CLINICAL STUDY

Time-dependent changes in the expression of thyroid hormone receptor α1 in the myocardium after acute myocardial infarction: possible implications in cardiac remodelling

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Abstract

The present study investigated whether changes in thyroid hormone (TH) signalling can occur after acute myocardial infarction (AMI) with possible physiological consequences on myocardial performance. TH may regulate several genes encoding important structural and regulatory proteins particularly through the TRα1 receptor which is predominant in the myocardium. AMI was induced in rats by ligating the left coronary artery while sham-operated animals served as controls. This resulted in impaired cardiac function in AMI animals after 2 and 13 weeks accompanied by a shift in myosin isoforms expression towards a fetal phenotype in the non-infarcted area. Cardiac hypertrophy was evident in AMI hearts after 13 weeks but not at 2 weeks. This response was associated with a differential pattern of TH changes at 2 and 13 weeks; T3 and T4 levels in plasma were not changed at 2 weeks but T3 was significantly lower and T4 remained unchanged at 13 weeks. A twofold increase in TRα1 expression was observed after 13 weeks in the non-infarcted area, P<0.05 versus sham operated, while TRα1 expression remained unchanged at 2 weeks. A 2.2-fold decrease in TRβ1 expression was found in the non-infarcted area at 13 weeks, P<0.05, while no change in TRβ1 expression was seen at 2 weeks. Parallel studies with neonatal cardiomyocytes showed that phenylephrine (PE) administration resulted in 4.5-fold increase in the expression of TRα1 and 1.6-fold decrease in TRβ1 expression versus untreated, P<0.05. In conclusion, cardiac dysfunction which occurs at late stages after AMI is associated with increased expression of TRα1 receptor and lower circulating tri-iodothyronine levels. Thus, apo-TRα1 receptor state may prevail contributing to cardiac fetal phenotype. Furthermore, down-regulation of TRβ1 also contributes to fetal phenotypic changes. α1-adrenergic signalling is, at least in part, involved in this response.

Introduction

Recent clinical and experimental observations provide substantial evidence that thyroid hormone (TH) may serve an important role in the pathophysiology of heart failure. Altered TH pattern is evident at a very early stage of the idiopathic left ventricular dysfunction and TH levels in plasma have been closely related to cardiac dysfunction and patient symptoms (1). Furthermore, in overt heart failure, low TH levels have been associated with increased mortality (2). Accordingly, experimental studies on animals have linked changes in thyroid signalling with cardiac dysfunction due to hypertrophy or heart failure. In fact, in a rat model of cardiac hypertrophy, mRNA levels of the three TR isoforms were found to be down-regulated with subsequent changes in TH target genes related to heart function (3). TH-degrading deiodinase activation was shown to be increased in rats with cardiac hypertrophy and failure, an effect which may contribute to local hypothyroid state (4). Furthermore, overexpression of the type 2 deiodinase in mouse heart (converting T4 to active T3), prevented pressure overload-induced cardiac dysfunction (5).

TH mediates its actions through the activation of nuclear receptors. Thyroid hormone nuclear receptors (TRs) are transcription factors with ligand-regulated activity. In the absence of TH, the unliganded receptors (apo-receptors) recruit co-repressors and repress the transcription of target genes. Upon hormone binding, the receptors (holo-receptors) exchange co-repressors for co-activators and activate transcription (6). TRα1 is the predominant TR isoform in the heart and plays an important role in determining cardiac function (7, 8). Furthermore, TRα1 is a molecular switch of cardiac function between fetal and post-natal life (9). In fact, under physiological fetal conditions, TRα1 is
overexpressed and circulating levels of TH are low (10, 11). At birth, a conversion of TRα1 occurs from apo-receptor into holo-receptor, upon changes in TH availability.

Cardiac fetal phenotype is recapitulated during cardiac remodelling following acute myocardial infarction (AMI). This process, not limited only to the infarcted area but also extending to the non-ischaemic region of the heart, results in progressive deterioration of cardiac function (12). Whether fetal phenotypic-like changes in TH–TH nuclear receptors axis could also occur during this process remains largely unknown. Thus, the present study investigated whether changes in TH signalling can occur at early and late stages in the course of cardiac remodelling following AMI in rats. Furthermore, possible underlying mechanisms were investigated in a cell-based model. This issue is of important therapeutic relevance, since thyroid analogues are now suggested as an effective treatment for heart illnesses (13, 14).

Materials and methods

Animals

Thirty male Wistar rats, 280–360 g, were used for this study. The rats were handled in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health Guide (NIH Pub. no. 83-23, Revised 1996).

Experimental model of myocardial infarction

Myocardial infarction was induced by ligation of the left coronary artery as previously described (15). Rats were anaesthetised with an i.p. injection of ketamine (70 mg/kg) and midazolame (0.1 mg/kg), intubated and ventilated via a tracheal cannula using a constant-volume rodent ventilator (Inspira, Harvard Apparatus, Holliston, MA, USA; 50 breaths/min, 1 ml/100 g tidal volume). Anaesthesia was maintained by inhalation of small doses of sevoﬂurane (1–2%). Left thoracotomy was performed at the fourth intercostal space followed by pericardiotomy. Left coronary artery was then ligated with a 6-0 silk round-bodied suture. The heart was quickly returned to the chest cavity, the chest was closed and rats were allowed to recover using assist mode ventilation. Atelectasis was prevented by producing positive end-expiratory pressure at the end of the surgical procedure. Continuous electrocardiographic recording was used to monitor heart rate and ECG ischaemic changes after coronary artery ligation. Body temperature was maintained at 37 °C using a heat blanket (Harvard Homeothermic Blanket, 50-7061). The mortality in the infarction group within the first 24 h was up to 40%. Myocardial infarctions which produced a scar area of 90–135 mm² were included in this study, corresponding to 35–55% of the left ventricle. The animals were left to recover for 2 or 13 weeks after myocardial infarction. These groups of animals were designated as AMI-2w, n = 6 and AMI-13w, n = 5 respectively. The same procedure was followed for sham-operated control animals, but the coronary artery was not ligated. These animals were designated as SHAM-2w, n = 6 and SHAM-13w, n = 6.

Echocardiography

Two weeks after coronary artery ligation, rats were sedated with ketamine hydrochloric acid (100 mg/kg) and heart function was evaluated by echocardiography. Short- and long-axis images were acquired using a digital ultrasound system (Sonosite 180Plus, 21919 30th Drive SE, Bothell, WA, USA) with a 7.5 MHz sector-array probe. A 7.5 MHz probe has been used effectively in several studies for echocardiographic analysis in rats (16–18). Sonosite is a fully digitalised system of the most recent generation with excellent image quality and during the analysis the image was presented in a 17” screen to increase resolution. A large number of consecutive measurements were performed and analysed by two independent operators. Representative images are shown in Fig. 1.

Left ventricular internal diameter at diastolic phase (LVIDd), LV internal diameter at systolic phase (LVIDs), posterior wall thickness at diastolic phase (LVPW) and systolic velocity of posterior wall radial displacement (SVPW) were measured. Ejection fraction was calculated as follows: EF% = ((LVIDd³ – LVIDs³)/LVIDd³) × 100. All measurements were averaged for at least three consecutive cardiac cycles. Heart rate (in beats per minute) was not different between groups during analysis and is shown in Table 1.

Isolated heart preparation

A non-ejecting isolated rat heart preparation was perfused at a constant flow according to the Langendorff technique (19, 20). An intraventricular balloon allowed measurement of contractility under isovolumic conditions. Left ventricular balloon volume was adjusted to produce an average initial left ventricular end-diastolic pressure of 6–7 mmHg in all groups and was held constant thereafter throughout the experiment.

Rats were anaesthetised with ketamine hydrochloric acid and heparin 1000 IU/kg was given intravenously before thoracotomy. The hearts were rapidly excised, placed in ice-cold Krebs–Henseleit buffer (composition in mmol/l: sodium chloride 118, potassium chloride 4.7, potassium phosphate monobasic 1.2, magnesium sulphate 1.2, calcium chloride 1.4, sodium bicarbonate 25 and glucose 11) and mounted on the aortic cannula of the Langendorff perfusion system. Perfusion with oxygenated Krebs–Henseleit buffer (95% O₂/5% CO₂) was established within 60 s after thoracotomy. The perfusion apparatus was heated to ensure a temperature...
Figure 1 Images obtained by echocardiography analysis are shown. Long-axis view (A), short-axis view at end-diastole (B) and end-systole (C), M-mode view in sham-operated (SHAM) and post-infarcted rat hearts (AMI) are presented. Note the increased dimensions of the left ventricle in AMI hearts.
Table 1 Functional contractile indexes.

<table>
<thead>
<tr>
<th></th>
<th>SHAM-2w</th>
<th>AMI-2w</th>
<th>SHAM-13w</th>
<th>AMI-13w</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate (bpm)</td>
<td>327 (8.2)</td>
<td>324 (13)</td>
<td>333 (10.8)</td>
<td>344 (15)</td>
</tr>
<tr>
<td>LVVIDd (mm)</td>
<td>6.9 (0.2)</td>
<td>9.1 (0.1)*</td>
<td>7.3 (0.3)</td>
<td>9.5 (0.3)*</td>
</tr>
<tr>
<td>LVVIDs (mm)</td>
<td>4.2 (0.2)</td>
<td>7.8 (0.2)*</td>
<td>4.8 (0.4)</td>
<td>8.2 (0.3)*</td>
</tr>
<tr>
<td>LVPW (mm)</td>
<td>1.9 (0.05)</td>
<td>2.0 (0.04)</td>
<td>2.0 (0.05)</td>
<td>2.4 (0.08)*</td>
</tr>
<tr>
<td>EF%</td>
<td>71.4 (3.5)</td>
<td>33 (2.8)*</td>
<td>67.4 (3.9)</td>
<td>34 (1.3)*</td>
</tr>
<tr>
<td>SVPW (cm/s)</td>
<td>3.3 (0.3)</td>
<td>2.3 (0.2)*</td>
<td>3.1 (0.2)</td>
<td>2.0 (0.05)*</td>
</tr>
<tr>
<td>LVDP (mmHg)</td>
<td>117 (3.0)</td>
<td>88 (9.2)*</td>
<td>125 (2.9)</td>
<td>72.4 (12)*</td>
</tr>
<tr>
<td>+dp/dt (mmHg/s)</td>
<td>3950 (429)</td>
<td>2350 (233)*</td>
<td>4450 (225)</td>
<td>2210 (400)*</td>
</tr>
<tr>
<td>−dp/dt (mmHg/s)</td>
<td>1850 (66)</td>
<td>1580 (117)*</td>
<td>2400 (98)</td>
<td>1480 (235)*</td>
</tr>
</tbody>
</table>

Echocardiographic measurements of LVVIDd and at systolic phase (LVVIDs), posterior wall thickness at diastolic phase (LVPW), EF and systolic velocity of posterior wall radial displacement (SVPW) and additional measurements of left ventricular developed pressure (LVDP) and the rate of increase and decrease of LVDP (+ dp/dt and − dp/dt) under isometric conditions in sham-operated (SHAM) and post-infarcted hearts (AMI) after 2 and 13 weeks are shown. The values are mean (i.e., M ± SE) versus SHAM-2w, *P < 0.05 versus SHAM-13w.

of 37 °C throughout the course of the experiment. Hearts were paced at 320 beats/min with a Harvard pacemaker. The water-filled balloon, connected to a pressure transducer and coupled to a Gould RS 3400 (Gould Inc., Cleveland, OH, USA) recorder, was advanced into the left ventricle through an incision in the left atrium. Pressure signal was transferred to a computer using a data analysis software (IOX, Emka Technologies, Paris, France) which allowed continuous monitoring and recording of heart function.

As intraventricular volume was maintained at a constant value, diastolic fibre length, which represented preload, did not change. Thus the left ventricular developed pressure (LVDP), defined as the difference of LVDP (− dp/dt and − dp/dt) under isometric conditions in sham-operated (SHAM) and post-infarcted hearts (AMI) after 2 and 13 weeks are shown. The values are mean (i.e., M ± SE), *P < 0.05 versus SHAM-2w, †P < 0.05 versus SHAM-13w.

Protein isolation, SDS-PAGE and immunodetection

Approximately 0.2 g left ventricular tissue was homogenised in ice-cold buffer (A) containing 10 mM HEPES (pH 7.8), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM PMSF, 1 mM DTT and 10 µg/ml leupeptin and 10% glycerol and samples were incubated at 4 °C for 60 min (under agitation) followed by centrifugation at 10 000 g for 5 min at 4 °C. The supernatant containing the nuclear fraction was separated and stored at −80 °C, while the pellet containing cellular debris and cytoskeleton was discarded (15). TRα1 and TRβ1 protein expression was determined in nuclear fraction. Protein concentrations were determined by the bichinonic acid method.

Samples were prepared for SDS-PAGE by boiling for 5 min in Laemmli sample buffer containing 5% 2-mercaptoethanol. Twenty micrograms (nuclear fraction) of total protein were loaded onto 10% (w/v) acrylamide gels and subjected to SDS-PAGE in a Bio-Rad Mini Protein gel apparatus. For western blotting, following SDS-PAGE, proteins were transferred electro-phoretically to a nitrocellulose membrane (Hybond ECL, Amersham) at 100 V and 4 °C, for 1.5 h using Towbin buffer. After western blotting, filters were probed with specific antibodies against TRα1 (Affinity Bioreagents, Bingham, Nottingham, UK; PAI-211A, dilution 1:1000, 2 h at 37 °C), TRβ1 (Affinity Bioreagents, MA1-216, dilution 1:1000, overnight at 4 °C) and histone H3 (Cell Signalling, Danvers, MA, USA, #9715, dilution 1:1000 overnight at 4 °C). Filters were incubated with appropriate anti-mouse (Amersham) or anti-rabbit (Cell Signalling) HRP secondary antibodies and immunoreactivity was detected by ECL using Lumiglo reagents (New England Biolabs, Ipswich, MA, USA) and exposed to Hyperfilm paper (Amersham). Five samples from each group were loaded on the same gel. Histone H3 protein expression was used in order to normalise slight variations in nuclear protein loading. Immunoblots were quantified using the AlphaScan Imaging Densitometer (Alpha Innotech Corporation, 14743, Catalina Street, San Leandro, CA, USA). To determine the linearity of the technique in preliminary studies, total protein (nuclear fraction) in the range of 10–50 µg was subjected to electrophoresis and western blotting analysis. The linearity of the assay was determined by densitometric scanning of the resulting film. The results of the assay were linear from 10 to 50 µg total protein for both TRα1 and TRβ1. Based on these results, 20 µg total protein (nuclear fraction) were used for western blotting analysis.

Measurement of myosin heavy-chain isofrom content

Homogenates of all samples were diluted 40-fold with Laemmli sample buffer containing 5% 2-mercaptoethanol. The composition and preparation of the gels was carried out as previously described (7, 21). Briefly, the stacking and separating gels consisted of 4 and 8% acrylamide (w/v) respectively, with acryl:bis-acryl in the ratio of 50:1. The stacking and separating gels included 5% (v/v) glycerol. The upper running buffer consisted of 0.1 M Tris (base), 150 mM glycine, 0.1% SDS and 2-mercaptoethanol at a final concentration of 10 mM.
The lower running buffer consisted of 0.05 M Tris (base), 75 mM glycine and 0.05% SDS. The gels were run in Bio-Rad Protein II xi electrophoresis unit at a constant voltage of 240 V for 22 h at 4 °C. The gels were fixed and silver stained (Bio-Rad silver stain kit). Gels were scanned and quantified using the AlphaScan Imaging Densitometer (Alpha Innotech Corporation).

**Cell culture**

Neonatal rat cardiomyocyte cultures were prepared as previously described (22, 23). Ventricles from Wistar rat pups (48–72 h after birth) were dissected, and the cells were dissociated in a spinner flask using collagenase (740 U/digestion; Worthington Biochemicals, NJ, USA), trypsin (370 U/digestion; Worthington Biochemicals) and DNase I (2880 U/digestion; Worthington Biochemicals). Myocytes were separated from non-muscle cells on a discontinuous Percoll gradient and plated at a density of 45 000 cells/cm² (on 35 mm dish) for fluorescent microscopy or 190 000 cells/cm² (on 60 mm dish) for electrophoresis and immunoblotting analysis. Fibroblast overgrowth was not found in our culture. There was a high percentage of beating cells in the culture, while after addition of phenylephrine (PE), beating was significantly enhanced. Furthermore, in a previous study in our laboratory, contamination with fibroblasts was not observed with this experimental technique as this was detected using staining with myosin heavy-chain antibody, which better distinguishes these two cell types (22). Jimenex et al. (24) have also reported that the same isolation and culture technique results in at least 90% cardiomyocytes after 7 days in culture.

Cells were initially plated in F10 medium (Gibco) containing 10% fetal bovine serum (Hyclone, Logan, UT, USA), 10% horse serum (Gibco) and antibiotics (100 U/ml penicillin and streptomycin; Gibco) for the first 18–22 h and then the culture medium was replaced with serum-free medium containing 10 µg/ml insulin, 10 µg/ml transferrin/selenium, 0.2% BSA and 20 µg/ml ascorbic acid. Cells were serum starved for 24 h and then treated with 20 µM PE (Sigma) or remained untreated for 5 days; PE-treated and non-treated groups respectively.

**Western blotting analysis in neonatal cardiomyocytes**

After washing twice with PBS, the cells were scraped into 400 µl lysis buffer containing 20 mM HEPES (pH 7.9), 10 mM KCl, 1 mM EDTA, 10% glycerol, 0.2% NP-40, 0.5 mM DTT, 0.5 mM PMSF, 5 µg/ml aprotinin, 5 µg/ml leupeptin and centrifuged at 12 000 g for 1 min at 4 °C. The nuclear fraction was prepared by resuspension of the pellet in buffer containing 20 mM HEPES (pH 7.9), 0.42 M NaCl, 0.2 mM EDTA, 1.5 mM MgCl₂, 25% glycerol, 0.5 mM DTT, 0.5 mM PMSF, 5 µg/ml aprotinin, 5 µg/ml leupeptin and incubated with agitation for 1 h at 4 °C before centrifugation for 10 min at 12 000 g. The resulting supernatants were collected and used for protein analysis of nuclear fraction.

Protein concentration of the supernatants was determined by the RC/DC protein assay (Bio-Rad) based on the Lowry assay. After boiling for 4 min (with 4% SDS, 2% mercaptoethanol and 0.004% bromophenol blue), a quantity of 20 µg protein of this fraction was separated on 10% SDS-PAGE using the Bio-Rad Mini-Protean gel apparatus. Western blotting and immunodetection for TRα1 and TRβ1 protein expression were performed as described previously. Four separate dishes (from each group were loaded on the same gel. Histone H3 protein expression was used in each filter to normalise slight variations for nuclear protein loading.

**Measurement of cellular protein content**

Total protein content of myocytes was measured as previously described (22, 25) with some modifications. Cells were plated at a density of 45 000 cells/cm² and cultured for 48 h. The cells were washed twice with PBS and 200 µl trypsin (0.25%) was added to each well and incubated at 37 °C until the cells detached. A solution of 10% FBS in PBS was added to each well to stop the reaction. The cells were harvested and a small fraction was used for determination of the cell number using a Neubauer haematocytometer (Fisher Scientific, Pittsburgh, PA, USA). Cells were then collected by centrifugation and incubated with 100 µl lysis buffer (250 mM Tris–HCl, 4% SDS, 10% glycerol (pH 6.8)) at 4 °C overnight. This mixture was then warmed at 37 °C, and protein concentration was determined using the DC protein assay (Bio-Rad) based on the Lowry method. Protein contents were normalised to the cell count.

**Fixation and staining**

Cardiomyocytes from non-treated and PE-treated groups cultured on collagen-coated (collagen type I, Upstate, Charlottesville, VA, USA) glass cover slides (Fisher Scientific) were fixed with 4% paraformaldehyde (Sigma) for 15 min at 4 °C and permeabilised with 0.1% Triton-X/PBS for 15 min at 4 °C. Subsequently, the cells were incubated with fluorescent phalloidin (Molecular Probes, Invitrogen) in 1% BSA/PBS for 20 min at room temperature. Between each step, cells were washed three times with PBS. Slides were mounted and examined by fluorescence phase-contrast microscopy (Zeiss Axiovert, Thornwood, NY, USA).

**Measurement of thyroid hormones**

Plasma i-thyroxine and 3,5,3’-tri-iodothyronine quantitative measurements were performed with ELISA, using
kits obtained from Alpha Diagnostic International, TX, USA (no. 1100 for total T₄ and no. 1700 for total T₃), as previously described (15). ³-Thyroxine and 3,5,3'-tri-iodothyronine levels were expressed as nmol per litre of plasma. Absorbance measurements were performed at 450 nm with Tecan Genios ELISA reader (Tecan, Austria).

**Statistical analysis**

Results are presented as mean (s.e.m.). Unpaired t-test and Mann–Whitney test were used to evaluate differences between groups. Significance was set at 0.05.

**Results**

**Cardiac hypertrophy**

Although scar area and weight were comparable between post-infarcted hearts at 2 and 13 weeks, viable left ventricular weight (LVW) was significantly decreased only in post-infarcted hearts at 2 weeks as compared with SHAM (Table 2). In addition, indexes of cardiac hypertrophy, such as total LVW and the ratio of LVW to body weight (BW in gram) in sham-operated (SHAM) and post-infarcted hearts (AMI) after 2 and 13 weeks.

**Echocardiographic study**

Echocardiographic measurements are shown in Table 1. Post-infarcted hearts at 2 and 13 weeks after coronary artery ligation showed significant dilation of the left ventricle with reduced contractile function, assessed by measurement of the EF. Systolic velocity of posterior wall (Fig. 2C).

**Contractile function assessed under isometric conditions**

Left ventricular developed pressure (LVDP) and the rate of increase and decrease of LVDP ( + dp/dt and − dp/dt) are shown in Table 1. LVDP decreased by 35% at 2 weeks and 43% at 13 weeks after myocardial infarction. In addition, + dp/dt and − dp/dt were found to be decreased by 40 and 15% at 2 weeks and 50 and 40% at 13 weeks after myocardial infarction respectively (Table 1).

**Plasma levels of T₃ and T₄, expression of TRα₁ and TRβ₁ and myosin isoform expression in non-infarcted myocardium**

T₄ and T₃ levels were found to be 38.8 (4.0) and 1.0 (0.01) nM in SHAM-2w and 37.4 (4.9) and 0.9 (0.04) nM in AMI-2w rats respectively, P=n.s. After 13 weeks of myocardial infarction, T₄ and T₃ levels were found to be 38.2 (1.8) and 0.94 (0.07) nM for SHAM-13w and 38.0 (4.7) and 0.58 (0.05) nM for AMI-13w rats respectively; P<0.05 for T₄ (Fig. 2A).

In AMI hearts after 2 weeks, TRα₁ protein expression was not changed, while after 13 weeks a twofold increase was observed in TRα₁ protein expression in AMI hearts as compared with SHAM, P<0.05 (Fig. 2B). Furthermore, no significant change in TRβ₁ expression was seen 2 weeks, while a 2.2-fold decrease in TRβ₁ expression was found in the non-infarcted area at 13 weeks in AMI as compared with SHAM hearts, P<0.05 (Fig. 2C).

A shift was observed in myosin isoform expression after myocardial infarction. In fact, the ratio of α₁-myosin heavy chain (MHC) (fast) to β-MHC (slow) expression was 1:1 in SHAM-2w and SHAM-13w hearts, while this ratio changed to 1:2 in AMI-2w and 1:4 in AMI-13w hearts, P<0.05 (Fig. 3).

**TRα₁ and TRβ₁ protein expression and morphological changes with PE administration in neonatal cardiomyocytes**

TRα₁ protein expression was found to increase 4.5-fold, while TRβ₁ protein expression was decreased 1.6-fold after PE treatment in neonatal cardiomyocytes, P<0.05 (Fig. 4A).

In addition, total protein expression (an index of hypertrophy) was increased 1.4-fold in PE-treated as compared with non-treated cardiomyocytes, P<0.05.

Staining of the actin cytoskeleton with phalloidin showed that non-treated cardiomyocytes were of almost circular shape with poorly organised cytoskeleton, while PE treatment resulted in large cardiomyocytes with disoriented, dense myofibrils with stellar shape (Fig. 4B).
Discussion

Although it has long been recognised that several genes encoding important regulatory and structural proteins in the myocardium are TH responsive, the role of TH signalling in the pathophysiology of heart diseases has attracted very little attention (26). The present study provides some evidence showing that changes in TH signalling may occur during cardiac remodelling in a time-dependent manner and may have potential physiological consequences.

In our experimental model of AMI in rats, tissue necrosis and cardiac remodelling of the non-infarcted myocardium (as indicated by changes in myosin isoform expression in the non-ischaemic region) resulted in depressed cardiac function both at 2 and 13 weeks, as indicated by functional indices measured either by echocardiography (EF%) or in the Langendorff preparation (±dp/dt). Such alterations in cardiac functional state were accompanied by a different pattern of changes in the TH–TH nuclear receptor axis; early in the course of cardiac remodelling, circulating T4 and T3 were not changed and no changes were seen in the expression of TRα1 or TRβ1 in the myocardium. At 13 weeks, the expression of TRα1 receptor was up-regulated in the non-ischaemic region and the circulating T3 was significantly decreased, while T4 remained unchanged. This situation closely resembles the prenatal stages of development where the TRα1 is overexpressed and relatively unliganded (due to the lower TH levels) and functions as an apo-receptor with subsequent repression of thyroid hormone target genes, such as the α-myosin isoform (9–11). In fact, an apo-receptor TRα1 activity was evident in this study, as indicated by the fetal-like pattern of myosin isoforms expression found in the non-infarcted myocardium. In fact, the ratio of α-MHC to β-MHC expression was 1:1 in SHAM-2w and SHAM-13w hearts, while this ratio changed to 1:2 in AMI-2w and 1:4 in AMI-13w hearts. It should be noted that the observed ratio of α-MHC to β-MHC was not considerably above 1 in SHAM rats, as previously reported for normal animals (7). This may be due to the surgical procedure and/or the age of the animals.

TRα1 receptor, in the absence of ligand, is now recognised to be implicated in cardiac cell growth (27) and in other tissues in cell proliferation (28), while liganded TRα1 promotes differentiation (29). This could provide an explanation for the fact that cardiac hypertrophy was developed at later stages after myocardial infarction in rats and not as early as 2 weeks. Furthermore, the expression of TRβ1 was found to be decreased contributing to fetal phenotype. In fact, low expression of TRβ1 is observed during fetal life,
C. Pantos and others

Cardiomyocytes with disoriented, dense myofibrils and stellular shape. Organised cytoskeleton, while PE treatment results in hypertrophied treated cardiomyocytes are of almost circular shape with poorly microscropy after staining of actin cytoskeleton with phalloidin. Non-PE-treated neonatal cardiomyocytes obtained by fluorescence

\[ \text{TR}_{\alpha 1} \text{ protein expression (Arbitrary units)} \]

\[ \text{TR}_{\beta 1} \text{ protein expression (Arbitrary units)} \]

Figure 4 (A) Densitometric assessment in arbitrary units and representative western blots of thyroid hormone receptor \( \alpha 1 \) (TR\( \alpha 1 \)) and \( \beta 1 \) (TR\( \beta 1 \)) protein expression in non-treated neonatal cardiomyocytes and cardiomyocytes treated with phenylephrine (PE, 20 \( \mu M \)) for 5 days (columns are means of optical ratios, bar = S.E.M.). \( *P < 0.05 \) versus non-treated. (B) Images of non-treated and PE-treated neonatal cardiomyocytes obtained by fluorescence microscopy after staining of actin cytoskeleton with phalloidin. Non-treated cardiomyocytes are of almost circular shape with poorly organised cytoskeleton, while PE treatment results in hypertrophied cardiomyocytes with disoriented, dense myofibrils and stellular shape.

Furthermore, it provides an explanation for the observed increased mortality in patients with cardiac dysfunction and low circulating TH (30) and for the beneficial effect of TH treatment in heart failure (31, 32). It should be noted that low serum T3, with unchanged T4, known as non-thyroidal illness (NTI), occurs during various disease states and particularly in myocardial infarction and heart failure (33). This response has been regarded until now as an adaptation in order to reduce metabolic rate and is thought to have minimal clinical significance.

The mechanisms through which the expression of TR\( \alpha 1 \) receptor is altered during cardiac remodelling are largely unknown. Several neurohormonal systems have been implicated in cardiac remodelling with the adrenergic system, particularly the \( \alpha 1 \) stimulation, to have a critical role in this response (34). Therefore, we used a cell-based model to investigate whether exposure to \( \alpha 1 \)-adrenergic stimulation could result in changes in the expression of TR\( \alpha 1 \) receptor. Interestingly, our study showed a marked increase in TR\( \alpha 1 \) protein expression after prolonged exposure of neonatal cardiac cells to PE. Furthermore, the expression of TR\( \beta 1 \) was found to be decreased. This response was associated with increased cellular growth (as indicated by the increased protein synthesis) and morphological changes indicative of de-differentiated cells (Fig. 4).

Our observations clearly indicate that cross-talk may exist between the \( \alpha 1 \)-adrenergic signalling and the TH receptor \( \alpha 1 \), with potential physiological consequences in situations where adrenergic over-activation occurs as in cardiac remodelling.

To our knowledge, the present study is the first to provide evidence of altered TH signalling during cardiac remodelling after AMI in rats. Previous studies have shown that, at the mRNA level, TR\( \alpha 1 \) and TR\( \beta 1 \) are down-regulated in the myocardium of rats with aortic constriction-induced cardiac hypertrophy and in neonatal cardiomyocytes after PE administration (3). Furthermore, in human failing hearts, TR\( \alpha 1 \) mRNA was found to be reduced, while TR\( \beta 1 \) mRNA was unchanged (35). The expression of TR\( \alpha 1 \) protein was not detected. Changes in mRNA though do not necessarily reflect changes in protein expression. However, an increased protein expression of TR\( \alpha 1 \) was found in samples from patients with end-stage heart failure due to ischaemic heart disease (36). Interestingly, this finding is consistent with our results.

In conclusion, cardiac dysfunction which occurs at late stages after AMI is associated with an increased expression of TR\( \alpha 1 \) receptor and reduced circulating levels of tri-iodothyronine. Thus, apo-TR\( \alpha 1 \) receptor state may prevail contributing to cardiac fetal phenotype. Furthermore, down-regulation of TR\( \beta 1 \) also contributes to fetal phenotypic changes. \( \alpha 1 \)-adrenergic signalling is, at least in part, involved in this response.

While marked changes in TR\( \beta 1 \) expression around birth may be important in relation to specific maturation events and the general increase in TR\( \beta 1 \) during development may be important in maintaining a basal level of TH cellular action (11).

Taken together, these data strongly indicate that changes in TH signalling may play a critical role in the pathophysiology of cardiac remodelling late after AMI.
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References


Chassande O. Do unliganded thyroid hormone receptors have physiological functions? Journal of Molecular Endocrinology 2003 31 9–20.
10 White P, Burton KA, Fowden AL & Dauncey MJ. Developmental expression analysis of thyroid hormone receptor isoforms reveals new insights into their essential functions in cardiac and skeletal muscles. FASEB Journal 2001 15 1367–1376.
14 Morkin E, Pennock GD, Spooner PH, Bahl JJ & Goldman S. Clinical and experimental studies on the use of 3,5-diiodothyropropionic acid, a thyroid hormone analogue, in heart failure. Thyroid 2002 12 527–533.
15 Pantos C, Mourouzis I, Saranteas T, Piaizs I, Xinaris C, Malliopoulos V & Cokkinos DV. Thyroid hormone receptors alpha1 and beta1 are downregulated in the post-infarcted rat heart: consequences on the response to ischaemia-reperfusion. Basic Research in Cardiology 2005 100 422–432.
27 Kinugawa K, Jeong MY, Bristow MR & Long CS. Thyroid hormone induces cardiac myocyte hypertrophy in a thyroid hormone receptor alpha1-specific manner that requires TAK1 and p38 mitogen-activated protein kinase. Molecular Endocrinology 2005 19 1618–1628.
28 Gonzalez-Sancho JM, Figueroa A, Lopez-Barahona M, Lopez E, Beug H & Munoz A. Inhibition of proliferation and expression of T1 and cyclin D1 genes by thyroid hormone in mammary epithelial cells. Molecular Carcinogenesis 2002 34 25–34.
31 Hamilton MA, Stevenson IJ, Fonarow GC, Steimle A, Goldhaber J, Child JS, Chopra IJ, Moriguchi JD & Hage A. Safety


34 Lamba S & Abraham WT. Alterations in adrenergic receptor signaling in heart failure. *Heart Failure Reviews* 2000 **5** 7–16.


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