Species Differences in Cardiac Thyroid Hormone Receptor Isoforms Protein Abundance

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Little is known about the cardiac expression of different thyroid hormone receptor (TR) isoforms. The aim of the study was to investigate such patterns of TR expression at the protein level in different species and in some human tissues. Western blot analysis with specific polyclonal rabbit antibodies to each TR isoform was performed with samples from myocardium of the left ventricle from man, dog, guinea pig, rat and mouse, as well as with samples from several human tissues such as heart, skeletal muscle, brain, liver and thyroid. The TR α 1 isoform was present in all of the species examined. The TR α 2 was recognized in human, dog and guinea pig heart, while no such band was recognized in rat and mouse hearts. TR β 1 was not detected in the human heart but in the other species. Similarly to TR α 1, TR β 2 was detected in all of the species examined. In the human tissues studied, TR α 1 was detected in heart and skeletal muscle, whereas TR α 2 was found only in the heart. TR β 1 was not detected in any of the examined human tissues, while TR β 2 was found in all of them. These results revealed unique distributions of TR variants and they demonstrate common epitopes in TR in the different species. For the first time, the presence of a TR β 2 isoform has been shown in human tissues. TR isoforms may have a tissue and species specific role in the regulation of gene expression and may in part explain variable tissue effects of thyroid hormones.

Key words thyroid hormone receptor; human; dog; guinea pig; rat; mouse

Thyroid hormone receptors (TR) are members of a superfamily of nuclear hormone receptors which play an important role in the growth, development and metabolism of the cell through the regulation of gene expression. In 1986, two thyroid hormone receptor *erb A* genes were described.^{1,2)} In human, TR α and TR β genes are located on chromosomes 17 and 3, respectively.^{2,3)} As the result of alternative splicing each of the TR genes expresses two isoforms, α 1 and α 2, and β 1 and β 2, respectively.^{2,4–6)} Whereas TR α 1 and both TR β isoforms have ligand-binding and DNA-binding domains, the TR α 2 isoform contains a C-terminus different from TR α 1. This results in a nonability to bind ligand and activate transcription.⁷⁾ Moreover, by binding to the same DNA sequence as TR α 1 and TR β , it may inhibit their inductive effect on gene expression.⁸⁾

To date, in several studies the tissue distribution of rat TR protein isoforms has been investigated. Thus, TR α 1 is mainly expressed in brown fat and skeletal muscle,^{9,10)} whereas TR α 2 is expressed in the majority of rat tissues.¹¹⁾ TR β 1 has been found to be widely expressed, while the presence of TR β 2 has been initially demonstrated only in the pituitary and in some regions of the central nervous system.^{12–15)} In a later study, TR β 2 was also demonstrated in rat liver, brain, heart and kidney.¹⁶⁾

However there is not much known about TR isoform expression at the protein level in cardiac tissues of different species and in human tissues. To address these issues, we decided to examine TR α 1, TR α 2, TR β 1 and TR β 2 protein distribution by Western blot analysis with specific polyclonal rabbit antibodies to each of the TR isoforms. This was done in left ventricular samples from a number of species, such as human, dog, guinea pig, rat and mouse, and in human heart, skeletal muscle, liver, brain and thyroid.

MATERIALS AND METHODS

Preparation of Protein Extracts Protein medley concentrates were isolated by Clontech Laboratories, Inc., U.S.A., from whole tissue homogenates under conditions designed to minimize proteolysis and to ensure maximal representation of tissue specific proteins. Concentrates were diluted in sodium dodecyl sulfate (SDS) buffer.

B6D2/F1 (Sachsen Labs, U.S.A.) strain mouse, Sprague Dawley (B and K Universal, Sweden) strain rat, Dankin Hartley (Møllegaard, Denmark) strain guinea pig and dog hearts were removed and immediately frozen at -70°C . Human myocardial samples were obtained from brain-dead donors within 20 h of the diagnosis of brain death. The samples were taken within 15–30 min after the heart had stopped beating, immediately frozen in liquid nitrogen and stored at -80°C until they were analyzed. Protein extracts from left ventricular animal hearts and human donor hearts were prepared as follows. Tissues were homogenized in EPG buffer (20 mM NaP pH 7.4, 0.4 M NaCl, 10% glycerol, 1 mM dithiothreitol, 10 mg/ml phenylmethylsulfonylfluoride) in ice, followed by centrifugation at 4°C . Supernatants were diluted with SDS electrophoresis sample buffer and boiled for 5 min. Protein concentrations in supernatants were determined using the assay set, "Protein Assay ESL" (Boehringer Mannheim GmbH).

Ethics The study was approved by the Ethics Committees of the Karolinska Hospital and Karolinska Institute, Stockholm, Sweden. The investigation conforms with the principles outlined in the Declaration of Helsinki (*Br. Med. J.*, 1964; ii: 177) and with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985).

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Western Blot Analysis of TR Proteins Western blot analysis using specific polyclonal rabbit antibodies to each TR isoform was performed. Amino acid sequences recognised by these antibodies in TR α 1, TR α 2, TR β 1 and TR β 2 proteins are a.a. 403–410, a.a. 431–451, a.a. 62–82 and a.a. 86–101, respectively. Equal amounts of total protein extract (75 μ g per lane from each tissue) were loaded on 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), then transferred electrophoretically to nitrocellulose, blocked with 10% nonfat milk at 4°C overnight and probed with anti-human (h) TR α 1, hTR α 2 and hTR β 1 rabbit polyclonal antibodies (Affinity BioReagents, Inc.) at 1:100 dilution or with anti-TR β 2 rabbit polyclonal antibodies¹⁵⁾ diluted 1:500 at 4°C overnight. Anti-TR β 2 rabbit polyclonal antibodies have been raised against rat peptide, but have been shown to recognize human TR β 2 protein as well.¹⁵⁾ Bound IgG molecules were visualized with alkaline phosphatase conjugated goat anti-rabbit IgG (H+L) secondary antibodies (Bio-Rad). Secondary antibodies were used at a 1:3000 dilution with the substrate nitro blue tetrazolium (SIGMA) and 5-bromo-4-chloro-3-indolyl phosphate (Bio-Rad).

RESULTS

TR Isoform Recognition among the Species TR α 1 ap-

Fig. 1. Western Blot Analysis of TR Proteins in Myocardium of the Left Ventricle

Protein extracts (75 μ g) from human (lane H), dog (lane D), guinea pig (lane G), rat (lane R) and mouse (lane M) hearts were analysed by Western blot using polyclonal rabbit antibodies raised against each TR isoform: A, TR α 1; B, TR α 2; C, TR β 1; D, TR β 2.

peared in human, dog, guinea pig, rat and mouse heart (Fig. 1 and Table 1). In the rat heart, a band of a slightly larger size than in the other species was detected. The TR α 2 isoform was recognized in human, dog and guinea pig heart, but with faint bands in dog and guinea pig hearts. Antibodies against TR β 1 revealed the presence of this isoform in the heart of dog, guinea pig, rat and with less abundance, in mouse heart, but not in the human heart. A prominent band corresponding to TR β 2 was found in all the species analysed.

TR Expression at the Protein Level in Human Heart, Skeletal Muscle, Liver, Brain and Thyroid Tissue distribution of TR isoforms is shown in Fig. 2 and summarized in Table 2. TR α 1 was demonstrated in heart and skeletal muscle, while TR α 2 was detected in the heart only. The TR β 1 isoform was not found in any of the tis-

Table 1. Detection of TR Isoforms in Myocardium of the Left Ventricle by Western Blot Analysis

Species	Isoforms			
	TR α 1	TR α 2	TR β 1	TR β 2
Human	+++	++	—	+++
Dog	++	+	+++	++
Guinea pig	++	+	+++	+++
Mouse	++(+)	—	(+)	+++
Rat	+++	—	+++	+++

Fig. 2. Western Blot Analysis of TR Proteins in Human Tissues

Protein extracts (75 μ g) from human heart (lane H), liver (lane L), brain (lane B), thyroid (lane T) and skeletal muscles (lane M) were analysed by Western blot using polyclonal rabbit antibodies raised against each TR isoform: A, TR α 1; B, TR α 2; C, TR β 1; D, TR β 2.

Table 2. Detection of TR Proteins in Human Tissues by Western Blot Analysis

Tissue	Isoforms			
	TR α 1	TR α 2	TR β 1	TR β 2
Heart	++	++	—	+++
Liver	—	—	—	+++
Brain	—	—	—	++
Thyroid	—	—	—	+
Skeletal Muscles	++	—	—	+

Fig. 3. Specificity of Anti-TR β 2 Antibodies

Peptides (5 μ g per line) were used as positive (+) and negative (—) controls against preimmune (A) and immune (B) anti-TR β 2 rabbit sera in Western blot analysis. Anti-TR β 2 antibodies were raised against the positive control peptide (a.a. 86–101). The negative control peptide (a.a. 286–305) is from the carboxyterminal end of TR.

sues analysed. The TR β 2 isoform was detected in all of the tissues studied, with the highest relative abundance in liver and heart.

Bands of 48 kDa, 58 kDa, 55 kDa and 62 kDa were identified as TR α 1, TR α 2, TR β 1 and TR β 2 variants, respectively (Figs. 1 and 2). In protein extracts from human heart and skeletal muscle (Clontech), a protein band of much higher molecular weight, about 70 kDa, was also detected with anti-TR α 1 antibodies.

Thus TR β 1 in human heart and TR α 2 in mouse and rat hearts are absent or present at very low levels. On the other hand, the TR β 2 isoform was found in all examined tissues. Comparison of TR isoform expression in human tissues revealed either the absence or very low expression levels of TR α 1 in liver, brain and thyroid, TR α 2 and TR β 1 in liver, brain, thyroid and skeletal muscles.

Specificity of Anti-TR β 2 Antibodies Unlike anti-TR α 1, TR α 2 and TR β 1 antibodies, which are commercially available and have been previously characterised,^{11,24} anti-TR β 2 antibodies were produced in our laboratory. To confirm their specificity, preimmune (A) and immune (B) rabbit sera were used in the Western blot analysis of a positive control peptide (a.a. 86–101), against which anti-TR β 2 antibodies have been raised, and a negative control peptide (a.a. 286–305) from the carboxyterminal end of TR. A band of expected size appeared only in the position of the positive control peptide, and only when the immune sera were used (Fig. 3).

DISCUSSION

To date, four isoforms of thyroid hormone receptor, TR α 1, TR α 2, TR β 1 and TR β 2, are known. In several laboratories, anti-TR antibodies that can not distinguish

between different TR variants^{17–21} or antibodies specific to some of the isoforms^{22,11,15,16,23} have been used. In the present study, we examined the distribution of TR isoforms by the Western blot technique using antibodies specific to each isoform. Results obtained in our study revealed protein bands of 48 kDa, 58 kDa and 55 kDa, corresponding to TR α 1, TR α 2 and TR β 1, respectively, in agreement with the report of the detection of *in vitro* translated TR¹¹ or recombinant TR expressed in *E. coli*,²⁴ with the difference that in our study, the TR β 1 variant appeared as a single band of 55 kDa. Antibodies against the TR β 2 isoform displayed a band of approximately 62 kDa in accordance with immunoprecipitation studies of the *in vitro* translated TR β 2 isoform.¹⁵

As the heart represents an important organ for TR action, we prepared protein extracts from human, dog, guinea pig, rat and mouse myocardium of the left ventricle to study species differences in the sense of the presence of similar epitopes within TR variants. Interestingly, while TR α 1 and TR β 2 isoforms were found in all the species studied, TR β 1 was not detected in human heart and TR α 2 was absent in mouse heart and rat heart, with low abundance in dog heart and guinea pig heart.

We also demonstrated diverse distribution of TR isoforms among several human tissues, such as heart, skeletal muscle, liver, brain and thyroid. Only the TR β 2 isoform was present in all of them. Our findings that TR α 1, TR α 2 and TR β 1 are absent or very poorly represented in human liver are in contrast to data of recently published on Western blot studies of the expression these TR isoforms in normal and chronically diseased human liver.²³ Such a discrepancy could be explained in part by the fact that Chamba *et al.* used nuclear protein extracts, whereas we used whole protein extracts, since presence of TR receptors has been demonstrated not only in the cell nucleus, but in mitochondria, too.²⁵ It should be also noticed, that the TR β 2 isoform was detected in all of the examined tissues. Moreover, our data are in agreement with both Sakurai *et al.*²⁶ and Chamba *et al.*²³ Northern blot results, where by hybridization of cDNAs to total RNA prepared from human liver, TR α 1, TR α 2 or TR β 1 mRNAs were not detected at all or were found at a very low level.

Analysis of TR α 1 distribution in protein extracts from human tissues (Clontech) revealed the presence of an additional band of a much higher molecular weight (70 kDa) than the main band (48 kDa) in heart and skeletal muscle. It could be considered as non-specific binding, resulting from differences between particular human material sources, since this band did not appear in protein extracts prepared from the left ventricular heart. Another possible explanation may be modifications, acetylation or phosphorylation, of the TR α 1 isoform in skeletal muscle and regions of human heart other than the left ventricle.

In summary, our study demonstrated unique TR isoform distribution among the species and among human tissues. At the present time the reason for such variety is unclear, but it suggests a species and tissue specific role of TR variants in regulation of gene expression and may be one of the causes of the diversity of thyroid hormone effects.

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