Signaling Pathways Responsible for Fetal Gene Induction in the Failing Human Heart
Evidence for Altered Thyroid Hormone Receptor Gene Expression

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Background—We have previously demonstrated that changes in myosin heavy chain (MHC) isoforms that occur in failing human hearts resemble the pattern produced in rodent myocardium in response to hypothyroidism. Because thyroid hormone status is usually within normal limits in these patients, we hypothesized that failing/hypertrophied human myocardium might have a defect in thyroid hormone signaling due to alterations in expression of thyroid hormone receptors (TRs).

Methods and Results—To examine this hypothesis, we used RNase protection assay to measure mRNA levels of TRs in failing left ventricles that exhibited a fetal pattern of gene expression, ie, decreased expression of α-MHC with increased β-MHC expression compared with left ventricles from age-matched controls. We detected expression of TR-α1, -α2, and -β1 isoforms in human left ventricles. In failing left ventricles, TR-α1 was downregulated, whereas TR-α2, a splice variant that does not bind thyroid hormone but inhibits responses to liganded TRs, was increased. Expression levels of TR-β1 did not differ significantly between the 2 groups. According to linear regression analysis, expression levels of TR-α1 and -α2 were positively and negatively correlated with those of α-MHC, respectively.

Conclusions—We conclude that decreases in TR-α1 and increases in TR-α2 may lead to local attenuation of thyroid hormone signaling in the failing human heart and that the resulting tissue-specific hypothyroidism is a candidate for the molecular mechanism that induces fetal gene expression in the failing human ventricle. (Circulation. 2001;103:1089-1094.)

Key Words: thyroid hormones receptors myosin heart failure

We have recently shown that a fetal gene program is activated in failing human hearts, which includes isoform changes in myosin heavy chain (MHC).1–3 Decreases in the fast-shortening-velocity isoform (α-MHC) coupled with increases in the slow-shortening-velocity isoform (β-MHC) may contribute to decreased contractile function in failing human ventricular myocardium.1–3 Therefore, it is important to explore possible signaling mechanisms responsible for altered MHC gene expression in the failing human heart.

There is compelling evidence, based on clinical studies in the human heart4 and extensive research on rodent myocardium,5,6 that hypothyroidism induces a fetal gene program in the heart in a pattern similar to hypertrophy or failure. These observations suggest that failing hearts may represent a state of dysfunction of thyroid hormone (TH) signaling. Because MHC/fetal gene induction was observed in explanted hearts removed from subjects who were clinically and chemically euthyroid,1–3 any decreases in TH signaling in these hearts would have to be at the level of tissue processing of TH or in TH receptor (TR) regulation.

TRs are ligand-dependent transcription factors that regulate transcription of a number of genes, including MHC, by binding to thyroid-responsive elements (TREs) in promoter regions. TRs have 4 isoforms, α1, α2, β1, and β2.7 TR-β2 exhibits restricted tissue distribution, being found mainly in the pituitary gland, but the other isoforms are expressed differentially but rather ubiquitously.7–9 TR-α1, -β1, and -β2

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isoforms have similar affinities for endogenous TH and characteristics for DNA binding. TR-α2 arises from alternative splicing of the 3’-most exon of the TR-α gene. TR-α2 fails to bind TH, because TR-α2 lacks the C-terminal 40 amino acids of TR-α1, which are critical for TR ligand binding. In general, unliganded TRs repress expression of genes regulated positively by TH, activate expression of genes that are negatively regulated by TH, and block actions of liganded TRs. TR-α2 appears to function like unliganded TRs and thus is called a dominant-negative receptor.

Targeted disruption of TR isoforms is now delineating isoform-specific biological and developmental roles. Studies with mice lacking TR-α1 indicate that this TR isoform is critical for proper cardiac pacemaking and ventricular repolarization. Conversely, when mutant TR-β is overexpressed in mouse hearts in a tissue-specific manner, the dominant MHC induction of a fetal pattern of gene expression. There are alterations in the gene expression of TRs in failing human ventricular myocardium, which in turn trigger the possibility that TR dysregulation occurs in this setting. In the present study, we tested the hypothesis that TRs and thus is called a dominant-negative receptor.

Methods

Experimental Human Hearts

Human hearts were provided by the University of Colorado and Utah Cardiac Transplant Programs. Left ventricles (LVs) from 12 control (nonfailing [NF]) organ donor candidates (6 male, 6 female, mean age 40.6 ± 15.4 years) and 9 age- and sex-matched subjects with idiopathic dilated cardiomyopathy (IDC) undergoing cardiac transplantation (5 male, 4 female, mean age 42.5 ± 16.7 years) were obtained. LVs from 6 transplant recipients with ischemic cardiomyopathy due to coronary artery disease (CAD; 5 male, 1 female, 60.3 ± 6.8 years old) were also analyzed for TR expression, although age and sex were not matched to NF controls. All NF subjects had no cardiac history and no significant structural abnormalities on cardiac donor screening by echocardiography and, in subjects > 50 years of age, no significant CAD on coronary angiography. In all NF hearts, the echocardiogram revealed normal LV systolic function (ejection fraction > 50%), whereas LV ejection fractions in all patients with IDC or CAD were < 20%. No patients were receiving amiodarone or medication for thyroid disorder. All patients had normal thyroid-stimulating hormone (TSH) levels.

Laboratory personnel generating gene expression data were blinded to clinical information relevant to subjects. In all cases, written informed consent was given by a family member or by the transplant patient for research use of the explanted hearts.

Tissue Preparation and Total RNA Extraction

Hearts were placed in ice-cold oxygenated physiological salt solution immediately after removal, and myocardial specimens were gathered within 10 minutes of explantation. One-gm transmural specimens were taken from the central portion of the LV free wall and immediately frozen in liquid nitrogen, then stored at −80°C until use. Total cellular RNA was isolated from frozen LV specimens with RNA STAT-60 (Tel-Test, Inc) as previously described. Human, Ambion), which protected a 120-bp fragment (546 to 666 nucleotides of M33197), was made to a specific activity of ~9 × 10^9 cpm/μg. A 5'-digested human GAPDH probe (pTR1-GAPDH-Human, Ambion), which protected a 120-bp fragment (546 to 666 nucleotides of M33197), was made to a specific activity of ~0.5 × 10^9 cpm/μg and was added to each sample as an internal control. Ten micrograms of total heart RNA or yeast RNA (as a negative control) was hybridized with these probes at 45°C overnight. RNA-probe mixture was then digested with RNase A/T1 (1:100, RPAII, Ambion) at 37°C for 30 minutes, and protected fragments were size-separated by 5% urea-denaturing PAGE. The
part of the gel corresponding to each specific band was cut out, and its radioactivity was counted by liquid scintillation counting. Radioactivity was corrected for background, and the number of expressed molecules of each mRNA species was calculated from the specific activity of probe, length of protected fragment, and ratio of uridine residues in the protected fragment over the full-length probe.

Statistics
Data from quantitative RT-PCR assays were expressed as mRNA molecules $\times 10^5 / \mu\text{g total RNA}$. Data from RPAs were expressed as molar ratio $\times 10^4$ of each mRNA species over GAPDH. One-way ANOVA was used for comparison between different groups. Linear regression analysis was used to relate TR to fetal gene expression. A value of $P<0.05$ in a 2-tailed distribution was considered statistically significant.

Results

MHC and ANP Gene Expression
As previously demonstrated,\textsuperscript{1-3} failing human LVs expressed lower levels of $\alpha$-MHC with markedly elevated ANP expression (Figure 2). Changes in $\beta$-MHC mRNA levels did not reach statistical significance, but there was a tendency for an increase in failing hearts. Thus, the failing human LV samples used in the study exhibited a “fetal” pattern of gene expression.

TR Gene Expression
To investigate gene expression of TRs in human hearts, we first determined which TR isoforms are expressed in LVs. We used a probe that recognized both TR-$\beta_1$ and $\beta_2$ (Figure 1b), which should result in protected 601-bp ($\beta_1$) and 336-bp ($\beta_2$) specific bands. As shown in Figure 3a, no TR-$\beta_2$ specific band was detected in either NF or failing LVs, even with 20 $\mu\text{g}$ of total RNA and maximum specific activity of the riboprobe. Therefore, we used a probe specific for the TR-$\beta_1$ (Figure 1c) for quantification.

We mixed the $\beta_1$-specific probe (Figure 1c) with an $\alpha_1/\alpha_2$ common probe (Figure 1a) and a GAPDH probe. We detected the following isoform-specific bands in human heart samples: 301 bp for TR-$\beta_1$, 246 bp for TR-$\alpha_1$, 154 bp for TR-$\alpha_2$, and 120 bp for GAPDH (Figure 3b, lane 8). All probes were completely digested, and there was no background in the negative control lane, which contained 10 $\mu\text{g}$ of yeast RNA (Figure 3b, lane 7).

As shown in Figure 4, in failing human LVs, expression of TR-$\alpha_2$ was increased, whereas TR-$\alpha_1$ was decreased. There were no significant changes in TR-$\beta_1$ expression between NF and IDC samples. Several known amounts of in vitro–synthesized RNA corresponding to the protected fragment of TR-$\alpha_1$ or TR-$\alpha_2$ were hybridized with the TR-$\alpha_1/\alpha_2$ probe.
We found that the radioactivities of the bands specific for TR-α1 and TR-α2 were linear to the actual amount of input RNA and consistent with the specific activity of the probe. According to the measured radioactivities of bands specific for TR-α1 or TR-α2, the molar expression of TR-α1 or TR-α2 mRNA in 10 μg of human heart total RNA was 2 to 4 × 10^{-18} moles. Quantified data for TR expression are shown in Figure 6. We observed the same pattern of changes in TR mRNA expression in CAD samples.

To investigate the molecular mechanism of the changes in TR-α1 and TR-α2, we measured combined expression of α1 + α2 in NF, IDC, and CAD, using a probe for the consensus sequence of TR-α1 and TR-α3 (Figure 6). There was no difference in the combined expression of α1 + α2. Using this riboprobe, we found only 590-bp specific fragments. N-terminally truncated TRs have been reported as possible dominant-negative TRs.26 They should yield 250-bp fragments, but we could not detect their expression in human heart by RPA. Next, we examined the expression levels of Ear-1,27 a human homologue of rat Rev-erbAa. Rev-erbAa has been reported to inhibit splicing into TR-α2.28,29 Ear-1 is an antisense transcript from the 3′-end of the TR-α gene and has a sequence homologous with part of exon 10 of TR-α2 (Figure 1d). We did not find any differences in Ear-1 expression among the 3 groups (Figure 5), suggesting that mechanisms other than an increase in Ear-1 expression are responsible for the altered TR-α splicing in failing human hearts.

Relationship of Altered TR Expression to Fetal Gene Induction

The alterations in TR-α expression are qualitatively capable of causing changes in fetal gene expression. Accordingly, we performed linear regression analysis between TR and fetal gene expression using the age-matched samples derived from NF and IDC hearts. As can be seen in the Table, TR-α1 expression was positively correlated to α-MHC expression, whereas TR-α2 was negatively correlated to α-MHC expression. In contrast, ANP expression had a negative correlation to TR-α1 expression and a positive correlation to TR-α2 expression. The calculated ratio of α1/(α1 + α2) or α1/α2 was positively correlated to α-MHC and negatively correlated to ANP. β-MHC had no significant correlation with TR gene expression.

Discussion

In this study, expression of TR genes was altered in failing human LVs. Although we found that overall expression of TR-α mRNA levels was unaffected by chronic myocardial failure, there was a specific decrease in the levels of TR-α1 mRNA whose product would be TH-responsive. We also found a reciprocal increase in mRNA levels for TR-α2, which could inhibit TH action. These findings are consistent with the hypothesis that failing human heart has a defect in TH signaling.

MHC and Heart Failure

In rodents, α-MHC is the major isoform in adults, whereas β-MHC is the major one during the fetal-neonatal period and is induced under pathological circumstances, such as hypertrophy/failure or hypothyroidism. In human hearts, the dominance of β-MHC in adulthood precludes an actual switch, but α-MHC does, in fact, decrease and β-MHC increases in gene1,2 and protein3 expression in the hypertrophied, failing ventricle. Based on a single subject reported,4 it is likely that hypothyroidism in humans is also associated with a decrease in α-MHC and an increase in β-MHC gene expression. Therefore, hypothyroidism and hypertrophy/failure resemble each other in terms of myocardial MHC isoform gene expression.

Most subjects with end-stage heart failure undergoing transplantation have normal thyroid function both clinically and chemically, because of the intense clinical screenings given to such patients. In this study, heart failure patients were continuously monitored for thyroid function. According
to this screening, all the subjects with failing hearts in this study had normal TSH levels. Thus, it would seem unlikely that systemic hypothyroidism could have produced the fetal gene program identified in this and other studies. We propose here that TR dysregulation may cause isoform changes in MHC gene expression, but myocardial tissue–restricted decreases in TH levels have not been ruled out by this study.

Previous Work on Cardiac TR Expression
Several reports have detected mRNAs for TR-α1, -α2, and -β1 in RNA extracted from myocardial tissues. With the exception of a single study, TR-β2 mRNA has been detected only with RT-PCR. We could not detect TR-β2 mRNA by RPA in human LVs, or we did not perform RT-PCR or measure TR-β2 protein. On the basis of the observation that the mRNA/protein ratio of TR-β2 is extremely low, it seems likely that TR-β2 protein is quite stable and therefore may be less likely to be regulated in myocardial disease processes.

It has been reported that chronic diabetes or chronic energy deprivation causes no changes in TR gene expression, although the isofrom switch from α-MHC to β-MHC is observed in these disorders. Chronic energy deprivation, however, has been associated with a decrease in TR protein expression. Recently, failing dog hearts with dilated cardiomyopathy or chronic valvular disease have been reported to upregulate TR-β1 and TR-β2 expression. Thus, there is limited information and no consensus regarding the regulation of TRs in myocardial disease states.

Only 1 previous study has reported mRNA expression of TRs in human hearts. Sylvén et al measured TR expression in LVs of NF and IDC, as we did in this study. They described a decrease in TR-α1 in LVs with IDC but no change in TR-α2. They also found considerable TR-β2 mRNA expression in human hearts by use of solution hybridization for detection. The discrepancy between the data of Sylvén et al and ours may be due to the following: (1) they performed solution hybridization for quantification, which may result in relatively high backgrounds; (2) they did not match subjects in terms of age and sex, which may provide a better means to compare TR mRNA levels in NF versus failing human hearts; and (3) in contrast to their study, we did measure expression of cardiac-specific genes in the same samples that we used for mRNA assessment of TRs, which confirmed the origin of samples, ie, from NF or failing hearts.

Regulation of TR-α Gene Expression
We could not find any differences among the 3 groups in total TR-α gene expression. These data suggest that the promoter activity of the TR-α gene, which is shared between TR-α1 and TR-α2, is not altered in failing human hearts. Our results, however, do not preclude a change in TR-α gene transcription rate balanced by an alteration in mRNA stability.

Our findings suggest that alternative splicing of the TR-α gene might be altered in failing hearts. Lazar’s group demonstrated that Rev-erbα, the antisense transcript of the TR-α gene, inhibited splicing into TR-α1. We could not find any differences, however, in mRNA expression of Ear-1, the human homologue of Rev-erbα, among NF, IDC, and CAD hearts. Mechanisms other than an increase in Ear-1 expression should therefore be considered as the explanation for the altered splicing of TR-α gene in failing hearts.

Relationship of TR Dysregulation to Fetal Gene Induction
On linear regression analysis, TR-α1 was positively and TR-α2 was negatively correlated with α-MHC gene expression. The 5′-flanking region of the human α-MHC gene has 2 characterized TREs, providing a molecular basis for these relationships. Interestingly, β-MHC gene expression was not significantly related to TR-α expression. This is surprising, because the human β-MHC promoter is negatively regulated by TH. As far as we know, most of the ligand-independent activation through negative TREs has been related to the TR-β isoform. TR-β-deficient mice have upregulated TSH levels. Overexpression of mutant TR-β in mouse hearts is associated with dominant β-MHC isoform. One possible explanation for the lack of a relationship between β-MHC and TR gene expression is that negative TRE of the β-MHC gene is regulated primarily by TR-β1, for which we could not find any significant changes between NF and failing hearts. These observations suggest that upregulation of the β-MHC gene in the failing human ventricle involves primarily mechanisms other than TR regulation.

Limitations and Future Directions
Several points need to be addressed in the future. First, we have not measured protein levels of TRs in the present studies with antibody detection methods. This is technically difficult, because commercially available antibodies seem to work only with in vitro–synthesized TRs. Second, because we used whole-heart RNA from tissue samples, we cannot document cell specificity of TR expression in myocardium. This point needs to be addressed in future studies by cell dissociation/isolation techniques and/or in situ detection methods. Third, the mechanism of altered splicing in the TR-α gene has not been elucidated and needs to be further investigated, because it may explain our results with altered TR-α1 versus -α2 expression.

Conclusions
We conclude that end-stage failing hearts express lower TR-α1 and higher TR-α2 steady-state mRNA abundance, which provides a potential explanation for the isoform changes of MHC noted in failing human hearts.

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