Expression pattern and ontogenesis of thyroid hormone receptor isoforms in the mouse heart

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Abstract

Nuclear thyroid hormone (T₃) receptors (TR) play a critical role in mediating the effects of T₃ on development, differentiation and normal physiology of many organs. The heart is a major target organ of T₃, and recent studies in knockout mice demonstrated distinct effects of the different TR isoforms on cardiac function, but the specific actions of TR isoforms and their specific localization in the heart remain unclear. We therefore studied the expression of TRα1, TRα2 and TRβ1 isoforms in the mouse heart at different stages of development, using monoclonal antibodies against TRα1, TRα2 and TRβ1. In order to identify distinct components of the embryonic heart, in situ hybridization for cardiac-specific markers was used with the expression pattern of sarcoplasmic reticulum calcium-ATPase 2a as a marker of myocardial structures, while the pattern of expression of connexin40 was used to indicate the developing chamber myocardium and peripheral ventricular conduction system. Here we show that in the ventricles of the adult heart the TRβ1 isoform is confined to the cells that form the peripheral ventricular conduction system. TRα1, on the other hand, is present in working myocardium as well as in the peripheral ventricular conduction system. In the atria and in the proximal conduction system (sinoatrial node, atrioventricular node), TRα1 and TRβ1 isoforms are co-expressed. We also found the heterogeneous expression of the TRα1, TRα2 and TRβ1 isoforms in the developing mouse heart, which, in the case of the TRβ1 isoform, gradually revealed a dynamic expression pattern. It was present in all cardiomyocytes at the early stages of cardiogenesis, but from embryonic day 11·5 and into adulthood, TRβ1 demonstrated a gradual confinement to the peripheral ventricular conduction system (PVCS), suggesting a specific role of this isoform in the formation of PVCS. Detailed knowledge of the distribution of TRα1 and TRβ1 in the heart is of importance for understanding not only their mechanism of action in the heart but also the design and (clinical) use of TR isoform-specific agonists and antagonists.


Introduction

Tri-iodothyronine (T₃) affects cardiac function mainly by exerting a direct effect on cardiac cells through binding to thyroid hormone receptors (TR), thus regulating several functionally important proteins responsible for myocardial excitation and contraction (Gloss et al. 2001, Klein & Ojamaa 2001, Mansen et al. 2001, Dillmann 2002). At present, at least five different TR isoforms (α1, α2, β1, β2 and β3) are known (O’Shea & Williams 2002, Flamant & Samarut 2003). Although heart has been termed a TR tissue, recent work on TR isoform-specific knockout mice has indicated that both TR isoforms α1 and β1 play their part and have distinct effects (Weiss et al. 1998, 2002, Wikstrom et al. 1998, Johansson et al. 1999). Whereas TRα1-deficient mice have bradycardia; prolonged PQ, QRS and Q–T end intervals in the electrocardiogram; and preserved response to stimulation with T₃, TRβ-deficient mice show tachycardia, shortened Q–T end interval and lack of response to T₃ administration (Weiss et al. 1998, 2002, Wikstrom et al. 1998, Johansson et al. 1999). Hence, TRα1 appears to be involved in determining basal heart rate and ventricular depolarization, while TRβ appears to have a major function in mediating T₃-induced increase in heart rate. The suggestion that activation of TRα1 or TRβ1 may have distinct effects also derives from studies on GC-1 (Trost et al. 2000, Baxter et al. 2001). This TRβ1-selective agonist showed no effect on heart rhythm, but it had a positive inotropic effect, again indicating that the receptor isoforms work in different environments. There are no data, however, relating the different inotropic and chronotropic effects of TR isoforms to their presence in different structural components of the heart. Very little is known about the expression

DOI: 10.1677/joe.1.06282

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patterns of the TR isoforms in adult and embryonic heart. In contrast, it is well known that TRs show spatiotemporal dynamics in expression in other organs (Perez-Castillo et al. 1985, Forrest et al. 1991, Bradley et al. 1994, Gauthier et al. 1999). We have recently shown that the TR isoforms are zonally expressed in rodent liver and will therefore act in only a subset of cells (Zandieh et al. 2002, Zandieh-Doulabi et al. 2003). In the brain, TRα1, TRα2 and TRβ1 isoforms have also distinct expression patterns (Alkemade et al. 2005). In view of this and the differential effects of the TR isoforms mentioned above, we set out to study by immunohistochemistry the distribution of TRα1 and TRβ1 in mouse heart in the adult and in different stages of the embryonic development.

Within the heart, two types of myocardial components are distinguished functionally: the conduction system (CS) and the working (atrial and ventricular) myocardium. The adult CS consists of separate components. The pacemaker sinoatrial (SAN) and atrioventricular (AVN) nodes are slow-conducting myocardial regions, whereas the atrioventricular bundle (AVB or bundle of His), the right and left bundle branches, and the peripheral ventricular conduction system (PVCS) are fast-conducting pathways. These components vary in their morphologic characteristics and in their molecular phenotype. This is exemplified by the expression pattern of the gap-junction proteins connexin 40 (Cx40) and 43 (Cx43) in the mammalian heart. Cx43 is expressed throughout the working myocardium and PVCS, but not in the SAN, AVN and atrioventricular bundle, whereas Cx40 is mainly expressed in the atrial working myocardium, atrioventricular bundle, bundle branches and peripheral ventricular conduction system (PVCS) (Moorman et al. 1998). Therefore, both Cx40 and Cx43 can be used as negative markers of the SAN, whereas Cx40 can be used as a positive marker for the AVB and PVCS. Since SERCA2a is less expressed in the PVCS of embryonic and fetal heart than in the working, compact myocardium, it has also been used to distinguish these two components of the heart (Moorman et al. 1995, 1998).

Here we show that both TRα1 and TRβ1 are expressed in the myocardium at embryonic day 9.5, but during the later cardiogenesis and in the adult, TRβ1 expression becomes confined to the atria and the ventricular subendocardial cells that constitute the PVCS. TRα1 is expressed in the atria and all cardiomycocytes of the working myocardium and ventricular conduction system. Furthermore, both TRα1 and TRβ1 are present in the SAN, AVN and AVB.

Materials and Methods

Animals

Mouse embryos from different ages (embryonic days 9.5, 11.5, 13.5, 15.5 and 17.5) or adult mice were killed, and their hearts were collected after removing the thoracic wall. The hearts were processed for immunohistochemistry and in situ hybridization by the same protocol of fixation (overnight (4°C) in 4% paraformaldehyde) and embedded in Paraplast Plus (Monoject, Kildare, Ireland). Serial sections of 10 μm were cut, mounted on RNase-free slides (Superfrost/Plus; Menzel-Gläser, Braunschweig, Germany) and stored at room temperature. All animal experiments were approved by the local animal welfare committee of the University of Amsterdam.

The liver and heart of the TRβ knockout mice used in control experiments were the kind gift of Prof B Vennstrom, Stockholm, Sweden.

Immunohistochemistry

Serial sections were incubated with a new set of specific monoclonal antibodies against TRβ1 (clone 152), TRα1 and TRα2 (Zandieh et al. 2002, Zandieh-Doulabi et al. 2003). The specificity of the TRβ1 monoclonal 152 is indicated in Fig. 1. Antibody binding was detected by a goat antimouse secondary antibody, conjugated to alkaline phosphatase, which was visualized by NBT/BCIP (Roche) as a substrate of the alkaline phosphatase. Paraplast-embedded embryonic hearts were cut into 10-μm-thick sections and pasted on Superfrost/Plus slides (Menzel-Gläser). Sections were deparaffinized before immunostaining, placed in PBS (pH 7.4) and boiled for 2 min in sodium citrate buffer (pH 6) to retrieve the antigenic epitope. After cooling to room temperature, the sections were washed in PBS and placed in a blocking solution TENG-T (0.01 M Tris, EDTA, NaCl, gelatin and Tween-20) for 60 min. The first antibodies were diluted in PBS (pH 7.4) and incubated first for 1 h at room temperature and then overnight at 4°C. The sections were washed with PBS (pH 7.4) for 10 min, and then incubated with the secondary antibody (conjugated with alkaline phosphatase) diluted in PBS (pH 7.4). After a washing period of 10 min in PBS, the sections were placed in alkaline phosphatase buffer (50 mM MgCl2, 100 mM NaCl and 100 mM Tris (pH 9.2)) for 10 min. The substrate NBT/BCIP (Roche) and 0.1 M levamisole (Sigma) (inhibitor) were diluted respectively 1:50 and 1:100 in alkaline phosphatase buffer and added to the sections for 30–90 min in the dark. The sections were washed in PBS and then processed in xylene. The sections were enclosed with Entellan (Merck) (Zandieh et al. 2002, Zandieh-Doulabi et al. 2003).

RNA probes and probe specification

Poly-A RNA was isolated from mouse liver samples with MagnaPure (Roche) according to the manufacturer’s protocol. Thereafter, cDNA was synthesized with the First Strand Synthesis cDNA kit with random primers (Roche). To amplify specific fragments of TRs with
attached T7 or T3 promoters, we performed PCR reactions with the following primers:

- **TRα1** (forward, 5'-GAAATTAACCCTCAGCTGATGAAAG-3'; reverse, 5'-GTAATACGACTCCTATAGGGGCGTGAGGCTTTAGACTTCCTGATC-3').
- **TRβ1** (forward, 5'-GGAATTAACCCTCAGCTGATGAAAG-3'; reverse, 5'-GTAATACGACTCCTATAGGGGCGTGAGGCTTTAGACTTCCTGATC-3').

The amplified PCR products were used as templates for the synthesis of single-stained, digoxigenin (DIG)-labeled RNA probes, which were made according to the manufacturer’s specifications (Roche) and were not subjected to alkaline hydrolysis, because this treatment had no or few adverse effects on the sensitivity of hybridization. Sense probes have been synthesized for a negative control staining with T3-RNA polymerase. The probe labeling and the hybridization conditions were as detailed elsewhere (Moorman et al. 2001). Sequence-specific probes against Cx40 and Cx43 have been described previously (Delorme et al. 1995, 1997, Moorman et al. 2001). Complementary RNA probes against bMHC and SERCA2a were used, as detailed elsewhere (Moorman et al. 2001).

**Nonradioactive in situ hybridization**

The pretreatment of the sections consisted only of proteolytic digestion for 14 min at 37 °C with 20 µg/ml proteinase K dissolved in PBS, followed by a 5-min rinse in 0.2% glycine/PBS and two 5-min rinses in PBS. Sections were then refixed for 20 min in 4% formaldehyde/0.2% glutaraldehyde dissolved in PBS to ensure firm attachment of the sections to the microscope slides, and washed twice in PBS for 5 min. Sections were prehybridized in hybridization mix without probe for 1 h at 70 °C and then hybridized overnight at 70 °C. The hybridization mix comprises 50% formamide, 5× SSC, 1% block solution (Roche), 5 mM EDTA, 0.1% Tween-20, 0.1% Chaps (Sigma), 0.1 ng/ml heparin (Becton-Dickinson, Mountain View, CA, USA) and 1 mg/ml yeast total RNA (Roche). Probe concentration was about 2 ng/µl. Approximately 10 µl hybridization mix were applied to the sections, and no cover slips were used. After hybridization, sections were rinsed in 2× SSC (pH 4.5), washed three times for 30 min at 65 °C in 50% formamide/2× SSC (pH 4.5), followed by three 5-min washes in PBST. Probe bound to the section was immunologically detected by sheep antidigoxigenin Fab fragment covalently coupled to alkaline phosphatase and NBT/BCIP as chromogenic substrate, essentially according to
the manufacturer’s protocol (Roche). Sections were washed with double-distilled water, dehydrated in a graded ethanol series and xylene, and embedded in Entellan (Moorman et al. 2001).

Photo imaging

Images of sections were taken with a digital Olympus DP12 camera coupled to a Zeiss Axiophoto microscope, equipped with differential interference contrast (DIC) optics. A flat-field correction was applied to all images with a user-written macro in PMIS 4·1 (www.gkrcc.com).

Nonradioactive in situ hybridization

The pretreatment of the sections consisted only of proteolytic digestion for 5–15 min at 37 °C with 20 µg/ml proteinase K dissolved in PBS, followed by a 5-min rinse in 0·2% glycine/PBS and two 5-min rinses in PBS. Sections were then refixed for 20 min in 4% formaldehyde/0·2% glutaraldehyde dissolved in PBS to ensure firm attachment of the sections to the microscope slides, and washed twice in PBS for 5 min. Sections were prehybridized in hybridization mix without probe for 1 h at 70 °C and then hybridized overnight at 70 °C. The hybridization mix is composed of 50% formamide, 5 × SSC, 1% block solution (Roche), 5 mM EDTA, 0·1% Tween-20, 0·1% Chaps (Sigma), 0·1 mg/ml heparin (Becton-Dickinson), and 1 mg/ml yeast total RNA (Roche). Probe concentration was about 1 ng/µl. Approximately 6 µl hybridization mix were applied to the sections, and no cover slips were used. After hybridization, sections were rinsed in 2 × SSC (pH 4·5) and washed three times for 30 min at 65 °C in 50% formamide/2 × SSC (pH 4·5), followed by three 5-min washes in PBST. Probe bound to the section was immunologically detected by sheep anti-

Nonradioactive in situ hybridization in the adult heart

To see whether the mRNA is translated and the proteins are also present, we used monoclonal antibodies against TRα1 or TRβ1. Monoclonal antibody 152 is specific for TRβ1, as Fig. 1 shows no staining in liver slices of TRβ1 knockout mice, and the TRβ1-specific band is not detected in whole-cell liver extracts of these mice. The immunohistochemistry with TR isoform-specific monoclonal antibodies confirmed that both studied isoforms are present in the heart, but have different expression patterns. TRα1 and TRβ1 were detected in atria, SAN, AVN and ventricle (Fig. 3). As with the mRNA level, TRβ1 showed a heterogeneous pattern of expression in the ventricles and was highly restricted to the cells of PVCS (Fig. 3). Staining was both nuclear and cytoplasmic, as reported before (Falcone et al. 1992, Zandieh et al. 2002). Valves did not stain and thus gave an additional indication of the specificity of the reaction with the antisera (data not shown). For further verification of the expression profiles, we stained hearts from TRβ knockout mice, where we did find staining of the PVCS structures for Cx40 and TRα1, but the TRβ1 antiserum, as expected, did not stain (Fig. 3, right panel) these structures.

Ontogeny

Immunohistochemistry with the TR isoform-specific monoclonal antibodies demonstrated that all TRα1, TRα2 and TRβ1 isoforms are present in the myocardium from embryonic day (E) 9·5 onward. Staining was both nuclear and cytoplasmic. The cardiac cushions did not stain, indicating the specificity of the reaction. The epicardium and the endocardium also showed no staining.
and were used as internal negative controls. The results are described per isofrom and the figures per developmental stage.

Again, in order to identify the distinct cardiac components, we performed in situ hybridization staining for Cx40 and SERCA2a (Moorman et al. 1995, 1998, Figure 2

**Figure 2** Localisation of TRα1 and TRβ1 mRNA in the adult mouse heart. Sinoatrial node (SAN) and atrium (a, d, g): in situ hybridisation with antisense probes demonstrates TRα1 and TRβ1 in consecutive sections and the presence of their mRNAs in the SAN and the atrial myocardium (d and g). The SAN is identified by the absence of connexin 43 staining (a). The SAN artery (arrow) is used as an additional morphologic marker of the SAN on the consecutive sections. As a control we hybridised a consecutive section with a TRα1 sense probe (SP; j). Peripheral ventricular conduction system (PVCS; b, e, h): in situ hybridization for connexion 40 (Cx40) identifies the cells that form the subendocardial PVCS (panel b). (e) shows the presence of the TRα1 mRNA in both PVCS (arrows) and working myocardium. (h) shows the presence of the TRβ1 mRNA only in cells that make up the PVCS (arrows). As a control we hybridized a consecutive section with a TRα1 sense probe (SP; k). Atrioventricular node (AVN; c, f, i): in situ hybridization with antisense probes detects TRα1 and TRβ1 on consecutive sections and shows mRNAs present in the AVN (f and i). The AVN (arrow) is identified by the absence of connexin 43 staining (c). TRα1 mRNA is also expressed in the interventricular septum (IVS; f). As a control we hybridized a consecutive section with a TRα1 sense probe (SP; l). All panels were photographed at the same microscope settings; bar = 0.1 mm. Ig, lung; scv, superior caval vein.
Christo\DJ els et al. 2000). Cx40 is restricted to the developing chambers at E 9·5, but, later in development, it becomes confined to the atria, atroventricular bundle (AVB), bundle branches (bb) and peripheral ventricular conduction system. Cx40 is not present in the sinoatrial node (SAN), the atrioventricular node (AVN) and the ventricular working myocardium (Figs 1–9). However, SERCA2a is expressed in all cardiomyocytes. Both immunohistochemistry and in situ hybridization for SERCA2a show stronger staining in the atria than in the ventricles. Moreover, in the ventricles, the compact myocardium is more stained than the PVCS (Figs 6 and 8). Therefore, Cx40 is used as a negative marker of the proximal conduction system (SAN and AVN) and a positive marker of the PVCS; SERCA2a is used as a positive marker of the myocardium.

TRα1

At E 9·5, TRα1 could already be detected in the entire heart tube. All components of the developing myocardium (inflow tract, developing chambers and outflow tract) at E 9·5 were homogeneously stained (Fig. 4). In all developmental stages TRα1 expression was restricted to the myocardium. Endocardium, epicardium, cardiac cushions and valves were negative (Figs 1–9).
Further immunohistochemical study showed that TRα1 is expressed in all cardiomyocytes at all developmental stages, including working myocardium and the conduction system (SAN, AVN, AVB, bb and PVCS) (Figs 1–9). Furthermore, TRα1 expression was higher in the atria than in the ventricles in all developmental stages.

TRα2

At E 9·5, TRα2 could also be detected in all components of the developing myocardium (Fig. 4). Later, TRα2 remained expressed throughout the entire myocardium at all developmental stages, the atria showing stronger expression than the ventricles, as with TRα1 (Figs 6–8). Both the working myocardium and conduction system (SAN, AVN, AVB, bb and PVCS) revealed expression of TRα2 too (Figs 6–8).

TRβ1

At E 9·5, the TRβ1 isoform showed a similar homogeneous pattern of expression to the other studied isoforms (Fig. 4). The expression of TRβ1 at E 11·5 was also similar to the other studied isoforms, showing higher expression in the atria than in the ventricles (Fig. 5).

From E 13·5 onward, TRβ1 showed a distinct pattern of expression, appearing mainly in the SAN, atria and developing PVCS (Fig. 6). The latter can be seen by comparing the expression of TRβ1 in the ventricles with that of Cx40, which is higher in the fast-conducting, trabecular structures than in the compact working myocardium (Fig. 6). The trabecular structures will contribute to the PVCS.

At E 13·5, the precise localization of the AVN cannot be assessed, as the fibrous insulation of the atrial and ventricular chambers is not complete and the atrioventricular canal (AVC) is still a distinct anatomic and functional entity. However, Cx40 allows identification of the developing left and right bundle branches along the left and right aspects of the ventricular septum (Figs 6 and 8). In summary, TRβ1 is present in the atria and all components of the proximal conduction system (SAN, AVC and AVB), showing difference with the expression of Cx40, which is expressed in the atria, but not in the nodes. Interestingly, the expression pattern of TRβ1 in the ventricles follows that of Cx40.

At E 15·5, the ventricular conduction system is entirely recognizable morphologically, although the process of maturation continues. At this stage, TRβ1 expression remained predominant in the PVCS, represented by subendocardial trabecular structures and distal bundle branches, following the pattern of Cx40 expression (Fig. 7).

At E 17·5, the differences in the expression patterns of the studied isoforms became clearer, and they did not change when compared with the previous embryonic period (Figs 8 and 9).
Discussion

Various developmental and morphologic studies have identified the heart as a complex organ composed of functionally and morphologically distinct components (Moorman et al. 1998, Christoffels et al. 2000, Franco & Icardo 2001). Based on the structural and functional heterogeneity of the heart, we hypothesized that the different thyroid hormone receptor isoforms could be differentially expressed in the cardiac components and that this can partly account for functional differences of the TR isoforms.

Here we show that TRβ1 expression in the adult is confined to the PVCS, a structure responsible for the fast, simultaneous excitation of the ventricular myocardium. This confined expression pattern is seen at both mRNA and protein level. The stronger immunohistochemical staining agrees with studies showing that, when comparing the embryonal with the adult heart, the total mRNA concentration decreases, whereas the total protein concentration increases almost threefold (van den Hoff & Moorman 1999). The expression of TRα1 throughout both the ventricular working myocardium and the conduction system confirms previous results (Wikstrom et al. 1998, Dillmann 2002, Weiss et al. 2002). Not only is TRα1 the most abundantly present TR isoform in the heart, but it is also responsible for most cardiac actions of T3 (Wikstrom et al. 1998, Gloss et al. 2001, Dillmann 2002, Weiss et al. 2002).

During the formation of the heart, we found that TRα1, TRα2 and TRβ1 isoforms are expressed in myocardium from E 9·5 onward. The cardiac expression of all isoforms was confined to the myocardium. The presence of the TR isoforms in early stages of heart development agrees with previous RNA-based studies (Forrest et al. 1991, Bradley et al. 1994, Brent 1994). In line with these observations, TRα knockout embryos display a cardiac phenotype already at E 9·5 (Mai et al. 2004).

Figure 5 Embryonic age 11·5 days. Consecutive sections (10 μm) stained with monoclonal antibodies against TRβ1 (a), TRα1 (c), TRα2 (d) (immunohistochemistry) and DIG-labeled RNA probe for Cx40 (b) (in situ hybridization). The atrioventricular canal is indicated by arrow. avc, atrioventricular canal; RA, right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle. Bar=0·2 mm.
Figure 6 Embryonic age 13·5 days. Consecutive sections (10 μm) stained with monoclonal antibodies directed against TRβ1 (a), TRα1 (c), TRα2 (e) (immunohistochemistry), and DIG-labeled RNA probe for Cx40 (b) and SERCA2a (d) (in situ hybridization; ISH). The arrows on the insets indicate the sinoatrial node (SAN). The comparison of the staining of consecutive slices with TRβ1 monoclonal antibody and Cx40 ISH indicates the predominant expression of TRβ1 in the peripheral ventricular subendocardial conduction system (PVCS). It can be seen that all TR isoforms are expressed in the SAN, in contrast to Cx40, which is present in the SAN artery, but not in the myocardium. In the ventricles, however, TRβ1 has a similar expression pattern to Cx40. RA, right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle; SCV, superior caval vein. Bar=100 μm.
Figure 7 Embryonic age 15·5 days. Consecutive sections (10 μm) stained with monoclonal antibodies directed against TRβ1 (a), TRα1 (c), TRα2 (d) (immunohistochemistry; IHC) and DIG-labeled RNA probe for Cx40 (b) (in situ hybridization). (e–g) indicate the distal atrioventricular node (AVN)/proximal atrioventricular bundle (AVB) (indicated by asterisk). (a’–d’) show peripheral ventricular conduction system (PVCS).

The comparison of the staining of consecutive slices with the TRβ1 monoclonal antibody and Cx40 IHC indicates the predominant expression of TRβ1 in the subendocardial conduction system. RA, right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle. Bar = 100 μm.
The presence of TRs in the heart and lung before the development of the thyroid gland and secretion of thyroid hormone, which occurs in mice after E 15, suggests that the TRs exert their action in a T₃-independent manner or that TRs can be activated by maternal T₃. The first hypothesis is supported by the finding that TRs can repress...
Peripheral ventricular conduction system (PVCS) at embryonic age 17·5 days (from Fig. 8). Only a few cells stained for Cx40 (b) and negative for SERCA2a (d) are identified as PVCS. The staining of a consecutive slice with the TRβ1 monoclonal antibody (a) shows that the same cells stain with this antibody, indicating the predominant expression of TRβ1 in the subendocardial conduction system. The figure shows consecutive sections (10 μm) stained with monoclonal antibodies against TRβ1 (a), TRα1 (c), TRα2 (e) (immunohistochemistry) and DIG-labeled RNA probe for Cx40 (b), and SERCA2a (d) (in situ hybridization). Bar=100 μm.

Figure 9. Peripheral ventricular conduction system (PVCS) at embryonic age 17·5 days (from Fig. 8). Only a few cells stained for Cx40 (b) and negative for SERCA2a (d) are identified as PVCS. The staining of a consecutive slice with the TRβ1 monoclonal antibody (a) shows that the same cells stain with this antibody, indicating the predominant expression of TRβ1 in the subendocardial conduction system. The figure shows consecutive sections (10 μm) stained with monoclonal antibodies against TRβ1 (a), TRα1 (c), TRα2 (e) (immunohistochemistry) and DIG-labeled RNA probe for Cx40 (b), and SERCA2a (d) (in situ hybridization). Bar=100 μm.
certain genes in the absence of T₃ and act as antagonists of retinoid acid receptors (RAR) (Brent et al. 1989, Brand 2003, Clabby et al. 2003). In addition, low levels of T₃ during pregnancy do not seem to affect prenatal heart development (van Tuyl et al. 2004). However, maternal hypothyroidism seriously affects the postnatal cardiac maturation, even at normal T₃ levels (van Tuyl et al. 2004). Although the exact functions of the distinct TRs at this developmental stage still remain unclear, their presence in the heart indicates that they have an important role in the early cardiogenesis and may imply that the thyroid status of the mother during pregnancy affects cardiogenesis.

We found also that each studied isoform has a distinct expression pattern and follows specific dynamics during heart ontogenesis. TRα₁ and TRα₂ were present in all cardiomyocytes in all studied embryonic stages, being more abundant in the atria than in the ventricles. TRβ₁ was also ubiquitously expressed at E 9.5, but later in development, namely, E 11.5, E 13.5 and E 15.5, it was progressively confined to the atria, SAN and ventricular trabecular structures that will become the peripheral ventricular conduction system. Our findings could be of great importance for understanding the specific actions of the TR isoforms, because the TR-dependent genes are heterogeneously expressed in distinct cardiac components, and they could be regulated specifically by TRα₁, TRβ₁ or both isoforms (Moorman et al. 1995, 1998, Franco & Icardo 2001). Moreover, the pleiotropic effect of the mutation in TRα₁ is consistent with the wide distribution of TRα₁, whereas the more limited alterations generated by the ablation of the TRβ₁ gene agree with the more restricted pattern of expression observed for TRβ₁ (Gloss et al. 2001).

The most significant effect of TRs in heart development is on the regulation of differential expression of α- and β-myosin heavy-chain isoforms in the rodent heart. In the prenatal period, the α-myosin heavy chain becomes confined to the atria, and the β-myosin heavy chain to the ventricles. After delivery, a peak of T₃ secretion leads to upregulation of the α-isofrom in the ventricles and a concomitant suppression of the β-isofrom. No significant increase of the α-isofrom in the atria is observed (Klein et al. 1992, Morkin 1993, 2000, Klein & Ojamaa 2001, Mansen et al. 2001, Dillmann 2002). The differences in response to T₃ action between the atria and the ventricles could be related to the higher expression of TRα₁ and TRβ₁ isoforms in the atria. Since all TRs show less expression in the ventricles than in the atria, it could be that high hormone levels are needed to stimulate the α-myosin heavy chain in the ventricles.

The regional expression of the TRs can also explain why phospholamban and SERCA2a are predominantly TRα₁, but not TRβ₁, regulated in vivo (Gloss et al. 2001, Klein & Ojamaa 2001, Dillmann 2002). Both proteins are highly expressed in the compact myocardium but significantly lower or absent in the trabeculae (Moorman et al. 1995). Therefore, the fact that TRβ₁ is present only in these trabecular structures goes a long way to explain the lack of TRβ₁ regulation of phospholamban and SERCA2 in vivo. That this lack of regulation is indeed distribution dependent follows from the fact that TRβ₁ can regulate both these genes in vitro in rat cardiomyocyte cell cultures when they are transfected with TRβ₁ (Moriscot et al. 1997, Gloss et al. 2001).

A novel finding in our experiments was that TRβ₁ is expressed mainly in the ventricular trabeculations after 13·5 E. It is known that these structures differentiate eventually into the peripheral ventricular conduction system (Moorman et al. 1998, Franco & Icardo 2001). Formation of the PVCS is stimulated by the endocardium-derived paracrine factors, neuregulin-1 (Rentschler et al. 2002) and endothelin (Yanagisawa et al. 1988, Masaki et al. 1991, Gourdie et al. 1998). The idea that the TRβ₁ isoform has a specific role in the PVCS because of its restricted expression is supported by data which show that the effect of endothelin is enhanced in rat cardiomyocyte cells transfected with TRβ₁, resulting in hypertrophy and expression of specific proteins (Liang et al. 2003). The distinct pattern of expression of TRβ₁ could help to clarify the different chronotropic and hypertrophic effects of T₃ on the heart.

Both TRα₁ and TRβ₁ are present in atria and SAN. The abundant presence of TRα₁ in the SAN agrees with the data on the role that this isoform plays in regulation of basic heart rate and expression of the guanine-nucleotide regulatory proteins (HCN2 and HCN4) responsible for the pacemaking current (Pachucki et al. 1999, Gloss et al. 2001). The presence of TRβ₁ protein in the SAN agrees with previous studies on RNA level (Gloss et al. 2001, Swanson et al. 2003) and suggests a role for TRβ₁ in pacemaking activity too. Support for this role can be found in a recent report that TRβ₁PV/PV mice, under euthyroid conditions, have decreased heart rate, demonstrating that despite a lower expression of TRβ than TRα₁ in the mouse heart, the homozygous negative PV/PV mutation of TRβ₁ can negatively interfere with TRα₁ signaling in this organ (Swanson et al. 2003). Furthermore, our data support the conclusions of Swanson et al. (2003) on the expression and abundance of TRα₁, but, on the ventricles, they do not support their conclusion that TRβ₁ is ubiquitously expressed in the heart.

Our results are also consistent with studies on T₃ antagonists and agonists. For instance, it was not clear why dronedarone, a selective TRα₁ antagonist, in contrast to amiodarone (a nonselective TR antagonist), causes ventricular arrhythmia in dogs as a side effect (Van Beeren et al. 1996, 2003, Varro et al. 2001). A possible unique function of TRβ₁ in the ventricular conduction system could provide the explanation. The predominant expression of TRα₁ in the myocardium, on the other hand, could be the reason that the selective TRβ₁ agonists GS-1 and KB-141 have little or no effect on cardiac.

These results are, to our knowledge, the first to show the heterogeneous distribution of TRα1 and TRβ1 in the cardiac conduction system and working myocardium. Knowledge of the distribution of these two isoforms, and especially the specific expression of TRβ1 in PVCs, will be of great importance not only for understanding their functions in the heart but also for the design and (clinical) use of TR isoform-specific agonists and antagonists.

Acknowledgements

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received in final form 19 December 2005 
Accepted 17 January 2006

Made available online as an Accepted Preprint 15 February 2006