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CHAPTER 3

Zonal Expression of the Thyroid Hormone Receptor-α Isoforms in Rodent Liver

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Abstract

Many metabolic processes occur simultaneously in the liver and occur in different locations along the porto-central axis of the liver units. These reactions are often dependent on hormones one of which is thyroid hormone, which depends on the presence of the different isoforms of the thyroid hormone receptor (TR). They are encoded by two genes: c-erbA-α which encodes TRα1 and TRα2 and c-erbA-β which encodes TRβ1, TRβ2 and TRβ3.

We recently found a zonal (pericentral) expression of and a diurnal variation in the TRβ1 in rat liver. We were therefore also interested to find out whether TRα1 and TRα2 expression showed similar characteristics. For this reason, we raised both polyclonal and monoclonal antibodies against TRα1 and TRα2 isoforms and characterised these. Antibody specificity was tested using Western blots and immunohistochemistry in liver of TR isoform specific knock-out animals. Using these antibodies we found that the TRα1 and TRα2 are zonally expressed around the central vein in rat liver. Moreover the expression of the TRα2 protein showed a diurnal variation with a peak in the afternoon when the animals are least active whereas no such variation was found for the TRβ1 protein. Immunohistochemistry shows that the gradient of TRα1 is broader than that of TRβ1.

From our data it appears that both the TRα1 and TRα2 isoforms show a zonal distribution in the liver. This finding, together with the observed diurnal rhythm, has major implications for interpreting and timing experiments concerning the TR and its downstream actions in liver.

Introduction

In the liver many metabolic processes occur simultaneously, but not all of them take place in every liver parenchymal cell. Instead the various metabolic reactions occur in different locations along the porto-central axis of the liver units (1,2). For instance, lipogenesis and glycolysis are predominant in liver cells around the central vein, whereas lipolysis and gluconeogenesis are found in the area around the portal vein. This so-called metabolic zonation can be of a stable or dynamic kind which means that the expression of certain enzymes is restricted to certain cells regardless of the metabolic or hormonal state, whereas the expression of other enzymes can expand or shrink along the porto-central axis depending on the metabolic state or time of the day. Enzymes with a stable distribution can be located pericentrally (GS) or periportally (fructose 1,6-bisphosphatase (3). Similarly, enzymes with a dynamic distribution can be located pericentrally (ornithine aminotransferase (4)) or periportally phosphoenolpyruvate carboxy-kinase: PEPCK (5)).

Thyroid hormone has many diverse actions in the body. These actions are dependent on the presence of the different isoforms of the TR (6). Thyroid hormone receptors belong to nuclear receptor super family and act mainly as transcription factors. They are encoded by two separate genes: c-erbA-α which encodes TRα1 and TRα2.
isoforms and c-erbA-β which encodes TRβ1, TRβ2 and TRβ3 isoforms. Thyroid hormone (T₃) signals its presence to the cell by binding to the thyroid hormone receptors which then interact with so-called thyroid hormone response elements (TRE) in the promoter of T₃-responsive genes and thereby activates or represses the genes. Thyroid hormone receptors are distributed differentially in various tissues and during developmental stages indicating distinct or specific functional roles. TRα1, TRα2 are mainly distributed in CNS and in muscle and TRβ1 is predominantly present in the liver. Studies using knock-out mice have shown that certain actions of thyroid hormone are dependent on a particular receptor isoform (7) and the expression patterns of some of these genes overlap with that of the TRβ1 they are dependent on (chapter 5). We recently found a zonal (pericentral) expression of and a diurnal variation in the TRβ1 in rat liver (8). We were therefore also interested to see whether TRα1 and TRα2 expression showed similar characteristics. For this reason we raised antibodies against TRα1 and TRα2 isoforms. We generated polyclonal and monoclonal antibodies against the TRα isoforms and characterised these. We tested antibody specificity using Western blots and immunohistochemistry in liver of TR isoform specific knock-out animals.

Materials and methods

Animals
Wistar rats (Harlan Sprague Dawley, Zeist, The Netherlands) were kept in 12h/12h, light-dark cycle with free access to food. All animal experiments were approved by our local Animal Welfare Committee. Light was switched on at 07:00 h in the morning. The liver of each animal was used for immunohistochemistry and Western blots.

Polyclonal antibodies
To produce anti-TR antisera two linear synthetic peptides coupled to keyhole limpet haemocyanine (KLH) were made, namely amino acids 402-410 (NH2-EVFEDQEV-COOH) for human TRα1 and amino acids 425-442 (NH2-LRGPVLQHQSPKSPQQR-COOH) for TRα2. Using these peptides, antisera were raised in New Zealand White rabbits and affinity purified by Eurogentec (Seraing, Belgium).

Monoclonal antibodies
The same KLH-conjugated linear synthetic peptides TRα1 and TRα2 described above were used for immunisations in mice with a total of four injections of 25 μg peptides each. The first injection was (subcutaneously in complete Freund’s adjuvant) seven weeks before sacrifice, the second and third injections were respectively two and four weeks after the first injection (intraperitoneally in complete Freund’s adjuvant) and finally the last injection was given intravenously. Five days after the last boost of immunization, the spleen cells from the immunized BALB/C mice were fused with myeloma (SP20) by means of Polyethylene Glycol 1500 (Roche Molecular Biochemicals-Germany). The fused cells were plated on 24-well plates in Hypoxanthine Aminopterin Thymidine (HAT)-DMEM/15% FCS/1% PenStrep (Sigma) in presence of 5% HCF
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(Hybridoma Cloning Factor, Sanvertech, The Netherlands). Fourteen days later, the supernatant of the hybridomas were screened by immunohistochemistry. Positive clones were sub-cloned in 96-well plates in Hypoxanthine Aminopetrine Thymidine (HAT) medium by limiting dilution. The supernatants from the positive sub-cloned hybridomas were used for further screening on the liver of a rat killed at 19:30 and in nuclear or whole cell extracts on Western blots.

The selected clones were grown in bulk. Supernatant of each monoclonal antibody was precipitated with half the starting volume of saturated ammonium sulphate (Merck, Germany), which was added dropwise and was then left stirring overnight at 4°C. The next morning after centrifugation at 3000g for 30 minutes, 20ml ammonium sulphate was added at 4°C for six hours. After centrifugation for 30 minutes at 3000g, the pellet was saved and dissolved in 0.1 volume (4ml) of PBS. The concentrated antibody was dialysed overnight at 4°C in a dialysis tube (cut-off 12.000M.W., Thomas, USA).

A mouse monoclonal antibody isotyping kit (Hycult Biotechnology, Uden, The Netherlands) was used to establish the isotype of the monoclonal antibodies.

Western blots
Whole cell extracts (WCE) from livers were prepared by homogenising in 0.25 M sucrose containing Complete protease inhibitor (Roche Molecular Biochemicals) using a polytron homogenizer for 10 seconds at maximum speed.

Protein concentration of the WCE was determined using Biorad Bradford reagent (Biorad, Germany). Subsequently, 25 µg protein was loaded on 10% SDS-PAGE gel. After electrophoresis onto membrane (Protran BA45, Schleicher &Schuell, Dassel, Germany), the blots were blocked for 45 minutes in TBS containing 1% (w:v) casein (Roche, Germany) and 0.1% (w:v) Tween-20 (blocking buffer). Next, the blots were incubated for 2 hours at 22°C with the primary antiserum (polyclonal antisera; 1:250 dilution and monoclonal antisera; 1:50 dilution). After 30 minutes of incubation with secondary antibodies (1:20,000 dilution) in blocking buffer, the blots were washed and Lumilight plus substrate (Roche Molecular Biochemicals) was added. The signals were visualised and quantified using a Lumilmarker (Roche Molecular Biochemicals).

Nuclei were isolated as was previously described (9). In short, 4 gram liver was homogenised in Sol A (20 mM Tris/HCl, 0.25 M sucrose, 2 mM CaCl₂, 1 mM MgCl₂, and 5%(v/v) glycerol pH=7.6). The nuclei were pelleted by ultracentrifugation (45000 g for 45 minutes at 4°C) and washed with Sol A containing 0.5% Triton X-100 and resuspended in Sol B (200 mM Tris, 5%(v/v) glycerol, 0.25 M sucrose, 1 mM EDTA and 50 mM NaCl) and stored in liquid nitrogen. The amount of protein in this nuclear preparation was determined using the Biorad protein assay. For Western blots 50 µg protein equivalent of nuclei was used.

Immunohistochemistry
Both paraffin-imbedded and frozen livers of several rats were used as tissue for immunohistochemistry. The frozen rat liver sections of 6 µm were fixed with different kinds of fixatives depending on the type of experiments being either, paraformaldehyde (PFA; Merck, Germany) 4% (w:v) in phosphate buffered salt (PBS); pH=7.4), acetone, methanol or ethanol for 15 minutes at 4°C. Paraffin-imbedded rat livers were fixed
for 16 hours at 4°C in PFA sections (6 μm) and pasted on Superfrost/Plus (Menzel-Gläser, Germany) slides and dewaxed prior to immunostaining. Sections were placed in Tris buffered salt (TBS, (pH=8.0)) and microwaved for 10 minutes for the paraffin and 5 minutes for the frozen sections to unmask the antigenic epitope. After cooling to room temperature, the sections were incubated in a blocking solution for 60 minutes (5% low fat milk (Campina, Netherlands) and a detergent either 0.1% (w:v) Triton X-100 (Sigma, Germany) or 0.5% (w:v) saponin (Sigma, Germany) diluted in TBS at pH=8.0. The first antibodies were diluted in the blocking solution, and incubated first for one hour at room temperature and then overnight at 4°C. The sections were washed with 0.5% (w:v) Triton X-100 in TBS (pH=8.0) for 10 minutes, and then incubated with the second antibody (conjugated with alkaline phosphatase) diluted 0.5% (w:v) Triton X-100 in TBS (pH=8.0). After a washing for 10 minutes in TBS, the sections were placed in alkaline phosphatase buffer (50mM of MgCl₂, 100mM of NaCl, and 100 mM Tris pH=9.2) for 10 minutes. The substrates NBT/BCIP (Roche Diagnostics, Germany) and 0.1M levamisole (Sigma, Germany) were diluted respectively to 1:50 and 1:100 in alkaline phosphatase buffer and added to the sections for 30 minutes to 90 minutes in the dark. The sections were washed in TBS and then placed in methanol (Merck, Germany) for at least 5 minutes to reduce background. The sections were mounted with Kaiser’s glycerol gelatine (Merck, Germany).

Data analysis
For immunohistochemistry each experiment was repeated twice. The staining intensity of each section was judged blindly by two different observers. In the figures, the staining intensity is represented in arbitrary units: 0, no staining; 1, weak staining; 2, intense staining; and 3, very intense staining. The curves shown in the figures were fitted by second degree polynome using Microsoft Excel. Variations between groups were evaluated with the unpaired, two-tailed t-test. A difference was considered statistically significant at p<0.05. All data were evaluated using SPSS version 9 (SPSS Inc, Chicago, IL).

Results

Specificity of antibodies on Western blots

Polycnkal TRα1 and TRα2 antibodies
The polyclonal antibodies were typed as IgG type antibodies. In liver whole cell extracts (WCE) and nuclear extracts a 47 kD band was detected using the TRα1 polyclonal antiserum and a 58 kD band was detected using the TRα2 polyclonal antiserum (figure 1A). As a control for the TRα1 polycional antiserum we used a Vaccinia expressed TRα1. We also tested both our polyclonal antibodies on Western blots of liver WCE of animals where either the TRα1 or TRα2 were knocked out (10,11). The TRα1 polyclonal antibody showed no signal at 47 kD in liver WCE from a TRα1−/TRβ1− animal and the TRα2 polyclonal did not show the 58 kD band which was seen in the WT animals (figure 1B). Furthermore no cross-reaction with TRα1 protein is found, since Vaccinia-TRα1 was not detected with this antibody (figure 1B).
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Figure 1. Specificity of the TRα1 and TRα2 antibodies tested on Western blots.
(A) Western blots were prepared using both nuclear (nuc) and whole cell extracts (WCE). These were incubated with either the TRα1 or TRα2 monoclonal or polyclonal antibodies as described in materials and methods. The arrow indicates the specific bands. Position of the protein markers is indicated alongside.
(B) Western blots were prepared with whole cell extracts from WT, TRα1/β1 and TRα2 mice and Vaccinia expressed TRα1. These blots were incubated with the polyclonal antibodies against TRα1 and TRα2 to check specificity. Position of the protein markers is indicated alongside.

Monoclonal TRα1 and TRα2 antibodies
The monoclonal antibody clones were typed as IgM. The specificity of the monoclonal antibodies was tested on liver slices derived from mice devoid of either TRα1 (TRα1-) or TRα2 (TRα2-/β1-). As can be seen in figure 2 no staining was observed with the TRα1 monoclonal antibody in a TRα1- liver. This was not a general loss of TR since the TRβ1 polyclonal was still able to detect the TRβ1 protein in the TRα1- animals (figure 2). TRα2 staining was absent in the TRα2-/β1- animals. In these mice TRα1 was detected in a control experiment (data not shown). On Western blots the monoclonal TRα1 antibodies detected bands of similar size (47 kD) to that detected with the polyclonal antibody but the signal was stronger in the nuclear preparation. With the TRα2 monoclonal, a strong band of about 58 kD was detected in the nuclear preparation at the same position as the bands found with the polyclonal TRα2 antibody.
Figure 2. Specificity of the TRα1 and TRα2 antibodies tested with immunohistochemistry
Slices from livers of wild type (WT) or TRα1⁻/⁻ mice were incubated with the monoclonal TRα1 antibody with the TRβ1 polyclonal as a control (left panel). The monoclonal TRα2 antibody was incubated with slices from livers of wild type (WT) and TR α2⁻/β⁻ mice using the TRβ1 polyclonal antibody as a control. Magnification 40x except for TRα1 WT in lower right panel stained with TRα1; magnification 100x.

Immunohistochemistry
In order to study the distribution of the TRα1 and TRα2 proteins in liver we set out to determine the optimal incubation conditions for our antibodies. The parameters of interest are microwave treatment before incubation with the first antibody, salt (NaCl) concentration and pH during the first antibody incubation. When performing immunohistochemistry on both frozen and paraffin embedded sections of the rat liver we found that microwave treatment diminished background and intensified staining (probably by unmasking the antigenic epitope) with both polyclonal and monoclonal antibodies on sections fixed with paraformaldehyde (PFA). We therefore used PFA as fixative and microwave treatment in all immunohistochemistry experiments using both polyclonal and monoclonal antibodies.
To find the optimum salt concentration during incubation with the first antibody at pH=8 we performed experiments with both frozen and paraffin embedded sections by increasing the salt concentration from 0 mM to 600 mM of NaCl. For both polyclonal and monoclonal TRα2 increasing the NaCl concentration up to 400mM had no effect on the staining. Surprisingly, the TRα1 monoclonal antibodies were very sensitive to an increase in the NaCl concentration. Since all different antibodies stained well at 150 mM NaCl this concentration was chosen in further experiments. Using this 150 mM NaCl concentration we next determined the pH optimum during incubation with the first antibody. For the TRα1 and TRα2 polyclonal antibodies and the TRα2 monoclonal antibody the pH optimum was pH=8. However in case of the monoclonal TRα1 the pH optimum was lower at pH=5 (figure 3).
Figure 3. pH dependence of the antibody reaction in immunohistochemistry
The signal intensity of the immunostaining measured in arbitrary units (AU) is indicated as a function of the pH of the incubation medium (see Materials and Methods). The curves of the individual antibodies are indicated in the figure by a circle and a name.

Since the monoclonal TRα1 antibody had a lower pH optimum we determined optimum salt concentration for this antibody at this pH and we found again that the antibody was very sensitive to the NaCl concentration. Moreover at this pH background was reduced using saponin in the incubation mixture.

Taking these results together we decided to use the monoclonal antibodies in the experiments aimed at studying the distribution and diurnal rhythm of the TRα isoforms. In these experiments the TRα1 monoclonal antibodies were diluted 1:50 in blocking buffer, 0.5% (w:v) saponin, pH=5 with no additional salt and the TRα2 monoclonal antibodies were diluted 1:50 in blocking buffer pH=8 with 150 mM NaCl.

Zonal distribution
Immunohistochemistry using the monoclonal antibodies on liver sections indicated that TRα1 and TRα2 were expressed around the central veins (figure 4). This expression pattern overlaps with that of the TRβ1 (8) and with the centrally expressed, T3-responsive gene GS, which we stained as a control on consecutive sections. However the area of expression of the TRα1 and TRα2 proteins extends further along the porto-central axis than that of TRβ1 and GS.
Figure 4. Zonal expression of the TRα1, TRα2 and TRβ1 isoforms
Liver slices were incubated with the monoclonal antibodies directed against TRα1 and TRα2 and the polyclonal anti TRβ1. Monoclonal anti-glutamine synthetase was used as a control to stain the central veins. Magnification 100x. P: portal vein, C: central vein.
**Diurnal variation of TR in the rat liver**
When rats were sacrificed at different time points during the day we found different staining intensities for the TRα's in liver slices. Especially TRα2 expression differed when the rats were sacrificed in the morning compared to those sacrificed in the afternoon. These results were confirmed using Western blotting where it can be seen that no change in expression level of TRα1 is found (figure 5A) but that there is a marked diurnal variation in the expression of the TRα2 protein (figure 5B) with a peak during the afternoon when the animals are least active. Immunohistochemistry showed that the expression of both TRα1 and TRα2 was of a stable kind.

![Figure 5](image_url)

*Figure 5. Diurnal variation of the TRα1, TRα2 isoform proteins*

The figure shows the signal intensities (expressed relative to an internal standard a band with constant expression level) derived from Western blots of whole cell extracts of rat liver incubated with monoclonal TRα1 (panel A) or TRα2 (panel B). Rats were killed (6 per time point) at the time points indicated in the figure. Light was on from 07:00 h to 19:00 h. Data are expressed as means ± SD and significant differences, when present, are indicated.
Discussion

Here we report the generation of a set of antibodies, both polyclonal and monoclonal, directed against the thyroid hormone receptor \( \alpha_1 \) and \( \alpha_2 \) isoforms which according to the criteria tested by us (Western blots with wt and specific knock-out WCE and staining on slices of WT and specific knock-out livers) are specific for these isoforms. Since the monoclonal antibodies gave a better signal on the Western blots we performed our studies on the zonal distribution and the diurnal variation of the TR\( \alpha_1 \) and TR\( \alpha_2 \) isoforms with the monoclonal antibodies. Several immunohistochemical studies have reported the presence of TR proteins in the liver of rodents and humans (12-16). Although not all hepatocytes were stained in these studies, none of these studies mention a topographical distribution of TR isoforms even though most known T\(_3\)-dependent genes are expressed in the liver in different zones. We showed recently that the TR\( \beta_1 \) protein was expressed in a rather small area around the central vein of rat liver and that its expression varies during the day (8). Moreover, certain TR\( \beta_1 \) dependent genes are expressed in the same area (chapter 5). Here we show that a similar stable zonal expression exists for two other TR isoforms namely TR\( \alpha_1 \) and TR\( \alpha_2 \). Although these two isoforms are also expressed around the central vein the extent of the zonal expression for both TR\( \alpha \) isoforms is wider then that of the TR\( \beta_1 \). This suggests the possibility that there may be genes solely dependent on TR\( \alpha_1 \) or TR\( \beta_1 \) whereas there will be another set which is specifically activated by one of the isoforms depending on its localisation along the porto-central axis of the liver unit. For instance, CYP7a is expressed mainly in the pericentral zone in conjunction with all receptors (17-19) while a gene encoding for example PEPCK is expressed towards the periportal zone where the TR\( \alpha \)’s are present. It can therefore be suggested based on the localisation of PEPCK that the TR\( \alpha_1 \) the most likely candidate to confer T\(_3\)-regulation on this gene.

From our results it is apparent that the expression of the TR\( \alpha_2 \) shows a diurnal rhythm whereas that of the TR\( \alpha_1 \) does not. This points to the possibility of regulated alternative splicing a phenomenon known to occur in other systems(20,21). The diurnal expression of the TR\( \beta_1 \) protein is the reverse of that of the TR\( \alpha_2 \) with maximum TR\( \beta_1 \) protein expression observed at the beginning of the dark period, when the rats are nutritionally active.

Whether the rhythm is dependent on the suprachiasmatic clock or on food intake is at presently unknown but the presence of a rhythm in the serum levels of thyroid hormones and in the expression of the TR isoforms together with the knowledge that thyroid hormone regulates many genes (esp. metabolic) in liver (22) points to the fact that thyroid hormone may play a role in mediating the clock signal originating in the superchiasmatic nucleus.

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