Cardiac induction of type III deiodinase in human ischemic heart disease


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Abstract

Type III deiodinase (DIO3) is a thyroid hormone (TH)-degrading enzyme, which activity is markedly and stably induced in the left ventricle (LV) following myocardial infarction in mice. The subsequent LV remodeling and the development of cardiac dysfunction is associated with decreased LV tissue TH content and decreased in vivo T3-dependent transcription activity in cardiomyocytes. This local hypothyroid condition may contribute to the development of heart failure, given the known regulation by TH of several key cardiac genes implicated in contractile dysfunction in heart failure. Here, we investigated whether DIO3 is also induced in the myocardium of patients with heart-failure from ischemic heart disease (ISHD).

Tissue samples from patients (n=36) were obtained during heart transplantation surgery and non-failing heart tissue was obtained from donor hearts (n=75). Paraffin sections of tissue microarrays comprising up to 120, 1 mm LV cores of patients and donors were analyzed for DIO3 expression using immunohistochemistry.

While little or no DIO3 staining was found in 72% of the donor samples, medium to very high DIO3 staining was found in 89% of the patient samples with only 11% showing low staining.

This study demonstrates for the first time that cardiac DIO3 expression is induced in ISHD. This suggests that the DIO3-dependent local hypothyroid condition of the heart found in a mouse model of ISHD may also apply to the human situation and contribute to the development of heart failure.
Cardiac DIO3 induction in ISHD

Introduction

In several animal models of heart failure the thyroid hormone (TH)-degrading enzyme type III deiodinase (DIO3) is induced in the heart. In models of pathological remodeling due to chronic pressure overload and myocardial infarction (MI) (Chapter 3), the induction of DIO3 was associated with decreased ventricular tissue TH content and decreased in vivo T3-dependent transcription activity in cardiomyocytes. This aspect of remodeling is expected to influence the progression of heart failure. Because the possible expression of cardiac DIO3 has not been addressed in human heart disease, we investigated DIO3 expression in myocardium of donors as well as of end-stage heart-failure patients who underwent cardiac transplantation.

Pathological ventricular remodeling resulting in congestive heart failure typically results from hypertension, valvular disease, or ischemic heart disease (ISHD). The chronic mechanical and metabolic stress imposed on the heart results in structural and functional changes and although initially adaptive, can progress to dysfunction and failure. A complex interplay of signal-transduction systems drives gene expression that results in the changes in myocyte morphology, contractile properties, metabolism and extracellular matrix composition that ultimately underlie dysfunction.

A partial recapitulation of the fetal gene program is a hallmark of pathological ventricular remodeling. Re-expression of this gene program is also an aspect of the hypothyroid heart, and consequently a role for impaired cardiac TH signaling in ventricular remodeling has been suggested. Examples of TH-regulated cardiac genes that show a hypothyroid profile during remodeling are sarco/endoplasmic reticulum Ca2+-ATPase, voltage-gated potassium channel, β-1 adrenergic receptor, phospholamban, the myosin heavy chain isoforms α and β, the Na+/K+-ATPase, and the Na+/Ca2+ exchanger.

The suggested reduction of TH signaling is supported by several studies showing changes in cardiac TH metabolism as well as alterations in TH receptor (TR) expression. With respect to the latter, both up- and down-regulation of receptor mRNA and protein has been reported, both in animal models and humans. These data indicate that possible involvement of TRs in altered TH action, apart from species differences, depends on the type and stage of pathological remodeling studied. In contrast, in all animal models studied thus far, up-regulation of DIO3 activity has been found. DIO3 converts thyroxine (T4) and 3,5,3′-triiodothyronine (T3) to the inactive metabolites 3,3′,5′-triiodothyronine (rT3) and 3,3′-diiodothyronine (T2), respectively, and it is primarily expressed in fetal tissues. With the exception of brain and skin, DIO3 is virtually absent in adult tissues. However, recent studies show that DIO3 can be up-regulated in conditions of cellular stress as well as in certain pathologies, both in rodents and humans, leading to a reduction of local TH levels. The relevance of DIO3 induction for cardiac TH metabolism was indicated by studies in rat and mouse showing substantial reduction of tissue T3 levels in failing RV and left ventricle (LV).
(Chapter 3[^1]), respectively, which were not related to changes in plasma TH levels. Moreover, the local hypothyroid condition was confirmed in these models by decreased in vivo T\textsubscript{3}-dependent transcription in cardiomyocytes.

Taken together, these data suggest that re-expression of DIO3 might also occur in human heart failure. In the present study, we therefore used explanted hearts of end-stage heart failure patients and unused donor hearts to study the expression of DIO3.

**Methods**

*Human myocardium*

Human cardiac tissue was obtained during heart transplantation surgery from patients with end-stage heart failure. LV free wall was dissected and tissue samples were frozen immediately and stored in liquid nitrogen, or fixed in 5% formaldehyde for examination by immunohistochemistry.

Non-failing cardiac tissue was obtained from potential but unused transplant donor hearts. Hearts were perfused with ice-cold cardioplegic solution, transported on ice to the University of Sydney usually within 2-3 hours of the declaration of brain death. Transmural sections of the free wall of the LV were frozen immediately and stored in liquid nitrogen. Part of the tissue was fixed in 5% buffered formaldehyde for examination by immunohistochemistry.

Samples were obtained with approval of the local ethical committees and in accordance with Australian and Dutch law. The investigation conforms to the principles outlined in the Declaration of Helsinki ([Cardiovascular Research 1997;35:2–4](#)).

*Tissue microarrays*

Five tissue microarrays (TMAs) were constructed as described by others.[^35] The Beecher-MTA1 instrument was used to make an assembly of up to 120 tissue samples from donor and patient hearts. Each heart was represented by two 1 mm diameter cores cut from each fixed-embedded tissue block. Tissue samples were paraformaldehyde (5%)-fixed and paraffin-embedded. Thus, our TMAs contained 60 duplicated samples from each patient or donor. Sections of the resulting TMA block were cut at 4 \(\mu\)m thickness and used for immunohistochemistry.

*Histochemistry*

For DIO3 immunohistochemistry, 4 \(\mu\)m TMA sections were deparaffinized and rehydrated, exposed to 0.02 M HCl (20 min) to block endogenous peroxidase activity and microwaved at 93°C for 10 min in 10 M citrate buffer (pH 6.0) for epitope retrieval. Sections were incubated with the validated, affinity-purified polyclonal rabbit anti-DIO3 antibody 718[^36] at 1:50 dilution for 1 hour at 37 °C and then processed with Envision®+ reagents (Dako, Glostrup, Denmark).
using 3,3′-diaminobenzidine as chromagen. Anti-DIO3 antibody was kindly provided by Dr. D. Salvatore (University of Naples Federico II, Department of Molecular and Clinical Endocrinology and Oncology, Naples, Italy). For the TMA sections, staining intensity of the blinded samples was scored by three investigators using a visual grading score of 1 to 4 (no staining to very high staining) and the scores of each core were averaged (Fig. 2B). The average DIO3 staining was categorized as low (1-2), medium (2.1-3), or high (3.1-4). Of all hearts, 63 were present on 1 TMA, 57 on two TMAs, and 15 on 3 TMAs. Damaged cores were not included in the analysis, and because of this, of 3 hearts only one core was analyzed, but on average 3 cores per heart (range 1 to 8) were scored for DIO3 staining and these scores were again averaged.

**Quantitative real-time PCR**

Total RNA was extracted from LV using TriPure (Roche Applied Science, Basel, Switzerland) and treated with DNase I (Invitrogen, Carlsbad, CA, USA). Two µg of total RNA was used to generate cDNA strands in a 20 µl reaction volume using the Cloned AMV First Strand Synthesis Kit (Invitrogen, Carlsbad, CA, USA). An equivalent of 31.25 ng of total RNA was subsequently used for the preamplification reaction with 15 nM primers for DIO3 (DIO3 sense primer, 5′-CCTGGGAC TCTGCTTCTGTAAC-3′; DIO3 anti-sense primer, 5′-GGGGTGTAAGAAATGCTGTAGAG-3′) and 1x MESA GREEN qPCR MasterMix Plus for SYBR assay (Eurogentec, Seraing, Belgium) in a total volume of 5 µl. Preamplification was performed under the following conditions: 2′ at 50 °C, 10′ at 95 °C, 14 cycles 15′ at 95°C and 4′ at 60°C. The preamplification product was diluted 3 x and 2.5 µl was used for the amplification reaction with 300 nM primers for DIO3 and 1x MESA GREEN qPCR MasterMix Plus for SYBR assay (Eurogentec, Seraing, Belgium) or 1x TaqMan Gene Expression assay for PPIA with VIC probe (Hs99999904_m1; Applied Biosystems, Foster City, CA, USA) and 1x Eurogentec qPCR MasterMix (Eurogentec, Seraing, Belgium) in a total volume of 10 µl, using standard cycle parameters. Both amplification reactions were performed on an Applied Biosystems model 7700 (Applied Biosystems, Foster City, CA, USA). Expression levels of Peptidylprolyl isomerase A (PPIA) were used for normalization.

**Control for effect of cardioplegic solution on DIO3 expression**

Housing of animals, and all experiments complied with the Guide for Care and Use of Laboratory Animals of the National Institutes of Health (NIH Publication no. 86-23, Revised 1996) and were approved by the Institutional Animal Care and Use Committee of VU University Medical Center Amsterdam.

A total of 7 male C57Bl/6J mice, between 11 and 13 weeks of age, were weighed, anesthetized with isoflurane [2.5–3.0% (vol/vol)] and MI was induced by permanent ligation of the left coronary artery (LCA) as described previously.\(^3\)\(^{37}\)\(^{38}\) Mice were sacrificed 8 days after the procedure when MI-induced DIO3 expression has been shown to be high. Animals were
anesthetized and the heart was excised. In 3 of the 5 animals that survived the MI surgery permanent ligation of the LCA resulted in an infarcted area of the LV of 30-50%.

The heart was briefly perfused in retrograde fashion via the aorta with ice-cold cardioplegic solution (119 nM NaCl, 1.2 mM NaH₂PO₄, 4 mM KCl, 25 mM NaHCO₃, 1.2 mM MgSO₄, 1 mM CaCl₂, 11 mM glucose). The LV noninfarcted tissue of each heart was cut in four pieces: one was frozen in liquid nitrogen and stored at -80°C until DIO3 activity analysis; one was fixed in 4% paraformaldehyde and processed for histochemistry; and two were incubated for 4 hours in cold cardioplegic solution after which one of the two was frozen in liquid nitrogen and stored at -80°C until DIO3 activity analysis and the other one was fixed in 4% paraformaldehyde and processed for histochemistry. The 4 hour incubation is the maximum time that donor hearts are kept in this solution.

For immunohistochemistry, 4 µm LV sections were stained for DIO3 as described above. DIO3 enzyme activity was determined in homogenates of LV tissue by analysis of inner-ring deiodination of radiolabeled T₃ essentially as described previously.³⁹

Statistics

Data analysis was performed with GraphPad Prism version 5.01 for Windows (GraphPad, San Diego, CA, USA) and SPSS 15.0 for Windows (SPSS Inc, Chicago, IL, USA). The Chi-square or Fisher’s exact test was used to evaluate the DIO3 immunostaining. For analyses of DIO3 mRNA of human heart samples unpaired t testing was used, with Grubbs’ test for outlier detection, and for analyses of DIO3 activity measurements of mouse heart samples paired t testing was used. Significance was accepted when P<0.05. Data are presented as means ± SEM of n observations.

Results

DIO3 expression

All ISHD patients (n= 36) undergoing transplant surgery suffered from end-stage heart failure. Characteristics of patients and donors are given in Table 1.

Immunohistochemical analysis of TMAs showed little or no DIO3 staining in most donor samples (Fig. 1A), with 54 out of 75 (72%) scoring 1 to 2. In contrast, medium to very high

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Data are means ± SEM.
DIO3 staining was found in most of the ISHD samples (Fig. 1A). Only 4 of the 36 patients had low staining while 13 scored 2 to 3 (36%) and 19 scored 3 to 4 (53%). Thus, the DIO3 staining was significantly higher in ISHD patients ($P<0.001$) compared to the donors.

In the majority of the positive ISHD samples, all cardiomyocytes showed high DIO3 expression (Fig. 1B-1), but in some samples both DIO3-positive and negative cardiomyocytes were present (Fig. 1B-2 and 1B-3), similar to what is found in remodeled mouse myocardium following MI (Chapter 3)\(^4\). Interstitial cells did not show DIO3 expression in any of the samples. Areas not containing cardiomyocytes, like the fibrotic areas annotated with an asterisk in Fig. 1A, were excluded when determining the score. Fig. 2 summarizes the DIO3 staining scores obtained for the donor and patient groups.

**Figure 1.** Typical examples of TMA elements of donor and ISHD patient LV samples stained for DIO3 (A). Details of cellular staining in ISHD sections shown in B, with high DIO3 staining in all cardiomyocytes in B1 and cells with high and low DIO3 staining in B2 and B3. Scale bar indicates 1 mm in A and 10 μm in B.

**Figure 2.** Distribution of patient and donor samples according to the DIO3 immunohistochemistry staining intensity (A). Staining intensity was scored on a scale of 1-4 by three observers as described in the Methods section using four reference samples (B). The average scores were categorized as follows: low staining: 1 to 2, medium staining: 2.1 to 3, and high staining: 3.1 to 4. Data are % of total number of donor or patient samples, for donor n = 75 and for ISHD n = 36.
**DIO3** mRNA expression was analyzed in LV tissue samples of 21 ISHD patients and 27 donors. Expression levels were found to be low and a preamplification of 14 cycles was needed to obtain Ct values of approximately 24. This is similar to the Ct values for **PPIA** obtained without preamplification and which were used for normalization. For two samples in each group **DIO3** mRNA expression could not be detected. Fig. 3 shows the absence of an effect of ISHD on the normalized expression of **DIO3** mRNA.

![Figure 3. RT-PCR analysis of mRNA levels of cardiac DIO3 in 24 ISHD samples (average DIO3 staining score 3.2±0.1) and 19 donor samples (average DIO3 staining score 1.7±0.1). DIO3 mRNA expression levels were normalized to those of

**Effect of cardioplegia on DIO3 expression**

LV tissue from explanted hearts is immediately frozen, whereas donor hearts are perfused with cold cardioplegic solution and kept on ice for up to 4 hours needed to transport the heart to the transplant theaters where the tissue was immediately dissected into small (about 1 g) pieces and frozen at -200 °C. To exclude the possibility that healthy hearts also express DIO3 but that the cardioplegic solution affects DIO3 staining, the effect of cardioplegia on DIO3 detection was tested on infarcted mouse hearts. DIO3 activity and DIO3 immunostaining were analyzed after brief perfusion with cold cardioplegic solution (t = 0) and after 4 hours of incubation on ice in this solution (t = 4). DIO3 activities at t = 0 and t = 4 were 0.8 ± 0.2 fmol/min/mg (n = 3) and 1.8 ± 0.5 fmol/min/mg (n = 3), respectively. This demonstrated that cold cardioplegic solution does not lower DIO3 activity. Accordingly, no difference was observed in the DIO3 immunostaining pattern or intensity between tissue sections of these hearts at t = 0 and t = 4 (data not shown).

**Discussion**

This study demonstrates for the first time that cardiac DIO3 expression is induced in human ischemic heart disease. This suggests that the DIO3-dependent local hypothyroid condition of the heart found in a mouse model of chronic heart failure following MI, also applies to human hearts and may contribute to the development of heart failure.

The immunostaining showed that DIO3 is expressed in cardiomyocytes and although we can not exclude that DIO3 is also expressed by other cell types, such as inflammatory cells.
which are known to be able to express DIO3, we did not observe DIO3 staining in cells other than cardiomyocytes in the immunohistochemistry images.

Although we found substantial cardiac expression of DIO3 in most ISHD patients with end-stage heart failure, some patients showed levels of DIO3 staining comparable to donor hearts. This may be explained by the limited number of randomly sampled tissue cores in combination with the heterogeneity of ventricular remodeling, i.e., the hearts of ISHD patients will also have areas with normally perfused, non-remodeling tissue as well as hibernating tissue distal to severe stenoses.

Despite the evident increase in DIO3 immunostaining in ISHD samples, DIO3 activity was not detectable in whole homogenates of cardiac tissue (data not shown). It should be noted that the significant DIO3 activity in freshly prepared mouse cardiac samples, showing similar DIO3 staining intensity, is in the lower fmol/min/mg range (Chapter 3). This is close to the detection limit of the current DIO3 assay and a likely explanation for the lack of DIO3 activity of the tissue used in the present study is that these samples have been stored in liquid nitrogen for at least one year. This does not affect the quality of mRNA, as normal levels of mRNA of house-keeping genes were found. The absence of a difference in the levels of DIO3 mRNA between the donor and the ISHD groups is in line with what is found in mice (Chapter 3). In this model DIO3 activity is stably induced for at least 8 weeks, whereas mRNA expression is only transiently stimulated after MI. This suggests that in later stages of remodeling post-transcriptional mechanisms are responsible for the increased DIO3 protein levels.

In animal models of heart failure cardiac DIO3 induction was associated with decreased tissue T3 levels and reduced T3-dependent transcription in vivo. The present data suggest that this may also be the case in human heart failure. Indeed, induction of DIO3 in human liver and skeletal muscle is a general response in critically ill patients. This was associated with decreased T3 and increased rT3 concentrations in plasma, which were prognostic for mortality. The prognostic value of low circulating T3 levels also applies to cardiac patients.

Whereas the induction of DIO3 expression in liver and skeletal muscle is a general response, the expression of DIO3 in some but not all cardiomyocytes in remodeled myocardium indicates regional or cell-specific mechanisms. Potential regulators of DIO3, which are also known to be involved in cardiac remodeling and hypertrophy, are transforming growth factor β, mitogen-activated protein kinases, sonic hedgehog, and hypoxia-inducible factor-1α (HIF-1α). The latter is a likely candidate regulating DIO3 induction during cardiac remodeling as it was shown to induce DIO3 in cardiomyocytes as well as in other cell types in response to hypoxia. In addition, HIF-1α interacts directly with the DIO3 gene. Furthermore, HIF-1α induction was found in several animal models post-MI and associated with DIO3 induction in a rat model of right ventricular hypertrophy and heart failure. HIF-1α expression is also up-regulated in ISHD as a result of the inadequate
myocardial perfusion and tissue hypoxia. In addition, increased ventricular wall tension may be an independent stimulus of HIF-1α signaling in the diseased heart.

A possible limitation of this study is that donor hearts underwent a different treatment than explanted failing hearts, which may have resulted in loss of any DIO3 activity present in healthy myocardium. Although DIO3 is a fetal gene and activity in adult heart may be assumed to be as low as in adult rodent heart, DIO3-expressing post-MI mouse hearts were treated as donor hearts to assess the effect of cardioplegia on DIO3 expression. Perfusion with cardioplegic solution and short term storage on ice did not affect DIO3 activity or immunostaining, excluding the procurement protocol as a cause of the low or absent DIO3 staining in donor tissue.

Tissue-specific induction of DIO3 has now been shown to occur in several pathological situations in animal models and in humans, affecting local and systemic TH metabolism. The present finding that cardiac DIO3 is also induced in ISHD patients, suggests that the impaired cardiac T3 signaling shown in animal models is present in human cardiac disease and may contribute to the development of heart failure.

Acknowledgments

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References


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