the left ventricle of the rat heart

	Control	5/6Nx	5/6Nx + T_4	Tx
miRNA-208 (RU)	4.21 ± 0.28	3.39 ± 0.29[b]	4.26 ± 0.37[c]	4.72 ± 0.32[c,d]
α-MHC (RU)	0.92 ± 0.06	1.14 ± 0.19[b]	1.03 ± 0.14[b]	0.81 ± 0.08[b,c,e]
β-MHC (RU)	0.92 ± 0.08	1.04 ± 0.09[b]	0.99 ± 0.11	0.86 ± 0.15[a,c]
TGF-β (RU)	0.84 ± 0.02	1.14 ± 0.17[b]	0.99 ± 0.10[b,d]	0.73 ± 0.05[b]

C, sham-operated rats; 5/6Nx, 5/6 nephrectomized rats; 5/6Nx+T_4, 5/6Nx, rats supplemented with L-thyroxine; Tx, thyroidectomized rats (n = 8). Each value represents mean ± SEM.

Data are expressed as relative units (RU). mRNA expression was normalized to GAPDH and mir-208 expression was normalized with U6 gene.

[a] p <0.05 vs. C.

[b] p <0.01 vs. C.

[c] p <0.01 vs. 5/6Nx.

[d] p <0.05 vs. 5/6Nx + T_4.

[e] p <0.01 vs. 5/6Nx + T_4.

appear moderate; however, it has been demonstrated that in addition to low hormone concentrations, CKD animals also show tissue resistance to thyroid hormones. Separately or together, they result in reductions of the activity of T_3-dependent hepatic enzymes (29,30), a biochemical evidence of hypothyroidism.

The macroscopic changes in the heart in the 5/6Nx group were evident and, as expected, associated with increments of creatinine levels and blood pressure. Supplementation with T_4 did not produce significant changes in these parameters; therefore, the effects of T_4 on heart should be considered independent of the degree of impairment of renal function or changes in blood pressure.

In thoracic aorta banding (TAB), in one of the models of myocardial hypertrophy, one of the most significant changes is the shift in the synthesis of α-MHC to β-MHC; this effect is mediated by mir-208. It is encoded as a part of α-MHC and they are expressed in parallel. As with other micro-RNAs, mir-208 impedes the synthesis of proteins, and one of its actions is to block β-MHC expression by binding to β-MHC mRNA and diminishing translation and allows that of β-MHC. On the other hand, the presence of mir-208 is necessary, given that it has been demonstrated that KO animals for mir-208 with TAB do not change their patterns of MHC. Nevertheless, the presence of mir-208 is not sufficient to generate hypertrophy or change the pattern of MHC by itself, given that animals with overexpression of mir-208 do not generate changes if there is no additional mechanical stimulus (31,32). Our results are congruent with this knowledge. In spite of moderate renal impairment and a moderate drop in T_3 and T_4, 5/6Nx animals had significantly low levels of mir-208 and increased β-MHC in comparison with C group, changes that were not present in 5/6Nx + T_4 group. Complete disappearance of mir-208 was not expected in this model because decrements in T_3 and T_4 were only moderate and because it is known that a mature form of mir-208 remains for long periods even when PTU is administered daily for several weeks (21,31). Remaining mir-208, together with myocardial stress originated by fluid and pressure overload, might allow increments of β-MHC as a manifestation of myocardial hypertrophy.

Profibrotic activity of CKD was described in the 1960s and seems to be a systemic condition. Serum from uremic patients promoted fibroblast growth and extracellular matrix protein deposit in cultured cells (32). Increased collagen content in the skin of CKD patients is now under study (33), and increased collagen content of the uremic heart has been recognized. However, the relationship of fibrosis with thyroid hormones has not been explored (34−36). Our data suggest that the existence of a deficient action of thyroid hormones at tissue level in 5/6Nx rats is a mechanism responsible for myocardial fibrosis. Increment in collagen content, both by histological and biochemical measurement, was seen in LV, an effect that could be prevented by T_4 supplementation. Increased synthesis seems to be the main effect of 5/6Nx because no changes in collagenase activity could be demonstrated. This finding does not discard changes in other collagenases or collagenase inhibitors. The inverse phenomenon, activation of myocardial matrix degradation, was seen in hyperthyroid rats (17).

Our data also suggest the participation of TGF-β in the observed changes because the TGF-β increment followed the same pattern as collagen detection. Other evidences support this relationship. The TGF-β gene has the elements for thyroid hormone responsiveness (37). Furthermore, circulating levels of TGF-β have been associated with cardiac fibrosis in rats with renal failure; however, local synthesis of TGF-β or its relationship with thyroid hormones has not been studied (38). There are alternative links between thyroid function and TGF-β. TSH stimulates growth factor synthesis (39), and reverse T_3, which is commonly elevated in CKD, has been found associated with high serum levels of TGF-β. We did not explore this fact because normal TSH is part of euthyroid sick syndrome and reverse T_3 was not measured.

Our study has some limitations. Post-transcriptional control of α- and β-MHC may be different in rodents than in humans. Other possible control mechanisms may be important. It has been reported that mir-208 also acts on β-MHC through blocking thyroid hormone receptor-associated protein 1 (THRAP1). This interesting process was not explored in this study. Changes observed in the 8-week follow-up period may be different in the long term or be modified by other comorbid conditions often seen in CKD such as inflammation and malnutrition. The model used explored a clean induction of CKD; natural kidney diseases may induce additional changes in heart remodeling.

In conclusion, rats with 5/6Nx showed decrements of thyroid hormones that were associated with abnormal myocardial remodeling such as increased expression of β-myosin heavy chains, TGF-β, and fibrosis, changes that were reversed after thyroxin supplementation. mir-208 seems to be an intermediary between decreased thyroid hormones and biochemical and molecular aspects of myocardial remodeling.

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