CHARACTERIZATION OF THYROID HORMONE UPTAKE IN HEART

Haidy van der Putten

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CHARACTERIZATION OF THYROID HORMONE UPTAKE IN HEART

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(met een samenvatting in het Nederlands)

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Haidy Hendrica Antonia Gerarda Maria van der Putten
geboren op 15 januari 1974, te Aarle-Rixtel

Promotoren

Prof. Dr. M.E. Everts
Department of Veterinary Anatomy and Physiology
Faculty of Veterinary Medicine, Utrecht University,
Yalelaan 1, P.O. box 80.157, 3508 TD Utrecht,
The Netherlands

Prof. Dr. Ir. T.J. Visser
Department of Internal Medicine
Erasmus University Medical Center
Dr. Molewaterplein 50, 3015 GE Rotterdam,
The Netherlands

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The Road goes ever on and on
Down from the door where it began
Now far ahead the Road has gone,
And I must follow if I can,
Pursuing it with eager feet,
Until it joins some larger way
Where many paths and errands meet.
And wither then? I cannot say.

(J.R.R. Tolkien)

voor Claudia en Anika

CONTENTS

CHAPTER 1

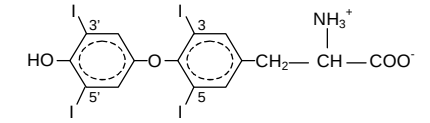
General Introduction

1.1 Thyroid hormone production and metabolism

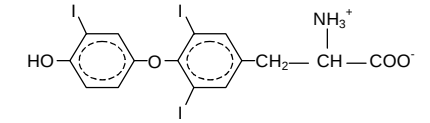
Thyroid hormones play an important role in overall body metabolism by regulation of oxygen consumption and heat production. The thyroid gland produces and secretes iodothyronines; about 90% of the output is 3,5,3',5'-tetraiodothyronine (thyroxine or T_4), while 3,5,3'-triiodothyronine (T_3) accounts for the remaining 10%. T_4 is considered to have little or no intrinsic bioactivity. T_3 is the most important bioactive thyroid hormone and virtually all the biological effects of T_4 elicited *in vivo* can be ascribed to its conversion into T_3. The total T_4 concentration in human serum is 75-135 nM, while total T_3 is present at a much lower concentration of 1.35–2.60 nM (Visser & de Jong, 1998). Finally, about 1% of the output of the thyroid gland is the inactive iodothyronine 3,3',5'-triiodothyronine (rT_3). Molecular structures of the iodothyronines are shown in Figure 1.

T_3 (Figure 2) and the other iodothyronines can be metabolized by deiodination, conjugation or oxidative deamination (Visser, 1988, 1994). Deiodination is quantitatively and qualitatively the most important pathway, which is catalyzed by a family of three iodothyronine deiodinases, all containing the amino acid selenocysteine in their catalytic site (Kohrle *et al.*, 1991; Bianco *et al.*, 2002). Deiodination of T_3 and rT_3 yields the diiodothyronine 3,3'-T_2. Conjugation, which mainly takes place in the liver, consists of either sulfation, catalyzed by sulfotransferases located in the cytoplasm or glucuronidation, catalyzed by enzymes located in the endoplasmic reticulum. Both reactions result in water-soluble derivatives (*e.g.* T_3 sulfate (T_3S)). Increased water-solubility facilitates the excretion in bile and urine. To a minor extent, thyroid hormones undergo oxidative deamination of the alanine side chain leading to iodothyroacetic acid derivatives *e.g.* triiodothyroacetic acid (Triac). Molecular structures of the metabolites are also shown in Figure 1.

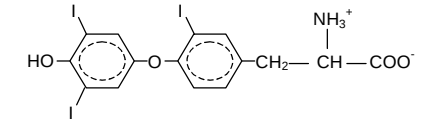
Functioning of the thyroid gland is controlled by a classic endocrine hypothalamus-pituitary axis. Upon stimulation by thermal and caloric signals the hypothalamus releases thyrotropin-releasing hormone (TRH), which activates the pituitary to produce and secrete a 30 kDa glycoprotein, thyroid-stimulating hormone (TSH) (Grossman *et al.*, 1997). TSH controls the functioning of the thyroid gland through interaction with the G-protein coupled TSH receptor.



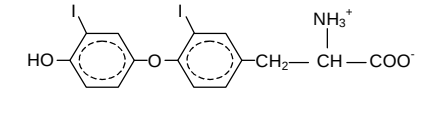
thyroxine (T_4)



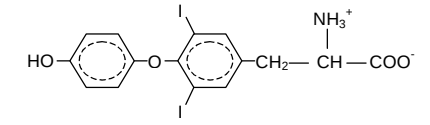
triiodothyronine (T_3)



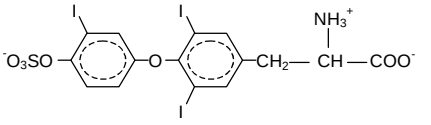
reverse T_3 (rT_3)



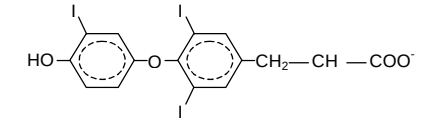
3,3'-diiodothyronine (3,3'-T_2)



3,5-diiodothyronine (3,5-T_2)



T_3 sulfate (T_3S)



triiodothyroacetic acid (Triac)

Figure 1 Structures of the iodothyronines

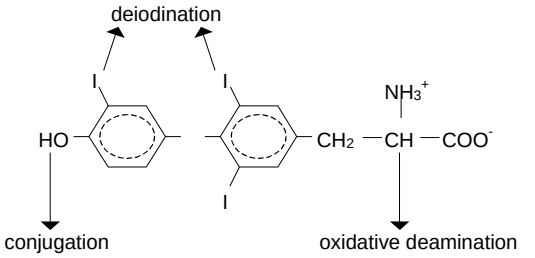


Figure 2 Three pathways for the metabolism of triiodothyronine (T_3).

1.2 Thyroid hormone action

Mechanism

The action of T_3 on metabolism includes an increase in the basal rate of oxygen consumption and heat production, that is generated via stimulation of ATP-requiring cellular processes such as the activity of enzymes and ion transport systems as well as the synthesis of DNA, RNA and other cellular components (Clausen *et al.*, 1991; Yen, 2001). T_3 is also essential for normal growth and development. The first step in thyroid hormone action and metabolism is transport across the cell membrane. In the cell, T_3 associates with cytosolic binding proteins, mitochondrial binding proteins and a specific nuclear receptor (Figure 3) (Ichikawa & Hashizume, 1995). Association with cytosolic proteins is regarded as an intracellular reservoir and also allows transfer of hormones in the cytosol. Most actions of T_3 are mediated via the nuclear receptor (TR). TR is a member of a large superfamily that includes receptors for steroids, retinoic acid, prostaglandins and fatty acids. In most cases TR acts as a heterodimer with the retinoic acid receptor (RXR). Furthermore, it is a ligand-modulated transcription factor capable to activate or repress gene expression (Muñoz & Bernal, 1997; Wu & Koenig, 2000). Several receptor subtypes have been described of which the α-receptor and β_1-receptor are expressed in the heart (Lazar, 1993; Muñoz & Bernal, 1997). Transcription is modulated by binding of the unliganded TR to specific DNA acceptor sites known as T_3 response elements (TRE), resulting in a repression of gene transcription. Association of T_3 with TR leads to a change in conformation,

thereby relieving the repression, resulting in induction of gene transcription.

In addition to the action of thyroid hormones mediated through binding to nuclear receptors, direct and non-genomic effects have been described. Segal *et al.* (1989a; 1989b) showed that T_3 increases cellular amino acid and sugar uptake in presence of a protein-synthesis inhibitor. These non-genomic actions occur primarily at the plasma membrane (Segal, 1990; Huang *et al.*, 1999) and the mitochondria (Wrutniak-Cabello *et al.*, 2001). In a study of Goglia *et al.* (1999), high affinity binding sites for T_3 have been identified in the mitochondria and it was shown that T_3 has a direct effect on mitochondrial ATP production.

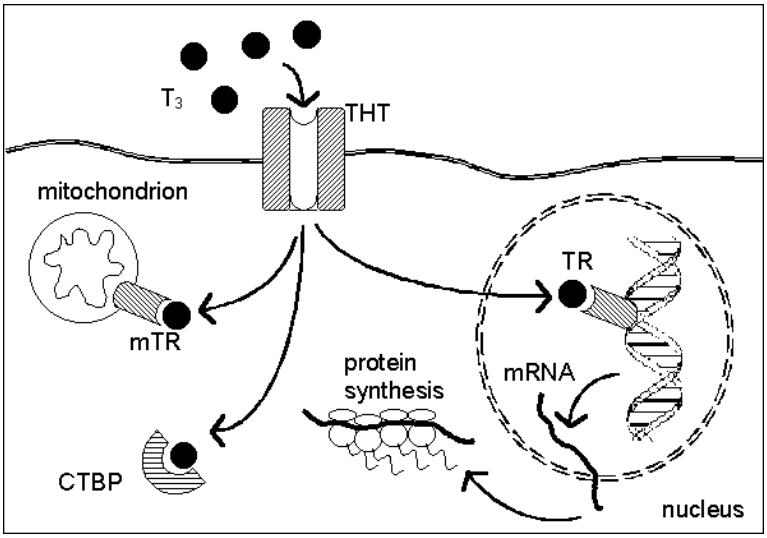


Figure 3 T_3 action in the cell. T_3 enters the cell via a carrier-mediated mechanism located in the plasma membrane, and binds to nuclear T_3 receptors to modulate transcription of genes. It can also associate with cytosolic binding proteins or with sites at the mitochondria. Redrawn after Ichiwaka *et al.* (1995). THT, plasma membrane thyroid hormone transporter; CTBP, cytosolic binding protein; TR, nuclear thyroid hormone receptor; mTR, mitochondrial thyroid hormone receptor

On the heart

The heart is very sensitive to the action of thyroid hormones. Measurable changes in cardiac performance are detected with small variations in thyroid hormone serum concentrations. Most patients and animals with hyperthyroidism experience cardiovascular manifestations, and the most serious complications of hyperthyroidism occur as a result of cardiac involvement (Rijnberk, 1996; Fadel *et al.*, 2000; Klein & Ojamaa, 2001). Alterations in cardiac performance include an increase in resting heart rate (a chronotropic effect), contractility (an inotropic effect), left ventricular muscle mass, and predisposition to atrial arrhythmias. These effects are mediated by thyroid hormone regulation of transcription of cardiac-specific genes (Polikar *et al.*, 1993; Klein & Ojamaa, 2001). T_3 administration in animals enhances myocardial contractility by stimulating the synthesis of the fast α myosin heavy chain and inhibiting the expression of the slow β isoform (Zähringer & Klaubert, 1982; Korecky *et al.*, 1987; Williams & Ianuzzo, 1988; Dillmann, 1990). It also causes an increase in sarcoplasmic reticulum (SR) Ca^{2+} adenosine triphosphatase (ATPase) and a decrease in the expression of the Ca^{2+} ATPase regulatory protein phospholamban (Zähringer & Klaubert, 1982; Simonides *et al.*, 1996; Muller *et al.*, 1997). The cardiac changes typical of hypothyroidism are opposite to those of hyperthyroidism, but they are accompanied by fewer signs and symptoms (Rijnberk, 1996; Klein & Ojamaa, 2001).

Finally, thyroid hormone induced cardiac hypertrophy can also result from changes in the blood pressure and flow rate (Simpson *et al.*, 1982; Klein *et al.*, 1992). Thyroid hormone decreases blood pressure and flow rate by dilating the resistance arterioles of the peripheral circulation via effects on the vascular smooth muscle cells and by increasing blood volume.

1.3 Transport of thyroid hormones

In blood

Thyroid hormones reach their sites of action bound to serum proteins. The three major binding proteins in serum are thyroxine-binding globulin (TBG), transthyretin (TTR) and albumin (Bartalena, 1990; Schussler, 2000). Human TBG circulates at a concentration of only 0.4 – 0.8 µM (Wiersinga & Krenning, 1998). Nevertheless, because of its high affinity, TBG carries most of the serum T_4 (75% in humans) and

T_3 (38-80% in humans). TTR, a binding protein of much more ancient lineage than TBG, is present in fish and higher vertebrates (Bartalena, 1990). TTR is synthesized in the liver and the concentration of TTR in plasma is 5 µM (Visser & de Jong, 1998). In addition, choroid plexus epithelial cells produce and secrete TTR, and it is suggested that this is an important source for brain T_4 (Bartalena, 1990; Schussler, 2000). Both TBG and TTR are considered to function as an extrathyroidal storage and as a buffer to protect cells from excessive hormone entry. The third binding protein for thyroid hormones and a variety of other compounds *e.g.* fatty acids (Curry *et al.*, 1998) in serum is albumin, it circulates at a concentration of 640 µM and binds T_4 with a 10-fold higher affinity than T_3. With the lowest affinity for thyroid hormones and the fastest thyroid hormone release compared to TBG and TTR, albumin may promote quick exchange of thyroid hormones with tissue sites (Krenning *et al.*, 1979; Pardridge, 1987).

In tissues

Forty years ago, it was postulated that thyroid hormones would pass the lipid bilayer of the cell membrane by passive diffusion (Lein & Dowben, 1961; Pardridge & Mietus, 1980). This was based on the fact that thyroid hormones are lipophilic and, as the plasma membrane is constituted of a lipid bilayer, there seemed no need for any other mechanism of translocation than that of diffusion. Today, it is clear that this assumption is not correct and that cells exhibit carrier-mediated mechanisms for the entry of thyroid hormones. The criteria for the existence of such mechanisms include: 1) The mechanisms are specific; only structurally related compounds are transported or compete with the mechanism; 2) it is saturable and has limited capacity; 3) there is no significant diffusion; 4) it is subject to regulation. The number of transporters, energy-status and possibly the Na^+ gradient may be important factors in regulation. Finally, 5) thyroid hormone uptake is rate limiting for subsequent metabolism. In patients with non-thyroidal illness (NTI) or during starvation, plasma T_3 production from T_4 in the liver is decreased (Docter & Krenning, 1990), due to reduced cellular uptake and not because of defective 5'-deiodination of T_4 (Hennemann *et al.*, 1993). Thus, transmembrane transport of thyroid hormones is important in the overall body thyroid hormone bioactivity.

The first report describing specific, saturable and energy-dependent uptake of thyroid hormones came from Rao *et al.* (1976). Since then, reports from several laboratories using cell types from various species have confirmed carrier-mediated, mostly energy- and Na^+-dependent uptake of iodothyronines (see for reviews: Kragie, 1994; Hennemann *et al.*, 2001). Hillier was the first to publish a series of studies on the uptake of T_3 and T_4 in an intact rat heart (Hillier, 1968a, 1968b, 1969). While he suggested that uptake is determined by the concentration of extra- and intracellular binding proteins, the more recent studies of Rosic *et al.* (1998; 2001) presented evidence for a specific uptake system for T_3 and T_4. The first reports on the presence of a T_3 transport mechanism in isolated heart cells came from our laboratory (Everts *et al.*, 1996b; Verhoeven *et al.*, 2001). The characterization of this T_3 transport system is the subject of the present thesis, as well as the question whether a comparable systems exists for T_4.

Sofar, no information is available about the molecular structure of thyroid hormone transporters. However, recently a number of plasma membrane transporters have been identified as potential mediators of thyroid hormone uptake, such as 1) a Na^+-dependent organic anion transporter (NTCP) (Friesema *et al.*, 1999), 2) members of Na^+-independent organic anion transporters (OATP) families (Abe *et al.*, 1996; Friesema *et al.*, 1999; Fujiwara *et al.*, 2001), and 3) the heterodimeric system L amino acid transporter consisting of the 4F2 heavy chain associated with LAT1 or LAT2 light chains (Friesema *et al.*, 2001). To what extent these transporters are relevant for the bioavailability of thyroid hormones *in vivo* is not known.

Efflux

Efflux of thyroid hormones has been studied in a number of cell types (Hennemann *et al.*, 2001). Efflux of T_3 from rat cardiomyocytes has been shown to be saturable, but not energy-dependent (Ribeiro *et al.*, 1996). The authors suggested that the efflux carrier for T_3 may be related to the multi drug resistance-related ATP-binding cassette (ABC) transporter family, but this has not been proven yet. Some reports show stimulation of efflux by aromatic amino acid counter transport (Osty *et al.*, 1990b) or by albumin (Benvenga & Robbins, 1998). The latter may be explained by facilitating diffusion of thyroid hormones through the water layer around the cell. T_4 and rT_3 efflux from choriocarcinoma cells (JAR) (Mitchell *et al.*, 1999) and human erythrocytes

(Osty *et al.*, 1990a), and T_4 efflux from rat hepatocytes (Hennemann *et al.*, 1984) showed no saturation. In conclusion, efflux of thyroid hormones may occur along various pathways. Whether this process requires a specific carrier remains to be established.

1.4 Physiological significance of thyroid hormone transport

In pathophysiological conditions

In patients with liver disease or chronic renal failure serum T_3 concentration is reduced, serum rT_3 concentration is increased, while serum T_4 concentration is usually normal and TSH does not rise (Docter *et al.*, 1993). This syndrome is known as NTI and is defined as any acute or chronic illness not related to the thyroid gland (Docter *et al.*, 1993). Like NTI, fasting is characterized by low serum concentration of T_3 (van der Heyden *et al.*, 1986). The low serum T_3 concentration has been ascribed to reduced activity of the type I deiodinase in the liver, responsible for the conversion of T_4 to T_3. However, since the activity of type I deiodinase also depends on the intracellular substrate concentration, transport of T_4 into the T_3-producing tissues is at least as important for peripheral production. This led to the hypothesis that the low serum T_3 concentration is caused by a reduction of T_4 transport into the liver (Vos *et al.*, 1995). Several reports underline this hypothesis. When human obese volunteers were subjected to a calorie restricted diet, T_4 kinetic studies revealed a 50% decrease in T_4 transport into tissues, that could account for half of the reduction in T_3 production (Docter *et al.*, 1993; Hennemann *et al.*, 1998). The effects of fasting on T_4 transport were also studied *in vitro* by incubating rat hepatocytes with serum from subjects before and after a 10-day period of calorie restriction (Lim *et al.*, 1994). This revealed a decreased iodide production from T_4 probably due to inhibition of T_4 transport. Similarly, when hepatocytes were incubated with serum of NTI patients, T_4 transport, but not type I deiodinase activity was impaired (Lim *et al.*, 1993). Identification of the factors in serum responsible for this inhibition, showed that several compounds are circulating at increased levels in serum of patients with NTI; among them 3-carboxy-4-methyl-5-propyl-2-furan propanoic acid (CMPF) and indoxylsulfate in patients with renal failure, and bilirubin and non-esterified fatty acids (NEFAs) in patients with liver disease (Docter *et al.*, 1993; Lim *et al.*, 1993). Serum levels of

NEFAs are also increased in mild NTI and during caloric deprivation to concentrations that inhibit T_4 transport (Lim *et al.*, 1994). Interestingly, in pituitary cells, where T_3 and T_4 probably share a common transport system (Everts *et al.*, 1994), T_3 and T_4 uptake were not inhibited by these compounds (Everts *et al.*, 1995; Wassen *et al.*, 2000), suggesting that this may be part of the mechanisms accounting for the absence of a rise in TSH in spite of the low serum T_3 in NTI patients (Everts *et al.*, 1996a; Hennemann *et al.*, 1998, 2001).

For treatment of heart disease

Patients undergoing coronary bypass surgery (Holland *et al.*, 1991; Klemperer *et al.*, 1995) and patients with congestive heart failure (Hamilton, 1993; Hamilton & Stevenson, 1996) may show the typical changes in serum thyroid hormone parameters known as NTI. It is still a matter of debate whether or not this condition should be treated (Klemperer *et al.*, 1995; Utiger, 1995; Camacho & Dwarkanathan, 1999). During the last 5 – 10 years clinical studies have explored the possibility of improving heart function by administration of T_4 or T_3 (Moruzzi *et al.*, 1996; Chowdhury *et al.*, 1999; Mullis-Jansson *et al.*, 1999) both in children with congestive heart failure and in adult patients undergoing coronary-artery bypass surgery. It has been shown that T_4 or T_3 administration decreased vascular resistance and improved cardiac performance in the early post-operative period. Though, administration over a longer period did not reduce the need for treatment with inotropic agents and mechanical devices like a pacemaker. Thus, it was concluded that T_4 or T_3 should not be used as a substitute for recommended drug therapy (Gomberg-Maitland & Frishman, 1998). Moreover, prolonged administration of T_4 or T_3 caused an increase in heart frequency, a chronotropic effect, which may be deleterious in patients with heart failure (Spooner *et al.*, 1999; Peters *et al.*, 2000). Since some of the effects of thyroid hormones could potentially benefit patients with heart failure, thyroid hormone analogs are being developed, which possibly have more inotropic than chronotropic properties (Morkin *et al.*, 1996; Spooner *et al.*, 1999; Pujol *et al.*, 2000; Trost *et al.*, 2000; Lameloise *et al.*, 2001). The propionic acid analog of 3,5-diiodothyronine, 3,5-diiodothyropropionic acid (DITPA) has been used to improve contractility of the heart after infarction, up to now only in animals (Spooner *et al.*, 1999). It has been shown that DITPA associates with bacterially expressed TRs (Morkin *et al.*, 1996). Furthermore, at the cellular

level, DITPA induces sarcoplasmic reticulum Ca^{2+} transport and protein expression, while it has been more complicated to interpret data on myosin heavy chain expression (Spooner *et al.*, 1999). From this point of view there is still a lack of understanding how this thyroid hormone analog works (Spooner *et al.*, 1999). In addition, DITPA shows no specificity with respect to receptor subtypes and the affinity is a 100-fold less that for T_3 (Morkin *et al.*, 1996). Recently, another thyroid hormone analog, the non-iodinated GC-1, has been developed (Chiellini *et al.*, 1998; Trost *et al.*, 2000). GC-1 has a high affinity for TR and shows receptor subtype specificity. It has a much greater potency in lowering cholesterol and TSH levels than T_3, but it has a smaller effect on heart rate (Trost *et al.*, 2000). These properties suggest that GC-1 may be a useful therapeutic agent.

In summary, the inotropic effects of thyroid hormone analogs could potentially benefit patients with heart failure. The mechanisms by which these analogs work are not fully understood and may involve tissue-selective uptake as suggested for GC-1 (Trost *et al.*, 2000). This stresses the importance of identification of thyroid hormone uptake mechanisms, which, on the other hand, may provide tools for development of drugs with tissue-selective thyromimetic activity, preferentially without chronotropic effects.

1.5 Aim, experimental models, and scope of the thesis

Aims of the study

There is abundant evidence that thyroid hormone transport across the plasma membrane occurs by facilitated mechanisms in many tissues and cell types. This thesis describes the characterization of thyroid hormone uptake mechanism(s) in heart cells and the attempts to identify a putative thyroid hormone transport protein. Cultured cells have proven to be a useful experimental model in the characterization of thyroid hormone transport mechanisms. We used neonatal rat cardiomyocytes and the embryonic heart cell line, H9c2(2-1). Furthermore, expression cloning in *Xenopus laevis* oocytes has proven to be a useful strategy in the cloning of a number of other transport proteins. In this thesis the following questions are addressed:

1. Are thyroid hormones transported by a specific transport mechanism(s) in cultured heart cells, and what are the characteristics of this mechanism,

2. What is the molecular structure of such a mechanism,
3. Are known transport proteins involved in uptake of thyroid hormones?

Experimental models

Three experimental models were used to address the questions described above.

Neonatal cardiomyocytes

Primary cultures of neonatal rat cardiomyocytes have proven to be a valid model in studies concerning intracellular signaling and gene transcription (van Heugten *et al.*, 1995; Muller *et al.*, 1997; Huang *et al.*, 1999; Sugden *et al.*, 1999; Gloss *et al.*, 2000). Moreover, studies to characterize uptake mechanisms for thyroid hormones and other compounds have successfully used this type of culture (Stremmel, 1988; Everts *et al.*, 1996b; Verhoeven *et al.*, 2001). In brief, cardiomyocytes are prepared from 2 to 3-day old Wistar rats by stepwise trypsinization (Everts *et al.*, 1996b), followed by a preplating step to obtain an enriched myocardial fraction consisting of more than 90% cardiomyocytes (Blondel *et al.*, 1971; van Heugten *et al.*, 1994). In our studies, the cells are plated at a density of 0.5 - 1.0 $\times$ 10^6 cells per well, and cultured in presence of 5% fetal calf serum (FCS) and 5% horse serum (HS). Experiments are routinely performed after 5 days at which time the cardiomyocytes form a spontaneously and synchronously contracting monolayer. It has been shown that these beating cells reflect the *in vivo* heart cells, since they generate action potentials and maintain their specificity towards drugs and hormones (Pinson, 1990).

The embryonic heart cell line H9c2(2-1)

We planned to continue the studies with neonatal rat cardiomyocytes with adult heart cells to be able to examine thyroid hormone transport during development and in pathophysiological conditions. For isolation of adult cardiomyocytes, we used a Langendorff perfusion method, in which the whole heart is perfused with proteolytic enzymes (Ikeda *et al.*, 1990; Piper *et al.*, 1990; Eppenberger *et al.*, 1994; Schlüter *et al.*, 1995). However, the quality of these adult cells was poor and the yield too low to perform experiments.

The myogenic cell line, H9c2(2-1), was established by Kimes and Brandt (1976) from embryonic rat heart tissue (ventricle). This cell line propagates as mononucleated

myoblasts, which are large, flat and spindle-shaped cells. Upon reduction of the serum concentration from 10 to 1%, the myoblasts organize themselves into linear parallel arrays and fuse to multinucleated myotubes. Several studies show that H9c2(2-1) cells possess properties of skeletal and cardiac cells (Ménard *et al.*, 1999; Wang *et al.*, 1999). Nevertheless, these cells have become an accepted model for heart cells to study the effects of ischaemia and diabetes (Eckel, 1996; Brostrom *et al.*, 2000; Wayman *et al.*, 2001) and we use this cell line to examine cardiac thyroid hormone uptake. Compared to primary cultures of neonatal rat cardiomyocytes, the H9c2(2-1) cells are phenotypically more homogenous and can be adapted to continuous cell culture conditions. Furthermore, the culture consists of pure myogenic cells. These advantages and the possibility to explore thyroid hormone uptake during differentiation from myoblast into myotube prompted us to use this cell line for continuation of our study on thyroid hormone transport mechanisms.

Xenopus laevis oocytes

Expression cloning using oocytes from the South African clawed toad, *Xenopus laevis*, has been shown to be an excellent tool for the structural/functional identification of various transport proteins (Wolkoff, 1996; Palacín *et al.*, 1998). The oocyte system, pioneered by Gurdon *et al.* (1971) and described in detail by Romero *et al.* (1998), offers several unique features (Wang *et al.*, 1991; Romero *et al.*, 1998). The oocyte is a relatively large cell (0.8–1.3 mm in diameter), making it convenient for micro-injection of RNA (either mRNA or cRNA). It is able to efficiently translate injected RNA, to correctly process proteins and to target them to subcellular compartments. This process takes about two to four days, after which functional expression of the protein can be examined by tracer uptake studies comparable to studies with cardiomyocytes or H9c2(2-1) cells. Furthermore, some advantages over other expression systems (*e.g.* eukaryotic cells) include simple handling of single cells, a maximal proportion of cells expressing transferred genetic information and good control of the environment of the oocytes.

Docter *et al.* (1997) adopted the expression cloning strategy in oocytes to characterize thyroid hormone transport in liver. Already in their studies, it appeared that the quantification of induction of thyroid hormone transport has difficulties, because oocytes possess an endogenous transport mechanism for thyroid hormones

and amino acids (van Winkle, 1993; Friesema *et al.*, 1998). Thyroid hormone uptake by such a mechanism may partly mask the uptake induced by injected RNA.

Scope of the thesis

Two studies with neonatal rat cardiomyocytes suggested the presence of a specific transport mechanism for T_3 (Everts *et al.*, 1996b; Verhoeven *et al.*, 2001). Based on these studies, we continued the characterization of T_3 uptake in neonatal rat cardiomyocytes, with emphasis on the cellular compartmentalization of T_3 in the cells. In addition, we tested the effects of the amino acids tryptophan and tyrosine for their effects on T_3 uptake (*Chapter 2*). To gain more insight in the specificity of plasma membrane transport, *Chapter 3* describes experiments in which we compared uptake of T_3 with that of Triac. Furthermore, we tested various analogs and metabolites for their effects on the uptake of T_3 in neonatal rat cardiomyocytes. In *Chapter 4*, we analyzed the uptake mechanism for T_3 and T_4 in H9c2(2-1) cells and investigated the possible role of thyroid hormone transport during the differentiation process. The results of the previous *Chapters* (2 – 4) suggested the existence of at least one uptake mechanism for thyroid hormones. Expression cloning was used to identify the molecular structure of these cardiac thyroid hormone transporter(s). *Xenopus laevis* oocytes were injected with rat heart mRNA (adult and neonatal). Experiments were performed in which T_3 and T_3 sulfamate (T_3NS) uptake by oocytes injected with heart mRNA was compared with that of water-injected controls (*Chapter 5*).

Apart from the involvement of amino acid transporters, fatty acid transporters could participate in thyroid hormone uptake in heart. The uptake of fatty acids, the preferred energy substrates for the heart, is mainly regulated by the fatty acid translocase (FAT). FAT cRNA was injected in *Xenopus laevis* oocytes. Its ability to transport iodothyronines was explored and compared with its functional expression as fatty acid transporter (*Chapter 6*). Finally, the findings of the preceding chapters are discussed in *Chapter 7*.

1.6 References

1. Abe T, Kakyo M, Sakagamii H, Tokui T, Nishio T, Tanemoto M, Nomura H, Hebert SC, Matsuno S, Kondo H & Yawo H 1996 Molecular characterization

and tissue distribution of a new organic anion transporter subtype (oatp3) that transports thyroid hormones and taurocholate and comparison with oatp2. *J Biol Chem* **273**:22395-22401

2. Bartalena L 1990 Recent achievements in studies on thyroid hormone-binding proteins. *Endocrine Rev* **11**:47-64
3. Benvenga S & Robbins J 1998 Thyroid hormone efflux from monolayer cultures of human fibroblasts and hepatocytes. Effect of lipoproteins and other thyroxine transport proteins. *Endocrinology* **139**:4311-4318
4. Bianco AC, Salvatore D, Gereben B, Berry MJ & Larsen PR 2002 Biochemistry, cellular and molecular biology, and physiological roles of the iodothyronine selenodeiodinases. *Endocr Rev* **23**:38-89
5. Blondel B, Roijen I & Cheneval JP 1971 Heart cells in culture: a simple method for increasing the proportion of myoblasts. *Experientia* **27**:356-358
6. Brostrom MA, Reilly BA, Wilson FJ & Brostrom CO 2000 Vasopressin-induced hypertrophy in H9c2 heart-derived myocytes. *Int J Biochem Cell Biol* **32**:993-1006
7. Camacho P & Dwarkanathan AA 1999 Sick euthyroid syndrome. What to do when thyroid function tests are abnormal in critically ill patients. *Postgraduate Medicine* **105**:215-219
8. Chiellini G, Apriletti JW, Al Yoshihara H, Baxter JD, Ribeiro RCJ & Scanlan TS 1998 A high-affinity subtype-selective agonist ligand for the thyroid hormone receptor. *Chem Biol* **5**:299-306
9. Chowdhury D, Parnell VA, Ojamaa K, Boxer R, Cooper R & Klein I 1999 Usefulness of triiodothyronine (T_3) treatment after surgery for complex congenital heart disease in infants and children. *Am J Card* **84**:1107
10. Clausen T, van Hardeveld C & Everts ME 1991 Significance of cation transport in control of energy metabolism and thermogenesis. *Physiol Rev* **71**:733-774
11. Curry S, Mandelkov H, Brick P & Franks N 1998 Crystal structure of human serum albumin complexed with fatty acid reveals an asymetric distribution of binding sites. *Nat Struct Biol* **5**:827-835
12. Dillmann WH 1990 Biochemical basis of thyroid hormone action in the heart. *Am J Med* **88**:626-630

13. Docter R & Krenning EP 1990 Role of cellular transport systems in the regulation of thyroid hormone bioactivity. In: Greer MA (ed) The Thyroid Gland. Raven Press, New York, pp. 233-254
14. Docter R, Krenning EP, de Jong M & Hennemann G 1993 The sick euthyroid syndrome: changes in thyroid hormone serum parameters and hormone metabolism. *Clin Endocrinol* **39**:499-518
15. Docter R, Friesema ECH, van Stralen PGJ, Krenning EP, Everts ME, Visser TJ & Hennemann G 1997 Expression of rat liver cell membrane transporters for thyroid hormone in *Xenopus laevis* oocytes. *Endocrinology* **138**:1841-1846
16. Eckel J 1996 Direct effects of glimepiride on protein expression of cardiac glucose transporters. *Horm Metab Res* **28**:508-11
17. Eppenberger HM, Hertig C & Eppenberger-Eberhardt M 1994 Adult rat cardiomyocytes in culture. A model system to study the plasticity of the differentiated cardiac phenotype at the molecular and cellular levels. *Trends Cardiovasc Med* **4**:187-193
18. Everts ME, Docter R, Moerings EPCM, van Koetsveld PM, Visser TJ, de Jong M, Krenning EP & Hennemann G 1994 Uptake of thyroxine in cultured anterior pituitary cells of euthyroid rats. *Endocrinology* **134**:2490-2497
19. Everts ME, Lim C, Moerings EPCM, Docter R, Visser TJ, de Jong M, Krenning EP & Hennemann G 1995 Effects of a furan fatty acid and indoxyl sulfate on thyroid hormone uptake in cultured anterior pituitary cells. *Am J Physiol* **268**:E974-E979
20. Everts ME, de Jong M, Lim C, Docter R, Krenning EP, Visser TJ & Hennemann G 1996a Different regulation of thyroid hormone transport in liver and pituitary: its possible role in the maintenance of low T_3 production during nonthyroidal illness and fasting in man. *Thyroid* **6**:357-366
21. Everts ME, Verhoeven FA, Bezstarosti K, Moerings EPCM, Hennemann G, Visser TJ & Lamers JMJ 1996b Uptake of thyroid hormones in neonatal rat cardiac myocytes. *Endocrinology* **137**:4235-4242
22. Fadel BM, Ellahham S, Ringel MD, Lindsay Jr. J, Wartofsky L & Burman KD 2000 Hyperthyroid heart disease. *Clin Cardiol* **23**:402-408
23. Friesema ECH, Docter R, Krenning EP, Everts ME, Hennemann G & Visser

TJ 1998 Rapid sulfation of 3,3',5'-triiodothyronine in native *Xenopus laevis* oocytes. *Endocrinology* **139**:596-600

24. Friesema ECH, Docter R, Moerings EPCM, Stieger B, Hagenbuch B, Meier PJ, Krenning EP, Hennemann G & Visser TJ 1999 Identification of thyroid hormone transporters. *Biochem Biophys Res Comm* **254**:497-501
25. Friesema ECH, Docter R, Moerings EPCM, Verrey F, Krenning EP, Hennemann G & Visser TJ 2001 Thyroid hormone transport by the heterodimeric human system L amino acid transporter. *Endocrinology* **142**:4339-4348
26. Fujiwara K, Adachi H, Nishio T, Unno M, Tokui T, Okabe M, Onogawa T, Suzuki T, Asano N, Tanemoto M, Seki M, Shiiba K, Suzuki M, Kondo Y, Nunoki K, Shimosegawa T, Ionuma K, Ito S, Matsuno S & Abe T 2001 Identification of thyroid hormone transporters in humans: different molecules are involved in a tissue-specific manner. *Endocrinology* **142**:2005-2012
27. Gloss B, Villegas S, Villiarreal FJ, Moriscot A & Dillmann WH 2000 Thyroid hormone-induced stimulation of the sarcoplasmic reticulum Ca^{2+} ATPase gene is inhibited by LIF and IL-6. *Am J Physiol Endocrinol Metab* **278**:E738-E743
28. Goglia F, Moreno M & Lanni A 1999 Action of thyroid hormones at the cellular level: the mitochondrial target. *FEBS Lett* **452**:115-120
29. Gomberg-Maitland M & Frishman WH 1998 Thyroid hormone and cardiovascular disease. *Am Heart J* **135**:187-196
30. Grossman M, Weintraub BD & Szkudlinski MW 1997 Novel insight into the molecular mechanism of human thyrotropin action: structural, physiological, and therapeutic implications for the glycoprotein hormone family. *Endocrine Rev* **18**:476-501
31. Gurdon JB, Lane CD & Woodland HR 1971 Use of frog eggs and oocytes for the study of messenger RNA and its translation in living cells. *Nature* **233**:177-182
32. Hamilton MA 1993 Prevalence and clinical implications of abnormal thyroid hormone metabolism in advanced heart failure. *Ann Thorac Surg* **56**:s48-s53
33. Hamilton MA & Stevenson LW 1996 Thyroid hormone abnormalities in heart failure: possibilities for therapy. *Thyroid* **6**:527-529

34. Hennemann G, Krenning EP, Bernard B, Huver F, Mol J, Docter R & Visser TJ 1984 Regulation of influx and efflux of thyroid hormones in rat hepatocytes: possible physiologic significance of the plasma membrane in the regulation of thyroid hormone activity. *Horm Metab Res* **14**:1-6

35. Hennemann G, Vos RA, de Jong M, Krenning EP & Docter R 1993 Decreased peripheral 3,5,3'-triiodothyronine (T_3) production from thyroxine (T_4): a syndrome of impaired thyroid hormone activation due to transport inhibition of T_4- into T_3-producing tissues. *J Clin Endocrinol Metab* **77**:1431-1435

36. Hennemann G, Everts ME, de Jong M, Lim C, Krenning EP & Docter R 1998 The significance of plasma membrane transport in the bioavailability of thyroid hormone. *Clin Endocrinol* **48**:1-8

37. Hennemann G, Docter R, Friesema ECH, de Jong M, Krenning EP & Visser TJ 2001 Plasma membrane transport of thyroid hormones and its role in thyroid hormone metabolism and bioavailability. *Endocrine Rev* **22**:451-476

38. van Heugten HAA, Bezstarosti K & Lamers JMJ 1994 Endothelin-1 and phenylephrine-induced activation of the phosphoinositide cycle increases cell injury of cultured cardiomyocytes exposed to hypoxia/reoxygenation. *J Mol Cell Cardiol* **26**:1513-1524

39. van Heugten HAA, de Jonge HW, Bezstarosti K, Sharma HS, Verdouw PD & Lamers JMJ 1995 Intracellular signaling and genetic reprogramming during development of hypertrophy in cultured cardiomyocytes. In: Dhella NS, Pierce GN, Ponegia V & Beemst RE (eds) Heart Hypertrophy and Failure. Kluwer Academic Publisher, Boston, pp. 79-92

40. van der Heyden JTM, Docter R, van Toor H, Wilson JHP, Hennemann G & Krenning EP 1986 Effects of caloric deprivation on thyroid hormone tissue uptake and generation of low T_3 syndrome. *Am J Physiol* **251**:E156-E163

41. Hillier AP 1968a The uptake and release of thyroxine and triiodothyronine by the perfused rat heart. *J Physiol* **199**:151-160

42. Hillier AP 1968b The effect of serum on the uptake of thyroid hormones by the perfused rat heart. *J Physiol* **199**:161-168

43. Hillier AP 1969 The uptake of thyroxine and tri-iodothyronine by perfused hearts. *J Physiol* **203**:665-674

44. Holland FW, Brown PS, Weintraub BD & Clark RE 1991 Cardiopulmonary

bypass and thyroid function: a "euthyroid sick syndrome". *Ann Thorac Surg* **52**:46-50

45. Huang CJ, Geller HM, Green WL & Craelius W 1999 Acute effects of thyroid hormone analogs on sodium currents in neonatal rat myocytes. *J Mol Cell Cardiol* **31**:881-893
46. Ichikawa K & Hashizume K 1995 Thyroid hormone action in the cell. *Endocr J* **42**:131-140
47. Ikeda U, Briggs GM, Allen PD, Sen L, Medford RM & Smith TW 1990 Properties of adult rat ventricular cells in long-term culture. *Cardioscience* **1**:225-233
48. Kimes BW & Brandt L 1976 Properties of a clonal muscle cell line from rat heart. *Exp Cell Res* **98**:367-381
49. Klein I, Ojamaa K, Samarel AM, Welikson R & Hong C 1992 Hemodynamic regulation of myosin heavy chain gene expression. Studies in the transplanted rat heart. *J Clin Invest* **89**:68-73
50. Klein I & Ojamaa K 2001 Thyroid hormone and the cardiovascular system. *N Engl J Med* **344**:501-509
51. Klemperer JD, Klein I, Gomez M, Helm RE, Ojamaa K, Thomas SJ, Isom W & Krieger K 1995 Thyroid hormone treatment after coronary-artery bypass surgery. *N Engl J Med* **333**:1522-1527
52. Kohrle J, Hesch RD & Leonard JL 1991 Intracellular pathways of iodothyronine metabolism. In: Braverman LE & Utiger RD (eds) The Thyroid. J.B. Lippincott Company, Philadelphia, pp. 144-189
53. Korecky B, Zak R, Schwartz K & Aschenbrenner V 1987 Role of thyroid hormone in regulation of isomyosin composition, contractility, and size of heterotopically isotransplanted rat heart. *Circ Res* **60**:824-830
54. Kragie L 1994 Membrane iodothyronine transporters. Part I: Review of physiology. *Endocr Res* **40**:319-341
55. Krenning EP, Docter R, Bernard HF, Visser TJ & Hennemann G 1979 The essential role of albumin in the active transport of thyroid hormones into primary cultured rat hepatocytes. *FEBS Lett* **107**:227-230
56. Lameloise N, Siegrist-Kaiser C, O'Connell M & Burger A 2001 Differences between the effects of thyroxine and tetraiodothyroacetic acid on TSH

suppression and cardiac hypertrophy. *Eur J Endocrinol* **144**:145-154

57. Lazar MA 1993 Thyroid hormone receptors: multiple forms, multiple possibilities. *Endocrine Rev* **14**:184-193
58. Lein A & Dowben RM 1961 Uptake and binding of thyroxine and triiodothyronine by rat diaphragm *in vitro*. *Am J Physiol* **200**:1029-1031
59. Lim CF, Docter R, Visser TJ, Krenning EP, Bernard B, van Toor H, de Jong M & Hennemann G 1993 Inhibition of thyroxine transport into cultured rat hepatocytes by serum of nonuremic critically ill patients: effects of bilirubin and nonesterified fatty acids. *J Clin Endocrinol Metab* **76**:1165-1172
60. Lim CF, Docter R, Krenning EP, van Toor H, Bernard B, de Jong M & Hennemann G 1994 Transport of thyroxine into cultured hepatocytes: effects of mild non-thyroidal illness and calorie restriction in obese subjects. *Clin Endocrinol* **40**:79-85
61. Ménard C, Pupier S, Mornet D, Kitzmann M, Nargeot J & Lory P 1999 Modulation of L-type calcium channel expression during retinoic acid-induced differentiation of H9c2 cardiac cells. *J Biol Chem* **274**:29063-29070
62. Mitchell AM, Rowan KA, Manley SW & Mortimer RH 1999 Comparison of mechanisms mediating uptake and efflux of thyroid hormones in the human choriocarcinoma cell line, JAR. *J Endocrinol* **161**:107-113
63. Morkin E, Pennock GD, Raya TE, Bahl JJ & Goldman S 1996 Development of a thyroid hormone analogue for the treatment of congestive heart failure. *Thyroid* **6**:521-526
64. Moruzzi P, Doria E & Agostoni PG 1996 Medium-term effectiveness of L-thyroxine treatment in idiopathic dilated cardiomyopathy. *Am J Med* **101**:461-467
65. Muller A, Zuidwijk MJ, Simonides WS & van Hardeveld C 1997 Modulation of SERCA2 expression by thyroid hormone and norepinephrine in cardiocytes: role of contractility. *Am J Physiol* **272**:H1876-H1885
66. Mullis-Jansson SL, Argenziano M, Homma S, Weinberg AD, Williams M, Rose EA & Smith CR 1999 A randomized double-blind study of the effect of triiodothyronine on cardiac function and morbidity after coronary bypass surgery. *J Thorac Cardiovasc Surg* **117**:1128-1135
67. Muñoz A & Bernal J 1997 Biological activities of thyroid hormone receptors.

Eur J Endocrinol **137**:433-445

68. Osty J, Valensi P, Samson M, Francon J & Blondeau JP 1990a Transport of thyroid hormones by human erythrocytes: kinetic characterization in adults and newborns. *J Clin Endocrinol Metab* **71**:1589-1595
69. Osty J, Zhou Y, Chantoux F, Francon J & Blondeau JP 1990b The triiodothyronine carrier of rat erythrocytes: asymmetry and mechanisms of *trans*-inhibition. *Biochem Biophys Acta* **1051**:46-51
70. Palacín M, Estévez R, Bertran J & Zorzano A 1998 Molecular biology of mammalian plasma membrane amino acid transporters. *Physiol Rev* **78**:969-1054
71. Pardridge WM & Mietus LJ 1980 Influx of thyroid hormones into rat liver *in vivo*. Differential availability of thyroxine and triiodothyronine bound by plasma proteins. *J Clin Invest* **66**:367-374
72. Pardridge WM 1987 Plasma protein mediated-transport of steroid and thyroid hormones. *Am J Physiol* **252**:E157-E164
73. Peters A, Ehlers M, Blank B, Exler D, Falk C, Kohlmann T, Fruehwald-Schultes B, Wellhoener P, Kerner W & Fehm HL 2000 Excess triiodothyronine as a risk factor of coronary events. *Arch Intern Med* **160**:1993-1999
74. Pinson 1990 Neonatal rat heart muscle cells. In: Piper HM (ed) Cell culture techniques in heart and vessel research. Springer, Berlin, pp. 20-35
75. Piper HM, Volz A & Schwartz P 1990 Adult ventricular rat heart muscle cells. In: Piper HM (ed) Cell culture techniques in heart and vessel research. Springer, Berlin, pp. 37-60
76. Polikar R, Burger MD, Scherrer U & Nicod P 1993 The thyroid and the heart. *Circulation* **87**:1435-1441
77. Pujol P, De Boisvilliers F, Bringer J & Jaffiol C 2000 Effects of thyrotropin suppression with tri-iodothyroacetic acid on radionuclide cardiovascular parameters. *Thyroid* **10**:1017-1018
78. Rao GS, Eckel J, Rao ML & Breuer H 1976 Uptake of thyroid hormone by isolated liver cells. *Biochem Biophys Res Comm* **73**:98-104
79. Ribeiro RCJ, Cavalieri RR, Lomri N, Rahmaoui CM, Baxter JD & Scharschmidt BF 1996 Thyroid hormone export regulates cellular hormone content and response. *J Biol Chem* **271**:17147-17151

80. Rijnberk A 1996 Thyroids. In: Rijnberk A (ed) Clinical endocrinology of dogs and cats. Kluwer Academic Publishers Inc., Dordrecht, pp. 35-59
81. Romero MF, Kanai Y, Gunshin H & Hediger MA 1998 Expression cloning using *Xenopus laevis* oocytes. In: Amara SG (ed) Methods Enzymol. Academic Press, San Diego, pp. 17-51
82. Rosic MA, Pantovic SB, Lucic AP, Ribarac-Stepic N, Trtic T, Andjelkovic I & Segal MB 1998 Triiodothyronine uptake by the isolated rat heart. *Pharmazie* **53**:351-352
83. Rosic MA, Pantovic SB, Lucic AP, Ribarac-Stepic N & Andjelkovic I 2001 Kinetics of thyroxine (T_4) and triiodothyronine (T_3) transport in the isolated rat heart. *Exp Physiol* **86**:13-18
84. Schlüter KD, Millar BC, McDermott BJ & Piper HM 1995 Regulation of protein synthesis and degradation in adult ventricular cardiomyocytes. *Am J Physiol* **269**:C1347-C1355
85. Schussler GC 2000 The thyroxine-binding proteins. *Thyroid* **10**:141-149
86. Segal J 1989a A rapid, extranuclear effect of 3,5,3'-triiodothyronine on sugar uptake by several tissues in the rat *in vivo*. Evidence for a physiological role for the thyroid hormone action at the level of the plasma membrane. *Endocrinology* **24**:2755-2764
87. Segal J 1989b Acute effect of thyroid hormone on the heart: an extranuclear increase in sugar uptake. *J Mol Cell Cardiol* **21**:323-334
88. Segal J 1990 Thyroid hormone action at the level of the plasma membrane. *Thyroid* **1**:83-86
89. Simonides WS, Brent GA, Thelen MHM, van der Linden CG, Larsen PR & van Hardeveld C 1996 Characterization of the promoter of the rat sarcoplasmic endoplasmic reticulum Ca^{2+}-ATPase gene and analysis of thyroid hormone responsiveness. *J Biol Chem* **271**:32048-32056
90. Simpson P, McGrath A & Savion S 1982 Myocyte hypertrophy in neonatal rat heart cultures and its regulation by serum and catecholamines. *Circ Res* **51**:787-801
91. Spooner PH, Morkin E & Goldman S 1999 Thyroid hormone and thyroid hormone analogues in the treatment of heart failure. *Cor Art Disease* **10**:395-399

92. Stremmel W 1988 Fatty acid uptake by isolated rat heart myocytes represents a carrier-mediated transport process. *J Clin Invest* **81**:844-852

93. Sugden MC, Priestman DA, Orfali KA & Holness MJ 1999 Hyperthyroidism facilitates cardiac fatty acid oxidation through altered regulation of cardiac carnitine palmitoyltransferase: studies *in vivo* and with cardiac myocytes. *Horm Metab Res* **31**:300-306

94. Trost SU, Swanson E, Gloss B, Wang-Iverson DB, Zhang H, Volodarsky T, Grover GJ, Baxter JD, Chiellini G, Scanlan TS & Dillmann WH 2000 The thyroid hormone receptor-β-selective antagonist GC-1 differentially affects plasma lipids and cardiac activity. *Endocrinology* **141**:3057-3064

95. Utiger RD 1995 Altered thyroid function in nonthyroidal illness and surgery. To treat or not to treat? *N Engl J Med* **333**:1562-1563

96. Verhoeven FA, Moerings EPCM, Lamers JMJ, Hennemann G, Visser TJ & Everts ME 2001 Inhibitory effects of calcium blockers on thyroid hormone uptake in neonatal rat cardiac myocytes. *Am J Physiol* **21**:H1985-H1991

97. Visser TJ 1988 Metabolism of thyroid hormone. In: Cooke BA, King RBJ & van der Molen HJ (eds) Hormones and Their Actions Part 1. Elsevier Science Publishers BV, Amsterdam, pp. 81-103

98. Visser TJ 1994 Sulfation and glucuronidation pathways of thyroid hormone metabolism. In: Wu SY & Visser TJ (eds) Thyroid hormone metabolism: molecular biology and alternate pathways. CRC Press, Boca Raton, pp. 85-117

99. Visser TJ & de Jong M 1998 Productie en metabolisme van schildklierhormoon. In: Wiersinga WM & Krenning EP (eds) Schildklierziekten. 2e ed. Bohn Stafleu Van Loghum, Houten, pp. 1-18

100. Vos RA, de Jong M, Bernard BF, Docter R, Krenning EP & Hennemann G 1995 Impaired thyroxine and 3,5,3'-triiodothyronine handling by rat hepatocytes in the presence of serum of patients with nonthyroidal illness. *J Clin Endocrinol Metab* **80**:2364-2370

101. Wang HC, Beer B, Sassano D, Blume AJ & Ziai MR 1991 Gene expression in *Xenopus* oocytes. *Int J Biochem* **3**:271-276

102. Wang W, Watanabe M, Nakamura T, Kudo Y & Ochi R 1999 Properties and expression of Ca^{2+}-activated K^{+}-channels in H9c2 cells derived from rat

ventricle. *Am J Physiol* **276**:H1559-H1566

103. Wassen FWJS, Moerings EPCM, van Toor H, Hennemann G & Everts ME 2000 Thyroid hormone uptake in cultured anterior pituitary cells: effects of energy status and bilirubin. *J Endocrinol* **165**:599-606
104. Wayman N, McDonald MC, Thompson AS, Threadgill MD & Thiemermann C 2001 5-aminoisoquinelinone, a potent inhibitor of poly (adenosine 5'-diphosphate ribose) polymerase, reduces myocardial infarct size. *Eur J Pharmacol* **430**:93-100
105. Wiersinga WM & Krenning EP 1998 Bijlage V. Normaalwaarden bij volwassenen. In: Wiersinga WM & Krenning EP (eds) Schildklierziekten. Bohn Stafleu Van Loghum, Houten, p 261
106. Williams HM & Ianuzzo CD 1988 The effects of triiodothyronine on cultured neonatal rat cardiac myocytes. *J Mol Cell Cardiol* **20**:689-699
107. van Winkle LJ 1993 Endogenous amino acid transport systems and expression of mammalian amino acid transport proteins in *Xenopus* oocytes. *Biochim Biophys Acta* **1154**:157-172
108. Wolkoff AW 1996 Hepatocellular sinusoidal membrane organic anion transport and transporters. *Sem Liver Dis* **16**:121-127
109. Wrutniak-Cabello C, Casas F & Cabello G 2001 Thyroid hormone action in mitochondria. *J Mol Endocrinol* **26**:67-77
110. Wu Y & Koenig RJ 2000 Gene regulation and thyroid hormone. *Trends Endocrinol Metab* **11**:207-211
111. Yen PM 2001 Physiological and molecular basis of thyroid hormone action. *Physiol Rev* **81**:1097-1141
112. Zähringer J & Klaubert A 1982 The effect of triiodothyronine on the cardiac mRNA. *J Mol Cell Cardiol* **14**:559-571

CHAPTER 2

T_3 transport in neonatal cardiomyocytes

Characterization of Uptake and Compartmentalization of 3,5,3'-Triiodothyronine in Cultured Neonatal Rat Cardiomyocytes

H.H.A.G.M. van der Putten, B.J.L.J. Joosten, P.H.M. Klaren and M.E. Everts.

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2.1 Abstract

The uptake of triiodothyronine (T_3) in cultured neonatal rat cardiomyocytes was investigated and compared with the uptake of reverse T_3 (rT_3) and thyroxine (T_4). Cellular compartmentalization of T_3 was studied by distinguishing T_3 activity associated with the plasma membrane from that in the cytosol or incorporated in the cell nucleus. T_3 and T_4 uptake displayed similar temperature dependencies which, in magnitude, differed from that of rT_3 uptake. T_3 uptake was Na^+ independent, and sensitive to oligomycin and monodansylcadaverine (42-49% and 25% inhibition of 15-min cellular uptake, respectively). Furthermore, T_3 uptake could be inhibited by tryptophan (20%) and tyrosine (12%), while 2-aminobicyclo-[2,2,1]heptane-carboxylic acid (BCH) had no effect. Co-incubation with tryptophan and oligomycin resulted in an additive inhibition of T_3 uptake (77%). We therefore conclude that (i) T_3 uptake is energy-dependent, (ii) receptor-mediated endocytosis may be involved and (iii) the aromatic amino acid transport system T may play a role, while system L is not involved in T_3 transport in cardiomyocytes. Co-incubation with unlabeled iodothyronines showed that 3,3'-di-iodothyronine and T_3 itself were the most effective inhibitors of T_3 uptake (30% and 36% inhibition of 15-min cellular uptake respectively). At 15-min incubation time, 38% of the total cell-associated T_3 was present in the cytosol and nucleus, and 62% remained associated to the plasma membrane. Unidirectional uptake rates did not saturate over a free T_3 concentration range up to 3.9 µM. We conclude that T_3 uptake in neonatal rat cardiomyocytes occurs by an energy- and temperature-dependent mechanism that may include endocytosis and amino acid transport system T, and is not sensitive to the Na^+ gradient. Elucidation of the molecular basis for the T_3 transporter is subject of current investigation.

Key words: hormone transport, thyroid hormone, triiodothyronine, cardiomyocytes

2.2 Introduction

The heart is a major target organ for thyroid hormone action. Thyroid hormone-induced changes in cardiac function are achieved through activation of iodothyronine-sensitive genes (Dillmann, 1990; Gloss *et al.*, 1999), or through indirect, non-genomic pathways (Craelius *et al.*, 1990; Davis & Davis, 1993). Genomic triiodothyronine (T_3)-mediated effects result from the association of the hormone to nuclear receptors that modify transcription of specific mRNAs, coding for, *e.g.* the α-isoform of myosin heavy chain or sarcoplasmic reticulum Ca^{2+}-ATPase (Rohrer *et al.*, 1991). A prerequisite for binding to the nuclear receptor is transmembrane transport of the hormone and, in the past, a non-protein-mediated diffusion pathway for T_3 transport has been proposed (Lein & Dowben, 1961; Pardridge & Mietus, 1980). However, a diffusion pathway is unlikely due to the lipophilic nature of thyroid hormones which makes it energetically unfavorable to cross the membrane unfacilitated, as evidenced by results from electron spin resonance studies and experiments on artificial lipid bilayers (Lai *et al.*, 1985; Chehín *et al.*, 1999). Moreover, a transport mechanism would confer a regulatory step in thyroid hormone metabolism (Hennemann *et al.*, 1993, 1998). For example, in patients with non-thyroidal illness or during starvation, plasma T_3 production by thyroxine (T_4) deiodination in the liver is decreased (Docter & Krenning, 1990), and Hennemann *et al.* (1993) showed that this resulted from a reduced cellular uptake of T_4, not from defective 5'-deiodination. Thus, the transmembrane transport of thyroid hormones is important in the overall body thyroid hormone bioactivity.

Although the heart is one of the most responsive target organs to thyroid hormones, little is known about the mechanisms of transport of these hormones across the plasma membrane of the cardiomyocyte. During the past decade evidence has been presented for T_3 uptake through a pathway mediated by a specific carrier protein (Oppenheimer & Schwartz, 1985; Docter *et al.*, 1987; Pontecorvi & Robbins, 1989; Kragie, 1994; Everts *et al.*, 1996b) and for receptor-mediated endocytosis (Cheng, 1983a). To our knowledge, only our previous report examines T_3 transport in cardiomyocytes (Everts *et al.*, 1996b). In the current report, we continue the investigation on the transport of T_3 in heart and its compartmentalization, using cultured neonatal cardiomyocytes of the rat as a model system. Since in some organs T_3 and T_4 share the same transporter (Everts *et al.*, 1996a), uptake of T_3 in cardiomyocytes was compared with that of T_4.

The substrate specificity of the putative transporter was further assessed by comparing the uptake of T_3 with that of reverse T_3 (rT_3), and by testing the effects of 3,5-di-iodothyronine (3,5-T_2) and 3,3'-di-iodothyronine (3,3'-T_2). Furthermore, a growing number of studies demonstrate that uptake systems for aromatic amino acids *e.g.* system L and system T, are involved in the transport of thyroid hormones (Everts *et al.*, 1994; Mitchell *et al.*, 1999; Ritchie *et al.*, 1999). In our previous report, we investigated the effect of tyrosine and tryptophan on T_3 uptake and suggested that part of the T_3 uptake may occur through these systems (Everts *et al.*, 1996b). In the present report, we have continued these studies by examination of the dose-dependency of the effect of tryptophan (Trp) and tyrosine (Tyr), and 2-aminobicyclo[2,2,1]heptane-carboxylic acid (BCH), a system L inhibitor, on T_3 uptake.

2.3 Materials and Methods

Animals

Wistar rats, 3-day-old and of both sexes, were obtained from laboratory stock (Utrecht University, the Netherlands). They were killed by decapitation, and hearts were quickly dissected and processed as described below to obtain primary cultures of cardiomyocytes.

Materials

All reagents used for cell isolation and cell culture were obtained from Life Technologies BV (Breda, the Netherlands), with the exception of trypsin and deoxyribonuclease which were purchased from Boehringer (Mannheim, Germany). Polystyrene culture dishes (24-wells) were obtained from Corning Costar Europe (Badhoevedorp, the Netherlands). Iodothyronines (T_4, T_3, 3,3'-T_2, 3,5-T_2), monodansylcadaverine (MDC), oligomycin, ouabain, Trp, Tyr, sulfobromophthalein (BSP), and BCH were purchased from Sigma Chemical Co. (St. Louis, MO, USA). [^{125}I]T_3 (81.4 TBq/mmol), [^{125}I]rT_3 (24.4 TBq/mmol) and [^{125}I]T_4 (4.3 TBq/mmol) were purchased from NEN Life Science Products, Inc. (Boston, MA, USA).

Cell culture

Primary cultures of neonatal rat cardiomyocytes were prepared using a trypsin digestion

method as described previously (Everts *et al.*, 1996b; Verhoeven *et al.*, 2001). An enriched myocardial fraction was obtained by including a preplating step (60 min at 37 °C; 5% CO_2; in 250 ml culture flasks) to remove fibroblasts (Blondel *et al.*, 1971). After this procedure, the preparation consists of more than 90% cardiomyocytes (van Heugten *et al.*, 1994). Cardiomyocytes were seeded into 24-well culture dishes at a density of 1.0×10^6 cells per well, and cultured at 37 °C in a 5% CO_2 atmosphere in 4:1 w/w Dulbecco's modified Eagle's medium (DMEM) – medium 199 (M199) supplemented with 5% horse serum (HS), 5% fetal calf serum (FCS), and 2% penicillin/ streptomycin. One day after isolation the culture medium was replaced by fresh medium. Experiments were routinely performed after 5 days of culture at which time the cardiomyocytes reached confluency and were in a spontaneously and synchronously contracting monolayer (Verhoeven *et al.*, 2001). Cell density was virtually constant, as the amount of total cellular protein per well (30 to 40 µg) varied only slightly between preparations. Cell viability on the day of experimentation, as judged by the ability of cells to exclude trypan blue, was virtually 100%. Although culture in the presence of serum might result in proliferation of fibroblasts that also show active uptake of [^{125}I]T_3 and [^{125}I]T_4 (Docter *et al.*, 1987), it has previously been shown that uptake of [^{125}I]T_3 and [^{125}I]T_4 was similar in cardiomyocytes cultured in the absence and presence of serum (Everts *et al.*, 1996b). Moreover, the uptake of [^{125}I]T_3 expressed per µg protein is more than tenfold higher in cardiomyocytes than in cardiac fibroblasts (S. van der Heide, personal communication).

Cellular uptake studies

After removal of the culture medium, cardiomyocytes were preincubated for 30 min at 37 °C in 0.5 ml Krebs-Ringer buffer (139 mM NaCl, 4.2 mM KCl, 1.5 mM $CaCl_2$, 1.4 mM KH_2PO_4, 1.4 mM $MgSO_4$, 5.0 mM glucose; prepared in Tris at pH 7.4) supplemented with 0.5% bovine serum albumin (BSA). Incubation was initiated by quickly replacing the preincubation medium with 0.5 ml Krebs-Ringer buffer containing the additions described below and either [^{125}I]T_3 (4×10^5 cpm/ml; 82 pM), [^{125}I]rT_3 (4×10^5 cpm/ml; 273 pM) or [^{125}I]T_4 (4×10^5 cpm/ml; 1.6 nM). The concentrations of BSA and iodothyronines in our incubation media were chosen so as to resemble free plasma hormone concentrations in neonatal rat. Incubations were performed at 37 °C, and the culture wells were mildly agitated. Uptake was terminated

by quick aspiration of the incubation medium, and culture wells were rinsed three times with 1 ml ice-cold saline. Cells were dissolved in 1 ml 0.1 M NaOH and analyzed for ^{125}I activity in a gamma-counter (Packard Cobra II, Packard Instruments Co., Meriden, CT, USA). The time-course of T_3 uptake was assayed in the presence or absence of sodium. Sodium-free conditions were attained by replacing sodium with choline on a mol-for-mol basis in the preincubation and incubation media. To test the temperature dependency of iodothyronine uptake, cardiomyocytes were pre-incubated and incubated at 0, 20 or 37 °C. Unidirectional iodothyronine uptake was measured by incubation of cardiomyocytes for 5 min in Krebs-Ringer buffer containing $[^{125}I]T_3$ and increasing concentrations of the unlabeled hormone (10 nM – 100 µM). Experiments in which the effect of analogues on 15-min T_3 uptake was measured were performed by adding 10 µM of the analogue to the Krebs-Ringer buffer containing 4×10^5 cpm/ml $[^{125}I]T_3$. In experiments in which the effects of oligomycin and MDC were investigated, they were added to both the preincubation and incubation medium. Possible involvement of other known transport systems in the uptake of $[^{125}I]T_3$ was examined by preincubation and incubation with 0.5 or 2 mM Tyr or Trp, 10 or 25 µM BSP, or 5 mM BCH.

The amount of $[^{125}I]T_3$, $[^{125}I]rT_3$ or $[^{125}I]T_4$ associated with the cells was expressed as either percentage of added ^{125}I activity (percentage of dose) or as pmol/10^6 cells. Results were corrected for the amount of ^{125}I activity associated with the walls of the culture wells by subtracting the amount of ^{125}I activity extracted from the wells incubated without cells.

Compartmentalization studies

To distinguish $[^{125}I]T_3$ activity partitioned within the plasma membrane from $[^{125}I]T_3$ activity present in the cytosol, cardiomyocytes were treated with detergent to release cytosolic tracer contents. After incubation in Krebs-Ringer buffer, as described above, cells were treated at room temperature with a permeabilization buffer containing 50 µg/ml saponin in 160 mM NaCl and 5 mM $MgCl_2$ (pH 7.4). During permeabilization, the incubation wells were mildly agitated to ensure efficient exchange of cellular contents with the overlying medium. Saponin treatment effectively permeabilized the cells, as judged by the failure of treated cells to exclude trypan blue. Total protein content of cardiomyocytes did not change after permeabilization, indicating that

saponin did not solubilize cells. After 30 min, the medium was aspirated and analyzed for [^{125}I]T_3 activity, which we interpreted to originate from the cytosol and, hence, resulting from the activity of the putative plasma membrane T_3 transport mechanism. After permeabilization, the remaining cellular debris was dissolved in 1 ml 0.1 M NaOH and analyzed for [^{125}I]T_3 activity. We interpreted the latter activity to be mainly associated to the plasma membrane.

To isolate cell nuclei, the cellular debris remaining after treatment with saponin was scraped from the well using a rubber spatula and collected in 1 ml ice-cold phosphate-buffered saline (PBS), pH 7.4 (42.5 mM Na_2HPO_4, 7.5 mM NaH_2PO_4, 150 mM NaCl). The well was rinsed once with 1 ml ice-cold PBS, and both 1-ml fractions were pooled and centrifuged at 300 × *g* for 7 min at 4 °C. The pellet thus obtained was dissolved and thoroughly mixed in 1 ml 0.5% (vol/vol) Triton X-100 in PBS and centrifuged at 900 × *g* for 5 min at 4 °C. This yielded a pellet consisting of nuclei with virtually no contamination by membrane or organelle fragments as judged from protein and DNA measurements. Pellet and supernatant were analyzed separately for [^{125}I]T_3 activity. All results were corrected for the amount of [^{125}I]T_3 activity collected from the wells in the absence of cells.

Protein and DNA measurements

Cellular protein was measured according to the method of Lowry *et al.* (1951). DNA was quantified using a Hoechst fluorescence staining method (Downs & Wilfinger, 1983).

Free hormone fraction measurements

Calculations of free T_3, rT_3 and T_4 concentrations were based on the determinations of the free hormone fraction by equilibrium dialysis (Sterling & Brenner, 1966). In Krebs-Ringer buffer containing 0.5% BSA and 82 pM [^{125}I]T_3, 273 pM [^{125}I]rT_3, or 1.6 nM [^{125}I]T_4, the free hormone fractions were 2.39 ± 0.2% (n = 6), 1.46 ± 0.02% (n = 3), and 0.58 ± 0.04% (n = 3), respectively (mean ± SD). Furthermore, the free [^{125}I]T_3 fractions in the presence of 1, 10 or 100 µM unlabeled T_3 were 2.54 ± 0.4%, 2.92 ± 0.5%, and 3.90 ± 0.4% (n = 3) respectively (mean ± SD). From the latter data a calibration curve was constructed to calculate unknown free [^{125}I]T_3 fractions by interpolation.

Statistics and calculations

A single experiment consisted of duplicate or triplicate observations. Data are presented as means ± SEM, unless stated otherwise. Statistical significance was evaluated by repeated measures ANOVA or Student's *t*-test, where appropriate. Statistical significance was accepted at $P < 0.05$. Transport data were analyzed using a non-linear regression data analysis program.

2.4 Results

[^{125}I]T_3 uptake and Na^+-dependency

Figure 1 shows the time-course of [^{125}I]T_3 uptake. Hormone uptake leveled off after 30 min. No significant difference was found between uptake of [^{125}I]T_3 in the presence of 139 mM Na^+ or an equimolar concentration choline in the incubation medium, indicating that T_3 uptake was not Na^+-dependent. This is corroborated by the observation that preincubation and incubation of cardiomyocytes with 1 mM ouabain, an inhibitor of the plasma membrane sodium pump, did not affect the uptake of T_3 (data not shown).

Effects of temperature on iodothyronine uptake

Figure 2 shows the temperature sensitivity of iodothyronine uptake by neonatal cardiomyocytes measured at 5-min incubation time. Cellular [^{125}I]T_3 uptake values, expressed as a percentage of the dose, increased 3.6- to 3.8-fold from 0 to 20 °C, and 2.2- to 2.8-fold from 20 to 37 °C ($P < 0.001$). Uptake of [^{125}I]rT_3 at 20 °C was increased compared with 0 °C; however, uptake did not rise significantly upon increasing the temperature from 20 to 37 °C (Fig. 2A).

Figure 2B shows that uptake of [^{125}I]T_4, expressed as a percentage of the dose, rose 2.8- and 2.1-fold upon increases in incubation temperature from 0 to 20 °C and from 20 to 37 °C, respectively, the only statistically significant increase being from 0 to 37 °C. Compared with [^{125}I]T_4, uptake of [^{125}I]T_3 at 37 °C was 2.3-fold higher.

Calculations of free T_3, rT_3 and T_4 concentrations were based on determinations of the free fractions by equilibrium dialysis performed at 37 °C. Uptakes at 37 °C of T_3, T_4 and rT_3 expressed in fmol/pM free hormone were 0.36 ± 0.12 (n=7), 0.70 ± 0.28 (n=4), and 0.21 ± 0.03 (n=3) respectively.

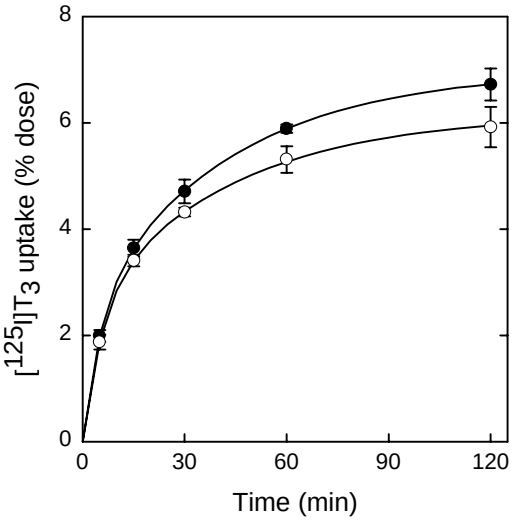

Figure 1 Time-course of $[^{125}I]T_3$ uptake in neonatal cardiomyocytes. Cardiomyocytes from 3-day-old rats were cultured at a density of 1×10^6 cells/well for 5 days in DMEM/M199 supplemented with 5% FCS-5% HS. The uptake of $[^{125}I]T_3$ (4×10^5 cpm/ml; 82 pM) was measured in Krebs-Ringer buffer with Na^+ (●) or in Krebs-Ringer buffer in which Na^+ was replaced by choline (O), both supplemented with 0.5% BSA. Incubations were performed at 37 °C. Mean ± SEM of three experiments are shown.

Effects of thyroid hormone analogues

Fifteen-minute uptake of $[^{125}I]T_3$ (total concentration: 82 pM) was significantly inhibited by 10 µM unlabeled T_3 (36%, $P < 0.05$, n = 5) and 10 µM 3,3'-T_2 (30%, $P < 0.05$, n = 4), while the effects of 3,5-T_2 (20%, n = 4) and T_4 (22%, n = 4) were not significant (data not shown).

Effects of inhibitors on $[^{125}I]T_3$ uptake

To test the energy dependence of $[^{125}I]T_3$ uptake and the possible involvement of endocytosis, cardiomyocytes were incubated for 5 or 15 min with either 10 or 50 µM oligomycin, or 100 µM MDC. Oligomycin at a concentration of 50 µM significantly

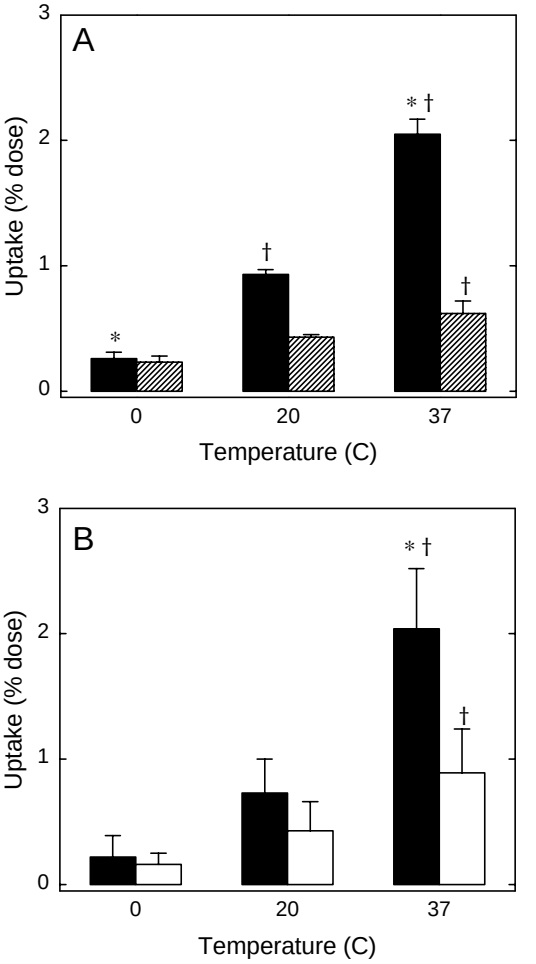

Figure 2 Effect of temperature in neonatal rat cardiomyocytes measured at 5-min incubation time of (A) [^{125}I]T_3 (solid bars) and [^{125}I]rT_3 (hatched bars) (n = 3) and (B) [^{125}I]T_3 (solid bars) and [^{125}I]T_4 (open bars) (n = 4). Uptake values are expressed as a percentage of the dose. † $P < 0.05$ vs 0 °C; * $P < 0.05$ vs 20 °C.

inhibited [^{125}I]T_3 uptake by 44% ($P < 0.05$) at 5 min and by 42 - 49% ($P < 0.001$) at 15 min (Fig. 3 and Table 1). MDC, an inhibitor of receptor-mediated endocytosis, reduced the uptake of [^{125}I]T_3 by 25% at 15 min ($P < 0.001$) (Table 1). To examine whether oligomycin and MDC affected T_3 transport through separate mechanisms, cardiomyocytes were preincubated and incubated for 15 min with 50 µM oligomycin together with 100 µM MDC. This co-incubation resulted in a 47% reduction of T_3 uptake, which was comparable to the effect of oligomycin alone (Table 1).

Participation of other transporters in [^{125}I]T_3 uptake; effects of Trp, Tyr, BCH and BSP

To test the possible involvement of amino acid transport system T and L in the uptake of [^{125}I]T_3, cardiomyocytes were preincubated and incubated (15 min) with 0.5 or 2 mM Trp, 0.5 or 2 mM Tyr (substrates for system T and L), or 5 mM BCH, a specific

Table 1 Effects of oligomycin, MDC, Trp and combinations of these compounds on 15-min uptake of [^{125}I]T_3. Data show the mean ± SEM of two to four experiments (shown in parentheses).

	[^{125}I]T_3 uptake (% dose)	Effect (%)	*P*
Experimental conditions			
No additions	3.82 ± 0.05 (4)		
+ 50 µM oligomycin	1.93 ± 0.13 (4)	-49	< 0.001
+ 100 µM MDC	2.87 ± 0.11 (4)	-25	< 0.001
+ 2 mM Trp	2.98 ± 0.07 (3)	-22	< 0.001
+ 50 µM oligomycin + 100 µM MDC	2.04 ± 0.17 (3)†	-47	< 0.001
+ 50 µM oligomycin + 2 mM Trp	0.87 ± 0.07 (2)∗	-77	< 0.001

The cardiomyocytes were preincubated (30 min) and incubated (15 min) in absence or presence of oligomycin, MDC, Trp, oligomycin with MDC or oligomycin with Trp. The inhibitory effect of these compounds on [^{125}I]T_3 uptake is compared with no additions and expressed as %. Statistical differences between additions and no additions are indicated. †$P < 0.001$, oligomycin + MDC *vs.* MDC alone; ∗$P < 0.001$, oligomycin + Trp *vs.* oligomycin or Trp alone.

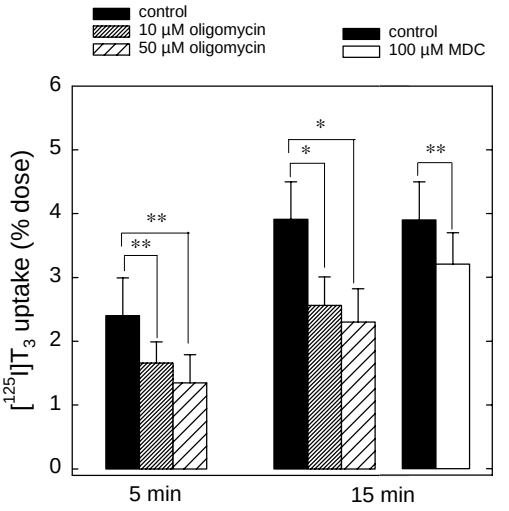

Figure 3 Effects of 10 and 50 µM oligomycin on 5-min (n = 3), and on 15-min [^{125}I]T_3 uptake (n = 4). Oligomycin was present during the preincubation (30 min) and incubation. * $P < 0.05$ *vs*. control; ** $P < 0.01$ *vs*. control.

system L inhibitor (Broer *et al.*, 1998) (Fig. 4). Trp and Tyr showed a dose-dependent inhibition of [^{125}I]T_3 uptake (20% and 12% maximal inhibition), of which the effect of Trp was significant (0.5 mM: $P < 0.05$; 2 mM: $P < 0.001$). Incubation with BCH showed no reduction of 15-min [^{125}I]T_3 uptake (Fig. 4) nor of 60-min [^{125}I]T_3 uptake (data not shown). The combined effect of 50 µM oligomycin and 2 mM Trp was tested in two experiments and showed a larger and additive reduction of [^{125}I]T_3-uptake (77%, $P < 0.001$) compared with the effects of the two compounds alone (49% and 22%, respectively) (Table 1). In addition, possible participation of an organic anion transport system in the uptake of [^{125}I]T_3 was examined in two independent experiments by preincubation and incubation for 5 min with 10 or 25 µM BSP. BSP is transported by members of the family of organic anion transporters (OATP family) (Wolkoff 1996). This gave the following values as percentage of the dose: controls, 2.36 ± 0.16 (n=8); 10 µM BSP, 2.51 ± 0.18 (n=4); 25 µM BSP, 2.22 ± 0.30 (n=4), showing that BSP does not inhibit T_3 uptake.

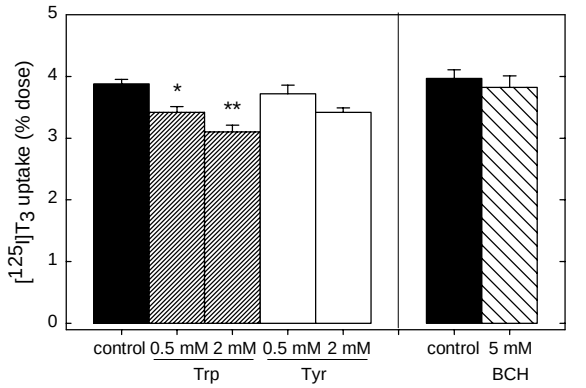

Figure 4 Effect of 0.5 and 2 mM tryptophan (Trp), 0.5 and 2 mM tyrosine (Tyr), and 5 mM BCH on 15-min uptake of [^{125}I]T_3. Substrates were present during preincubation and incubation. Data show mean ± SEM of two to four experiments. * $P < 0.05$ *vs.* control; ** $P < 0.001$ *vs.* control.

Substrate dependent unidirectional uptake

From Fig. 1 we chose a time-point of 5 min to measure unidirectional T_3 uptake rates. Figure 5A shows that unidirectional uptake rate of T_3, although not adequately described by a straight line, does not saturate over a total T_3 concentration ranging from 10 nM to 100 µM. When T_3 uptake data were analyzed as a function of the free T_3 concentration, the curve describing cellular uptake deviated notably from a straight line (Fig. 5B). Although uptake data appeared to converge on a rectangular hyperbola, the curve could not be adequately described by simple saturation kinetics.

Compartmentalization of T_3

Our observation of the apparent non-saturability of cellular T_3 uptake rates prompted us to investigate the passive incorporation of the lipophilic triiodothyronine in the plasma membrane as a possible confounding factor in our uptake assays. After an incubation period of 15 min and following detergent treatment of the cardiomyocytes, 35% of the total [^{125}I]T_3 activity in the cells was retrieved in the cytosolic fraction.

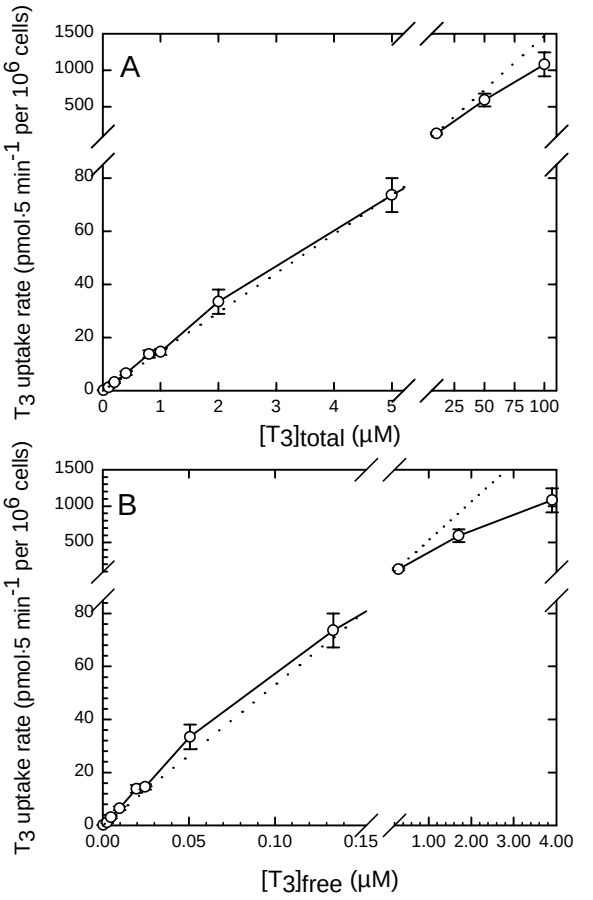

Figure 5 Unidirectional T_3 uptake rates as a function of (A) the total T_3 concentration, and (B) the calculated free T_3 concentration. The dotted line shows the result of a non-linear regression analysis of the data points to the linear function y = ax + b, indicating the deviation of the data from a straight line. Data points represent the mean ± SEM of three to seven experiments. Please note the different abscissa ranges in panels A and B.

The plasma membrane fraction contained 62% of the total [^{125}I]T_3 activity, and 3% was incorporated in the nuclei. After an incubation period of 120 min, total cellular [^{125}I]T_3 activity was increased 2.2-fold compared to 15 min. Now, 50% of the total [^{125}I]T_3 activity was associated with the membrane, and 47% was recovered from the cytosolic fraction, while 3% was incorporated into the nuclei (Table 2). In one experiment we determined the recovery of DNA in the nuclear fraction. As the DNA content of intact cells amounted to 6.8 ± 0.6 µg DNA/0.5 × 10^6 cells (n=3), DNA content of the isolated nuclear fractions after 15 and 120 min amounted to 4.9 ± 0.3 µg DNA/0.5 × 10^6 cells (n=3) (72% recovery) and to 5.0 ± 0.1 µg DNA/0.5 × 10^6 cells (n=3) (74% recovery) respectively.

Table 2 Compartmentalization of T_3 in membrane, cytosol and nucleus of cardiomyocytes. Cardiomyocytes were incubated and treated with saponin as described in Materials and Methods. Data show the mean ± SD of two (15-min uptake) to four (120-min uptake) different preparations.

	[^{125}I]T_3 uptake (% dose)	% of whole cellular uptake
Fraction		
15 min		
Membrane	1.4 ± 0.03	62 ± 0.1
Cytosol	0.8 ± 0.02	35 ± 0.1
Nucleus	0.06 ± 0.001	3 ± 0.1
Total	2.2 ± 0.06	100
120 min		
Membrane	2.5 ± 0.5	50 ± 6
Cytosol	2.3 ± 0.2	47 ± 6
Nucleus	0.17 ± 0.03	3 ± 0.4
Total	5.0 ± 0.43	100

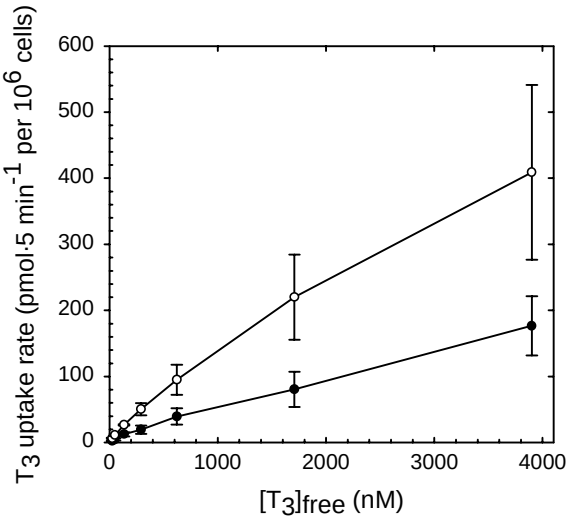

Figure 6 Unidirectional T_3 uptake rates in the membrane-bound (O) and cytosolic (●) fractions of cultured neonatal cardiomyocytes as a function of the free T_3 concentration. Data points represent the mean ± SEM of three experiments.

We again determined the unidirectional T_3 uptake rate as a function of the free T_3 concentration, but now measuring the [^{125}I]T_3 activity released from the cytosol by detergent treatment and the [^{125}I]T_3 activity remaining in the plasma membrane after permeabilization. Figure 6 shows that the uptake rates measured in the membrane-bound and cytosolic fraction did not saturate over a free T_3 concentration ranging from 25 nM to 3.9 µM.

2.5 Discussion

The main outcome of this study was that the uptake of T_3 in neonatal rat cardiomyocytes (cultured with serum) is temperature dependent, MDC, oligomycin and Trp sensitive, but not dependent on the Na^+ gradient. Apart from the Na^+ dependency (see below),

this confirms our observations in cardiomyocytes cultured in absence of serum (Everts *et al.*, 1996b). The present study also included examination of the cellular compartmentalization of T_3 showing that, although a high amount of T_3 is associated with the plasma membrane, T_3 is transported into the cytosol and to the nucleus. Finally, T_3 uptake did not saturate at very high free hormone concentrations.

In our first study on thyroid hormone uptake in cardiomyocytes (Everts *et al.*, 1996b), T_3 uptake was found partly to be dependent on the Na^+ gradient as judged from the inhibitory effects (20-30%) of 10 µM monensin. In the present study where Na^+ dependency was judged from incubations with choline buffer, no such Na^+ dependency could be demonstrated. A similar discrepancy was previously observed when [^{125}I]T_4 uptake by cultured anterior pituitary cells was examined (Everts *et al.*, 1994). While [^{125}I]T_4 uptake was reduced by 40% in presence of 10 µM monensin, it was reduced by only 20% in choline containing buffer (Everts *et al.*, 1994). Probably, by inducing the Na^+ influx, monensin causes a more pronounced rundown of the Na^+ gradient across the plasma membrane, as compared to replacing the Na^+ by choline.

The present results indicate that neonatal rat cardiomyocytes possess a specific T_3 uptake system, as evidenced by the fact that T_3 itself is the most potent inhibitor of [^{125}I]T_3 uptake and by the difference in temperature sensitivities between the uptake of T_3 and its analogue rT_3. A plasma membrane receptor or an integral membrane carrier protein would confer substrate specificity to the T_3 transport mechanism but, to date, no such protein has been identified. It has been found, however, that a monoclonal antibody directed against a M_r 52 000 membrane protein inhibited T_3 and T_4 uptake in rat liver cells (Mol *et al.*, 1986). This coincides remarkably with the observation that two M_r 53 000 and 55 000 membrane-associated proteins were sensitive to *N*-bromoacetyl-[^{125}I]T_3 affinity labeling in cultured mouse fibroblasts (Cheng, 1983b), a cell type shown to take up T_3 by receptor-mediated endocytosis (Cheng, 1983a). It remains to be investigated whether these observations are the manifestations of the putative T_3 transporter/receptor.

In whole cell uptake experiments, a large amount of T_3 activity was associated to the plasma membrane. Similar findings were reported by others (Yusta *et al.*, 1988; Chehín *et al.*, 1999), and this is in line with the known lipophilicity of native iodothyronines (Pontecorvi & Robbins, 1989). The presence of this (non)-specific binding of substrate to the plasma membrane could explain why the time-course of T_3

uptake could not be adequately described by a single exponential. These results would imply that passive partitioning of the substrate in the lipid bilayer of the plasma membrane masks the uptake performed by a specific transport mechanism, thus confounding the results of whole cell uptake studies. In addition, several groups described presence of receptors at the plasma membrane which initiate non-genomic effects *e.g.* modulation of Na^+ channels (Craelius *et al.*, 1990; Davis & Davis, 1993). Association of T_3 with these receptors presumably excludes uptake of the hormone. This warrants the use of a method which distinguishes membrane associated (non)-specific activity from that translocated across the plasma membrane by a transport system. We used a detergent treatment to determine this non-specific activity, resulting in values comparable to those reported by others (Cheng, 1983a; Pontecorvi & Robbins, 1989). It is noteworthy to remark that the magnitude of the unlabeled T_3-inhibitable component in whole cell uptake (*i.e.* 36%, see results) is comparable to the fraction of T_3 activity released from cardiomyocytes by detergent treatment (*i.e.* 35%, see Table 2), both measured at 15 min. Measurements after 2 h of incubation showed similar values (37%) for reduction of whole cell uptake by unlabeled T_3 (Verhoeven *et al.*, 2001). However, the large effect (80%) on nuclear T_3 binding after this incubation period (Verhoeven *et al.*, 2001) and the increase of T_3 activity in the cytosol (this report) indicate that T_3 uptake is regulated by a specific mechanism at the plasma membrane.

Data on plasma thyroid hormone, especially rT_3, concentrations in neonatal rat are scant. From data in the literature we estimated values of 2-7 pM free T_3, 2-17 pM free T_4 and 1 pM free rT_3 respectively (Rutgers *et al.*, 1987; Rondeel *et al.*, 1988; Aláez *et al.*, 1992; Schröder-van der Elst & van der Heide, 1992), and we chose the composition of our incubation media to approximate to the free plasma hormone concentrations in neonatal rat. The cardiomyocytes were exposed to 2 pM free T_3, 10 pM free T_4, or 4 pM rT_3. This allowed us to compare thyroid hormone uptake in cardiomyocytes under physiological conditions. T_3 uptake at 37 °C, expressed as percentage of dose, was higher than the uptake of T_4 and rT_3 (around two- and fourfold, respectively). These results indicate that, under physiological conditions *in-vitro*, cardiomyocytes take up thyroid hormone with a distinct preference for T_3 over T_4 and rT_3. Taking into account the differences in experimental conditions (*e.g.* different BSA and total hormone concentrations, seeded cell densities), the present uptake

values for T_4 and T_3 correspond well with those reported in our previous study (Everts *et al.*, 1996b).

The inhibition of T_3 uptake by MDC hints at the involvement of receptor-mediated endocytosis in the transmembrane transport of the hormone in cardiomyocytes. This is corroborated by results from other studies presenting evidence for a physiological role for endocytosis in the uptake of substrates in rat cardiomyocytes (Iida & Shibata, 1989; Page *et al.*, 1994). Not only in our cardiomyocyte preparation but also in other cell types, MDC was found to inhibit the uptake of T_3 (Goncalves *et al.*, 1989; Pontecorvi & Robbins, 1989; Everts *et al.*, 1993; McLeese & Eales, 1996), suggesting that receptor-mediated endocytosis participates in cellular T_3 uptake. The inhibitory effects of MDC and oligomycin on T_3 uptake were not additive. Oligomycin, affects the energy status of the cardiomyocyte by reducing its ATP content. Receptor-mediated endocytosis is an energy-dependent process (Barouch *et al.*, 1994), and this would explain the inhibition by oligomycin. Further examination of the involvement of this process is the subject of current studies.

Neither in the presence nor in the absence of Na^+ could the uptake of T_3 be adequately described by a single exponential, indicating the involvement of more than one component in T_3 uptake by cardiomyocytes. Interestingly, the effects of co-incubation with oligomycin and Trp on T_3 uptake were additive. It has been shown previously that members of the organic anion transporter family (rat Oatp1 – 3, human OATP-C or liver-specific organic anion transporter (LST-1)) transport thyroid hormones in a Na^+-independent manner (Abe *et al.*, 1996; Friesema *et al.*, 1999). Expression profiles show that some members of the OATP family are localized in heart (Tamai *et al.*, 2000). Furthermore, Abe *et al.* (1996) showed that BSP, which is an organic anion, inhibits OATP-mediated uptake of thyroid hormones. T_3 uptake in neonatal cardiomyocytes was not affected by BSP, indicating that this type of transporters is not involved in uptake of thyroid hormones in rat heart. Other likely candidates for a putative accessory transport system could be members of amino acid transport systems T or L. Both system T and L are Na^+ independent, and both systems transport aromatic amino acids (Kragie, 1994; Palacín *et al.*, 1998). It has been shown that the aromatic amino acids Tyr and Trp, which can be transported by both system L and T, inhibit the uptake of T_3 in neonatal cardiomyocytes and transfected *Xenopus* oocytes (Everts *et al.*, 1996b; Ritchie *et al.*, 1999). Only system L is sensitive to the

synthetic amino acid analogue BCH (Broer *et al.*, 1998; Ritchie *et al.*, 1999). Our results show that uptake of T_3 was not sensitive to BCH, from which we conclude that system L does not participate in uptake of T_3 in neonatal rat cardiomyocytes. However, based on the inhibition of T_3 uptake by Trp and Tyr shown in this report and the previous study in neonatal rat cardiomyocytes (Everts *et al.*, 1996b) we cannot exclude system T as a candidate for a putative accessory transport system., We therefore plan to explore the participation of system T in uptake of thyroid hormones in cardiomyocytes further.

In summary, the results of our study on neonatal cardiomyocytes indicate the presence of an energy- and temperature-dependent uptake mechanism for T_3 that is not dependent on the transmembrane Na^+ gradient. Based on the inhibitory effect of MDC, an endocytosis-mediated uptake mechanism cannot be excluded. Our data also suggest the presence an additional uptake system, *e.g.* amino acid transport system T. We are currently deploying an expression-cloning strategy to further characterize the T_3 uptake mechanism in neonatal rat cardiomyocytes.

2.6 Acknowledgements

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2.7 References

1. Abe T, Kakyo M, Sakagamii H, Tokui T, Nishio T, Tanemoto M, Nomura H, Hebert SC, Matsuno S, Kondo H & Yawo H 1996 Molecular characterization and tissue distribution of a new organic anion transporter subtype (oatp3) that transports thyroid hormones and taurocholate and comparison with oatp2. *J Biol Chem* **273**:22395-22401
2. Aláez C, Calvo R, Obregon MJ & Pascual-Leone AM 1992 Thyroid hormones and 5'-deiodinase activity in neonatal undernourished rats. *Endocrinology* **130**:773-779
3. Barouch W, Prasad K, Greene LE & Eisenberg E 1994 ATPase activity associated

with the uncoating of clathrin baskets by Hsp70. *J Biol Chem* **269**:28563-28568

4. Blondel B, Roijen I & Cheneval JP 1971 Heart cells in culture: a simple method for increasing the proportion of myoblasts. *Experientia* **27**:356-358
5. Broer A, Hamprecht B & Broer S 1998 Discrimination of two amino acid transport activities in 4F2 heavy chain-expressing *Xenopus laevis* oocytes. *Biochem J* **333**:549-554
6. Chehín RN, Issé BG, Rintoul MR & Farías RN 1999 Differential transmembrane diffusion of triiodothyronine and thyroxine in liposomes: Regulation by lipid composition. *J Membrane Biol* **167**:251-256
7. Cheng SY 1983a Characterization of binding and uptake of 3,3',5-L-triiodothyronine in cultured mouse fibroblasts. *Endocrinology* **112**:1754-1762
8. Cheng SY 1983b Structural similarities in the plasmamembrane 3,3',5-triiodo-L-thyronine receptors from human, rat and mouse cultured cells. Analysis by affinity labeling. *Endocrinology* **113**:1155-1157
9. Craelius W, Green WL & Harris DR 1990 Acute effects of thyroid hormone on sodium currents in neonatal myocytes. *Biosci Rep* **10**:309-315
10. Davis PJ & Davis FB 1993 Acute cellular actions of thyroid hormone and myocardial function. *Ann Thorac Surg* **56**:S16-S23
11. Dillmann WH 1990 Biochemical basis of thyroid hormone action in the heart. *Am J Med* **88**:626-630
12. Docter R, Krenning EP, Bernard HF & Hennemann G 1987 Active transport of iodothyronines into human cultured fibroblasts. *J Clin Endocrinol Metab* **65**:104-107
13. Docter R & Krenning EP 1990 Role of cellular transport systems in the regulation of thyroid hormone bioactivity. In: Greer MA (ed) The Thyroid Gland. Raven Press, New York, pp. 233-254
14. Downs TR & Wilfinger WW 1983 Fluorometric quantification of DNA in cells and tissues. *Anal Biochem* **131**:538-547
15. Everts ME, Docter R, van Buuren JCJ, van Koetsveld PM, Hofland LJ, de Jong M, Krenning EP & Hennemann G 1993 Evidence for carrier-mediated uptake of triiodothyronine in cultured anterior pituitary cells of euthyroid rats. *Endocrinology* **132**:1278-1285
16. Everts ME, Docter R, Moerings EPCM, van Koetsveld PM, Visser TJ, de Jong M, Krenning EP & Hennemann G 1994 Uptake of thyroxine in cultured anterior pituitary cells of euthyroid rats. *Endocrinology* **134**:2490-2497
17. Everts ME, de Jong M, Lim C, Docter R, Krenning EP, Visser TJ & Hennemann G 1996a Different regulation of thyroid hormone transport in liver and pituitary: its

possible role in the maintenance of low T_3 production during nonthyroidal illness and fasting in man. *Thyroid* **6**:357-366

18. Everts ME, Verhoeven FA, Bezstarosti K, Moerings EPCM, Hennemann G, Visser TJ & Lamers JMJ 1996b Uptake of thyroid hormones in neonatal rat cardiac myocytes. *Endocrinology* **137**:4235-4242
19. Friesema ECH, Docter R, Moerings EPCM, Stieger B, Hagenbuch B, Meier PJ, Krenning EP, Hennemann G & Visser TJ 1999 Identification of thyroid hormone transporters. *Biochem Biophys Res Comm* **254**:497-501
20. Gloss B, Sayen MR, Trost SU, Bluhm WF, Meyer M, Swanson EA, Usala SJ & Dillmann WH 1999 Altered cardiac phenotype in transgenic mice carrying the delta337 threonine thyroid hormone receptor β mutant derived from the S family. *Endocrinology* **140**:897-902
21. Goncalves E, Lakshmanan M & Robbins J 1989 Triiodothyronine transport into differentiated and undifferentiated mouse neuroblastoma cells (NB41A3). *Endocrinology* **124**:293-300
22. Hennemann G, Vos RA, de Jong M, Krenning EP & Docter R 1993 Decreased peripheral 3,5,3'-triiodothyronine (T_3) production from thyroxine (T_4): a syndrome of impaired thyroid hormone activation due to transport inhibition of T_4- into T_3-producing tissues. *J Clin Endocrinol Metab* **77**:1431-1435
23. Hennemann G, Everts ME, de Jong M, Lim C, Krenning EP & Docter R 1998 The significance of plasma membrane transport in the bioavailability of thyroid hormone. *Clin Endocrinol* **48**:1-8
24. van Heugten HAA, Bezstarosti K & Lamers JMJ 1994 Endothelin-1 and phenylephrine-induced activation of the phosphoinositide cycle increases cell injury of cultured cardiomyocytes exposed to hypoxia/reoxygenation. *J Mol Cell Cardiol* **26**:1513-1524
25. Iida H & Shibata Y 1989 Delivery of lectin-labeled membrane to the trans-Golgi network and secretory granules in cultured atrial myocytes. *J Histochem Cytochem* **37**:1885-1892
26. Kragie L 1994 Membrane iodothyronine transporters. Part I: Review of physiology. *Endocr Res* **40**:319-341
27. Lai CS, Korytowski W, Niu CH & Cheng SY 1985 Transverse motion of spin-labeled 3,3',5-triiodo-L-thyronine in phospholipid bilayers. *Biochem Biophys Res Comm* **131**:408-412
28. Lein A & Dowben RM 1961 Uptake and binding of thyroxine and triiodothyronine by rat diaphragm *in vitro*. *Am J Physiol* **200**:1029-1031
29. Lowry OH, Rosebrough NJ, Farr AL & Randall RJ 1951 Protein measurement with

the Folin phenol reagent. *J Biol Chem* **193**:265-275

30. McLeese JM & Eales JG 1996 Characteristics of the uptake of 3,5,3'-triiodo-L-thyronine and L-thyroxine into red blood cells of rainbow trout (*Oncorhynchus mykiss*). *Gen Comp Endocrinol* **103**:200-208
31. Mitchell AM, Rowan KA, Manley SW & Mortimer RH 1999 Comparison of mechanisms mediating uptake and efflux of thyroid hormones in the human choriocarcinoma cell line, JAR. *J Endocrinol* **161**:107-113
32. Mol JA, Krenning EP, Docter R, Rozing J & Hennemann G 1986 Inhibition of iodothyronine transport into rat liver cells by a monoclonal antibody. *J Biol Chem* **261**:7640-7643
33. Oppenheimer JH & Schwartz HL 1985 Stereospecific transport of triiodothyronine from plasma to cytosol and from cytosol to nucleus in rat liver, kidney, brain, and heart. *J Clin Invest* **75**:147-154
34. Page E, Goings GE, Upshaw-Earley J & Hanck DA 1994 Endocytosis and uptake of lucifer yellow by cultured atrial myocytes and isolated intact atria from adult rats. *Circ Res* **75**:335-346
35. Palacín M, Estévez R, Bertran J & Zorzano A 1998 Molecular biology of mammalian plasma membrane amino acid transporters. *Physiol Rev* **78**:969-1054
36. Pardridge WM & Mietus LJ 1980 Influx of thyroid hormones into rat liver *in vivo*. Differential availability of thyroxine and triiodothyronine bound by plasma proteins. *J Clin Invest* **66**:367-374
37. Pontecorvi A & Robbins J 1989 The plasma membrane and thyroid hormone entry into cells. *Trends Endocrinol Med* **1**:90-94
38. Ritchie JW, Peter GJ, Shi YB & Taylor PM 1999 Thyroid hormone transport by 4F2hc-IU12 heterodimers expressed in *Xenopus* oocytes. *J Endocrinol* **163**:R5-R9
39. Rohrer DK, Hartong R & Dillman WH 1991 Influence of thyroid hormone and retinoic acid on slow sarcoplasmic reticulum Ca^{2+}-ATPase and myosin heavy chain α gene expression in cardiac myocytes. *J Biol Chem* **266**:8638-8646
40. Rondeel JM, de Greef WJ, van der Schoot P, Karels B, Klootwijk W & Visser TJ 1988 Effect of thyroid status and paraventricular area lesions on the release of thyrotropin-releasing hormone and catecholamines into hypophysial portal blood. *Endocrinology* **123**:523-527
41. Rutgers M, Bonthuis F, de Herder WW & Visser TJ 1987 Accumulation of plasma triiodothyronine sulfate in rats treated with propylthiouracil. *J Clin Invest* **80**:758-762
42. Schröder-van der Elst JP & van der Heide D 1992 Effects of streptozocin-induced diabetes and food restriction on quantities and source of T_4 and T_3 in rat tissues.

Diabetes **41**:147-152

43. Sterling K & Brenner MA 1966 Free thyroxine in human serum: simplified measurement with the aid of magnesium precipitation. *J Clin Invest* **45**:153-163
44. Tamai I, Nezu J, Uchino H, Sai Y, Oku A, Shimane M & Tsuji A 2000 Molecular identification and characterization of novel members of the human organic anion transporter (OATP) family. *Biochem Biophys Res Comm* **273**:251-260
45. Verhoeven FA, Moerings EPCM, Lamers JMJ, Hennemann G, Visser TJ & Everts ME 2001 Inhibitory effects of calcium blockers on thyroid hormone uptake in neonatal rat cardiac myocytes. *Am J Physiol* **21**:H1985-H1991
46. Yusta B, Ortiz-Caro J, Pascual A & Aranda A 1988 Mechanism of L-triiodothyronine (T_3) uptake by glial C6 cells: regulation by butyrate. *Mol Cell Endocrinol* **58**:191-198

CHAPTER 3

[^{125}I]T_3 and [^{125}I]Triac uptake in cardiomyocytes

Uptake of Triiodothyronine and Triiodothyroacetic acid in Neonatal Rat Cardiomyocytes: Effects of Metabolites and Analogs

F. A.Verhoeven, H.H.A.G.M. van der Putten, G. Hennemann, J.M.J. Lamers, T.J. Visser and M.E. Everts.

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3.1 Abstract

Cellular and nuclear uptake of [^{125}I]triiodothyronine (T_3) and [^{125}I]triiodothyroacetic acid (Triac) were compared in cardiomyocytes of 2-3 day old rats, and the effect of thyroid hormone analogues on cellular T_3 uptake was measured. Cells (5-10 $\times$ 10^5 per well) were cultured in DMEM-M199 with 5% horse serum and 5% FCS. Incubations were performed for 15 min to 24 h at 37 ^{o}C in the same medium, 0.5% BSA and [^{125}I]T_3 (100 pM), or [^{125}I]Triac (240 pM). Expressed as % dose, T_3 uptake was five times Triac uptake, but expressed as fmol/pM free hormone, Triac uptake was at least 30% (P<0.001) higher than T_3 uptake, while the relative nuclear binding of the two tracers was comparable. The 15 min uptake of [^{125}I]T_3 was competitively inhibited by 10 µM unlabeled T_3 (45-52%; P<0.001) or 3,3´-T_2 (52%; P<0.001), and to a smaller extent by T_4 (27%; 0.05<P<0.1). In contrast, 10 µM 3,5-T_2 , Triac, or tetraiodothyroacetic acid (Tetrac) did not affect T_3 uptake after 15 min or after 24 h. Diiodothyropropionic acid (DITPA) (10 µM) reduced 15-min T_3 uptake by around 24% (P<0.05), but it had a greater effect after 4 h (56%, P<0.001). Exposure to 10 nM DITPA during culture reduced cellular T_3 uptake, as did 10 nM T_3, suggesting down-regulation of the plasma membrane T_3 transporters. We conclude that i) Triac is taken up by cardiomyocytes; ii) 3,3´-T_2, and, to a lesser extent, DITPA and T_4 interfere with plasma membrane transport of T_3, whereas 3,5-T_2, Triac, or Tetrac do not; iii) the transport mechanism for Triac is probably different from that for T_3.

Key words: neonatal rat, cardiomyocytes, culture, 3,3',5-triiodothyronine uptake, 3,3',5-triiodothyroacetic acid uptake, free hormone fraction, thyroid hormone metabolites and analogues

3.2 Introduction

Thyroid hormone induces an increase in cardiac contractility and frequency, resulting in a greater cardiac output (Polikar *et al.*, 1993; Toft & Boon, 2000; Klein & Ojamaa, 2001), and a proportional change in energy turnover (Clausen *et al.*, 1991). Thyroid hormone also induces relaxation of vascular smooth muscle (Ojamaa *et al.*, 1996), and it is very effective in reducing serum cholesterol (Staels *et al.*, 1990). Recently, the concept has been discussed that initiation of thyroxine (T_4) replacement therapy may precipitate angina pectoris (Gammage & Franklyn, 1997), whereas increased serum triiodothyronine (T_3) at the time of admission to hospital is associated with a greater risk for the development of myocardial ischemia (Peters *et al.*, 2000). This underscores the importance of developing thyromimetic compounds with lipid-decreasing activity, without cardiovascular and thermogenic effects (Boyd & Oliver, 1960; Underwood *et al.*, 1986; Stephan *et al.*, 1996; Ichikawa *et al.*, 2000; Trost *et al.*, 2000).

Patients undergoing coronary bypass surgery (Holland *et al.*, 1991; Klemperer *et al.*, 1995) and patients with congestive heart failure (Hamilton, 1993; Hamilton & Stevenson, 1996) may show typical changes in serum thyroid hormone parameters — low serum T_3, high serum reverse T_3 and normal T_4 and thyrotropin (TSH) levels — known as the euthyroid sick syndrome (Docter *et al.*, 1993). Whether or not this condition should be treated remains a matter of debate (Klemperer *et al.*, 1995; Utiger, 1995; Camacho & Dwarkanathan, 1999). However, during the past 5-10 years clinical studies have explored the possibility of improving heart function using treatment with T_3 or T_4 (Moruzzi *et al.*, 1996; Chowdhury *et al.*, 1999; Mullis-Jansson *et al.*, 1999) both in children with congenital heart disease and in elderly patients with congestive heart failure. To date, the thyroid hormone analogue diiodothyropropionic acid (DITPA), which possibly has a greater effect on cardiac contractility than on frequency has been applied in animal studies (Pennock *et al.*, 1993; Morkin *et al.*, 1996; Spooner *et al.*, 1999).

With the purpose of suppressing thyrotropin (TSH) secretion in patients with thyroid cancer (Pujol *et al.*, 2000), triiodothyroacetic acid (Triac) has been tested for specificity at the pituitary and the peripheral level (Beck-Peccoz *et al.*, 1988; Sherman & Ladenson, 1992; Everts *et al.*, 1994; Sherman *et al.*, 1997). In addition,

tetraiodothyroacetic acid (Tetrac) was more potent than T_4 in reducing TSH release in isolated pituitary cells (Everts *et al.*, 1995) and in hypothyroid rats (Lameloise *et al.*, 2001), but it was less efficient in inducing cardiac hypertrophy (Lameloise *et al.*, 2001). Recent work on mitochondria indicates that T_3 metabolites such as 3,5-T_2 may have thermogenic effects (Goglia *et al.*, 1999). Finally, several studies have demonstrated acute effects of T_3 (Davis & Davis, 1993) and 3,5-T_2 (Huang *et al.*, 1999) on the heart, in particular on ion channels.

In view of the significance of plasma membrane transport for the bioavailability of thyroid hormones (Hennemann *et al.*, 1998, 2001), and the possible existence of different transport systems in different tissues (Kragie, 1994; Everts *et al.*, 1996a), we have previously explored the thyroid hormone transport system in heart (Everts *et al.*, 1996b; van der Putten *et al.*, 2001; Verhoeven *et al.*, 2001). In the present study we continued this work by comparing the uptake of [^{125}I]T_3 with that of [^{125}I]Triac in neonatal rat cardiomyocytes. Furthermore, we tested the effects of T_4, the analog DITPA, and the metabolites 3,3'-T_2, 3,5-T_2, Triac, and Tetrac on the uptake of [^{125}I]T_3.

3.3 Materials and methods

Animals

All experiments were performed using 2-3-day-old Wistar rats of both sexes, obtained from laboratory stock. Rats were killed by decapitation, and hearts were quickly dissected and processed as described below, to isolate cardiomyocytes.

Cell culture

Primary cultures of neonatal rat cardiomyocytes were prepared as described in detail previously (Blondel *et al.*, 1971) with some modifications (van Heugten *et al.*, 1994; Everts *et al.*, 1996b; Verhoeven *et al.*, 2001). In brief, hearts were cut for 1 min and dissociated with 0.1% trypsin for 10 min at 33 °C. Cells from the first treatment with trypsin were decanted and discarded, and the remaining tissue was further digested with fresh enzyme and decanted. DMEM with medium 199 (M199) (4:1), 5% FCS, 5% horse serum, and 2% penicillin/streptomycin was then added to the suspended cells. This procedure was repeated seven times until all tissue was dissociated. Deoxyribonuclease (20 U/ml) was added, and the remaining tissue from the last step

together with the trypsinized cells was centrifuged (100 *g*) for 5 min. The supernatant was discarded, and the cells were resuspended in 30 ml DMEM-M199 with 5% FCS, 5% horse serum, and 2% penicillin/streptomycin. The cell suspension was passed through nylon mesh, and the dispersed cells were preplated (Blondel *et al.*, 1971) into 250 ml culture flasks for 60 min (37 °C, 5% CO_2) to remove fibroblasts. After this procedure, the preparation consists of more than 90% cardiomyocytes (van Heugten *et al.*, 1994). Cells of the enriched cardiomyocyte fraction were plated into 48-well culture dishes at subconfluent density (5×10^5 cells/well) in 1-ml volumes of DMEM-M199 with 5% FCS-5% horse serum-2% penicillin/streptomycin, and in some cases at a density of 10^6 cells/well in 2-ml volumes in 24-well culture dishes.

The cells were incubated in a humidified 5% CO_2 atmosphere at 37 °C for 1 day. After 1 day, the cells revealed spontaneous and synchronous beating, and the medium was replaced by the original culture medium. Experiments were routinely performed after 5 days. In a series of experiments, the cultured cells were exposed to 10 nM T_3 or DITPA from the start of culture.

Although culturing cardiomyocytes in the presence of serum may result in proliferation of contaminating fibroblasts that also show thyroid hormone transport (Docter *et al.*, 1987), we have previously shown that uptake of thyroid hormones is comparable in cardiomyocytes cultured for 5 days in absence or presence of serum (Everts *et al.*, 1996b; Verhoeven *et al.*, 2001). Moreover, the uptake of [^{125}I]T_3, expressed per μg protein, is more than 10-fold greater in cardiomyocytes than in cardiac fibroblasts (SM van der Heide, personal communication).

Cellular uptake of [^{125}I]T_3 and [^{125}I]Triac

The incubation medium was identical to the culture medium, except that serum was replaced by 0.5% BSA for measurements of [^{125}I]T_3 or [^{125}I]Triac uptake (Everts *et al.*, 1994, 1996b). Cells were preincubated (0.5 ml) for 30 min and incubated (0.25 ml) for 15 min-24 h at 37 °C without or with 10 μM unlabeled T_3, Triac, Tetrac, 3,3'-T_2, 3,5-T_2, or 1 nM-10 μM DITPA, T_3, T_4 or Triac, and in all cases [^{125}I]T_3 (100,000 cpm; 100 pM) or [^{125}I]Triac (200,000 cpm; 240 pM).

After incubation, the medium was removed and the cells were washed with 1 ml ice-cold saline to remove tracer not bound to the cells. This washing procedure proved to be sufficient (Verhoeven *et al.*, 2001). Cells were dissolved in 1 ml 0.1 N NaOH

and counted for iodine-125 activity in a 16-channel gamma-counter (NE 1600, Nuclear Enterprises, Edinburgh, UK). The amount of [^{125}I]T_3 or [^{125}I]Triac taken up was expressed as a percentage of the added radioactivity (percentage of the dose). The same procedure was applied to incubations without cells (blanks). All results were corrected for the amount of radioactivity recovered from the wells without cells.

Nuclear [^{125}I]T_3 and [^{125}I]Triac binding

Cells were cultured at a density of 10^6 cells/well in 24-well culture dishes as described above. Preincubation (30 min) and incubation (2 h) were performed at 37 °C in a volume of 0.5 ml, and [^{125}I]T_3 (200,000 cpm; 100 pM) or [^{125}I]Triac (500,000 cpm; 300 pM) during incubation. Unlabeled T_3 or Triac (both 10 μM) were present only during incubation. After incubation, cells were washed once with 2 ml ice-cold saline. The cells were scraped from the wells with a rubber policeman in 1 ml PBS (on ice) and the wells were washed with 0.5 ml PBS. These two aliquots were combined and centrifuged (300 *g* at 4 °C for 7 min), the cell pellet counted (30 sec), and solubilized in 1 ml PBS containing 0.5% Triton X-100 (Everts *et al.*, 1996b; Verhoeven *et al.*, 2001). After 2 min of continuous vortexing, nuclei were spun down (900 *g* at 4 °C for 5 min) and washed once with 1 ml PBS containing 0.5% Triton X-100. The nuclear pellets were counted for 5 min and frozen for DNA determination.

Free hormone concentrations

Calculation of the free hormone concentration was based on determination of the free fraction by equilibrium dialysis (Sterling & Brenner, 1966). As shown previously (Everts *et al.*, 1994), the free fractions of T_3 and Triac in medium with 0.5% BSA were 3.45 ± 0.19% (n=8) and 0.41 ± 0.03% (n=5), respectively — a ninefold difference. In the present study, the free T_3 fraction was not changed by addition of 10 μM unlabeled T_3, 3,3'-T_2, 3,5-T_2, Triac or Tetrac, and only slightly by 10 uM T_4 (4.21%; n=2). Upon addition of 0, 1 nM, 10 nM, 100 nM, 1 μM or 10 μM DITPA, the free T_3 fraction also showed a slight increase: from 3.22 ± 0.08% (n=5) to 3.61% (n=2), 3.49% (n=2), 3.45% (n=2), 3.78% (n=2) and 4.36 ± 0.06% (n=3), respectively. The free Triac fraction was 0.38 ± 0.02% (n=6), and did not change with 10 μM unlabeled Triac.

DNA and protein determinations

DNA content was determined using a fluorimetric method (Downs & Wilfinger, 1983). The cellular protein content was determined with the Bio-Rad Protein Assay-Kit (Bio-Rad, München, Germany). Cells plated at a density of 5×10^5 cells/well contained around 0.1 mg protein/well after 5 days of culture.

Chemicals and isotopes

All reagents used for cell isolation and cell culture were obtained from Gibco Europe (Breda, The Netherlands), with the exception of trypsin and deoxyribonuclease (Boehringer, Mannheim, Germany). Culture dishes (48 and 24 wells) were obtained from Costar (Cambridge, MA, USA). All iodothyronines, 3,5-diiodothyroacetic acid (3,5-Diac), Triac, and Tetrac were obtained from Henning Berlin (Berlin, Germany). Bovine serum albumin (BSA) (fraction V) and 3,5-diiodothyropropionic acid (DITPA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). [3'-^{125}I]T_3 (3070 μCi/μg) was purchased from Amersham International (Aylesbury, UK). [3'-^{125}I]Triac (2730 μCi/μg) was prepared from Na^{125}I and 3,5-Diac using the chloramine-T method (Rutgers *et al.*, 1989). Sephadex LH-20 was from Pharmacia (Uppsala, Sweden). All other reagents were of the highest purity available.

Calculations and statistics

On the basis of the free hormone fractions in the incubation buffer with 0.5% BSA, the chemical concentration of T_3 (100 pM) or Triac (240 pM) in the buffer, and the incubation volume, the counts per min (percentage added dose) were converted to fmol/pM free hormone. In case the free hormone fraction was unknown — for example in the cellular pellet and the nuclei (Table 1) —, data were only expressed as percentage of the added radioactivity .

The statistical significance of any of the tested compounds on [^{125}I]T_3 or [^{125}I]Triac uptake was evaluated by Student's t-test or by one-way analysis of variance and Bonferroni's test for multiple comparisons. $P<0.05$ was regarded statistically significant.

3.4 Results

Comparison of [^{125}I]T_3 and [^{125}I]Triac uptake

Time course of cellular uptake Figure 1 shows the time course of [^{125}I]T_3 uptake (O) compared with that of [^{125}I]Triac (●) in cardiomyocytes, expressed as percentage of the dose (Fig. 1A) and as fmol/pM free hormone (Fig. 1B). Both uptakes showed a steep phase up to 1 h of incubation. Between 1 and 4 h of incubation, [^{125}I]Triac uptake reached equilibrium, whereas [^{125}I]T_3 uptake showed a further increase. As can be seen in Fig. 1A, [^{125}I]T_3 uptake was fivefold higher than that of [^{125}I]Triac, when expressed as percentage of the dose. However, when expressed per pM free hormone (Fig. 1B), the uptake of [^{125}I]Triac was 30% greater than that of [^{125}I]T_3.The free fraction of the two hormones in buffer with 0.5% BSA was 3.5% for [^{125}I]T_3 and 0.4% for [^{125}I]Triac — a ninefold difference.

Nuclear binding To assess nuclear binding of T_3 and Triac, cardiomyocytes were preincubated for 30 min, and incubated for 2 h with [^{125}I]T_3 or [^{125}I]Triac in the absence or presence of 10 μM unlabeled T_3 or Triac (Table 1). Again, cellular uptake expressed as percentage of the dose of [^{125}I]T_3 was fivefold greater than that of [^{125}I]Triac. Incubation with 10 μM unlabeled T_3 or Triac inhibited cellular binding of [^{125}I]T_3 and [^{125}I]Triac, but this effect was smaller than the inhibition of nuclear binding: 19% compared with 72% for T_3, and 34% compared with 60% for Triac. The DNA contents of the nuclear pellets in absence or presence of 10 μM of the unlabeled hormone were the same.

Effect of DITPA [^{125}I]T_3 uptake was also measured over a 4-h incubation period in the absence or presence of 10 μM DITPA (Fig. 2). Results are expressed as fmol/pM free T_3. At incubation times 15 min, 1 h, and 4 h, the presence of 10 μM DITPA reduced [^{125}I]T_3 uptake by 28%, 45% and 56% (all n=6; P<0.05), respectively.

Plasma membrane transport of [^{125}]T_3

Effects of T_3, T_4, Triac, DITPA, Tetrac 3,3'-T_2, and 3,5-T_2 Figure 3 shows the competitive effects of increasing concentrations of unlabeled T_3, T_4, Triac and DITPA on the 15-min uptake of [^{125}I]T_3, expressed as fmol/pM free T_3. T_3 itself resulted in a

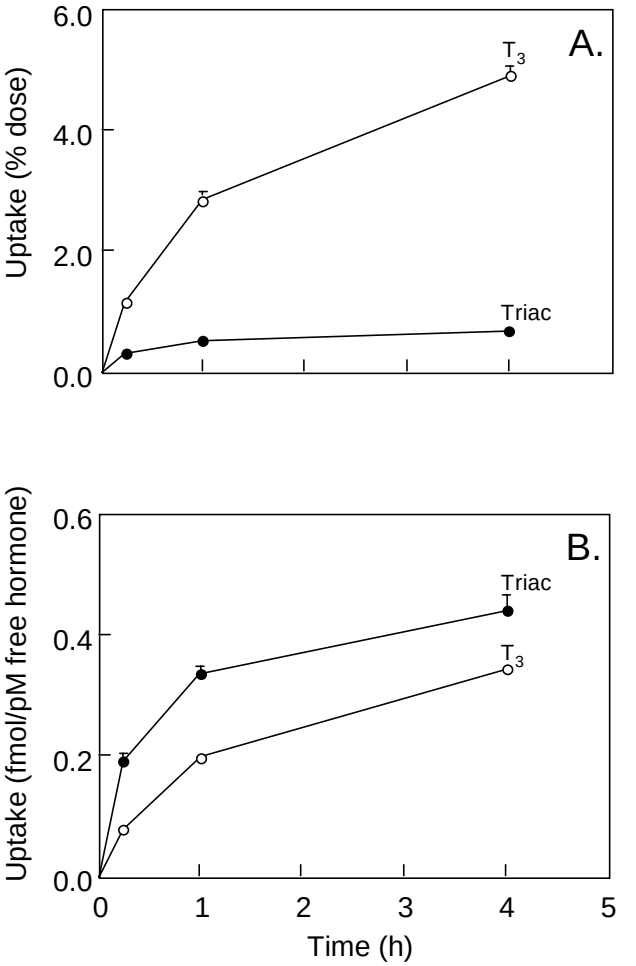

Figure 1 Time course of uptake of [125I]T_3 (o) and [125I]Triac (●) in neonatal rat cardiomyocytes expressed as % dose (A) and as fmol/pM free hormone (B). Cardiomyocytes were cultured for 5 days at a density of 500.000 cells/well. Thereafter, culture medium was removed and cells were preincubated for 30 min in incubation medium with 0.5% BSA, followed by incubation in the same medium with [125I]T_3 (100.000 cpm/well) or [125I]Triac (200.000 cpm/well) for periods of 15 min-4 h. Data represent mean ± SE of six observations from two independent experiments.

Table 1 Cellular (A) and nuclear (B) uptake of [^{125}I]T_3 and [^{125}I]Triac in cultured rat cardiomyocytes

	[^{125}I]T_3 or [^{125}]Triac activity (% dose)			
	Cell pellet (A)	Nuclear pellet (B)	(B/A) x 100%	DNA content (ng)
Experimental conditions				
[^{125}I]T_3 (5)	2.20 ± 0.25	0.18 ± 0.04	10.2 ± 2.4	904 ± 118
+ 10 μM T_3 (6)	1.78 ± 0.25	0.05 ± 0.01	3.1 ± 0.9	1122 ± 110
[^{125}I]Triac (6)	0.41 ± 0.03	0.05 ± 0.01	11.1 ± 3.0	1394 ± 163
+ 10 μM Triac (6)	0.27 ± 0.01	0.02 ± 0.00	5.6 ± 0.9	904 ± 118

Data show mean ± SE of triplicate observations of 2 experiments. Cardiomyocytes were prepared from 2-day old rats, and cultured for one day in 24-wells dishes in DMEM-M199/5% FCS/5% HORSE SERUM at a density of around 10^6 cells/well. After one day the culture medium was changed, and culture was continued for 4 days. Cells were incubated at 37 C for 2 h with [^{125}I]T_3 (200,000 cpm) or [^{125}I]Triac (500,000 cpm) in medium with 0.5% BSA. Cells were scraped from the wells in PBS, centrifuged and the cell pellet (A) counted. The nuclear pellet (B) was obtained after treatment with Triton X-100. The third column shows nuclear [^{125}I]T_3 or [^{125}I]Triac uptake relative to cellular [^{125}I]T_3 or [^{125}I]Triac uptake. The fourth column represents the DNA content of the nuclear pellet.

clear dose-dependent inhibition, and the maximum effect was seen with 10 μM (45%; $P<0.01$) (Fig. 3A). T_4 showed a dose-dependent inhibition, but the maximum effect at 10 μM (27%) was not significant (Fig. 3B). Exposure to Triac did not result in reduction of [^{125}I]T_3 uptake (Fig. 3C), whereas DITPA showed a stepwise reduction with increasing concentrations (Fig. 3D), but the maximum effect was now 20% (NS) as compared to 28% in Fig. 2.

The effects of a number of metabolites tested at a concentration of 10 μM on [^{125}I]T_3 uptake at 15 min (Fig. 4A) and 24 h (Fig. 4B) of incubation are shown in Fig. 4. The uptake curve for [^{125}I]T_3 increased up to 4 h of incubation and reached equilibrium between 4 and 24 h. Under control conditions, [^{125}I]T_3 uptake at 15 min,

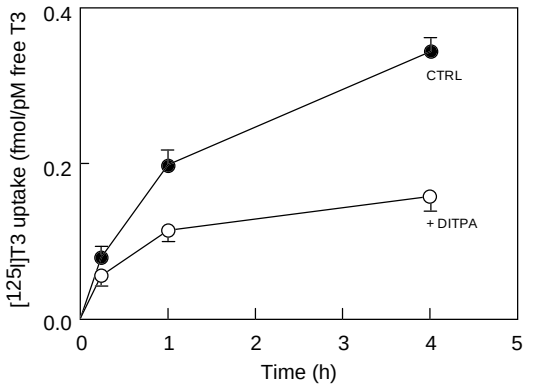

Figure 2 Time course of the effect of 10 μM DITPA on the uptake of [^{125}I]T_3 in cardiomyocytes. Cells were cultured, preincubated and incubated as described in legend to Figure 1, with 10 μM DITPA present during incubation. Data are expressed as fmol/pM free T_3, and represent mean ± SE of six observations from two independent experiments.

1 h, 4 h, and 24 h was 0.089 ± 0.017 (n=9), 0.222 ± 0.017 (n=9), 0.433 ± 0.038 (n=9), and 0.352 ± 0.038 (n=9) fmol/pM free T_3, respectively. After 15 min, [^{125}I]T_3 uptake was inhibited by 52% (n=9; P<0.001) in the presence of 10 μM 3,3'-T_2, and by 17% (n=9; NS) in the presence of 10 μM 3,5-T_2, whereas 10 μM Triac or Tetrac had no effect at all (both n=9; NS). After 24 h of incubation, the relative effect of 3,3'-T_2 was less (26%; n=9; P<0.02) than that observed after 15 min of incubation, whereas the effects of 3,5-T_2 (13%), Triac and Tetrac (both 19%) were still not significant. The effects of 10 μM unlabeled T_3 are given for comparison, and amounted to 52% (P<0.001) and 63% (P<0.001) after 15 min and 24 h, respectively.

Presence of DITPA and T_3 during culture

A second approach to evaluate possible effects of DITPA on the T_3 uptake system was to add 10 nM DITPA during culture for 5 days and compare its effect with the presence of 10 nM T_3. This experiment was performed twice, and after culture, the time course of [^{125}I]T_3 uptake was tested for 15 min to 24 h in absence or presence of 10 μM unlabeled T_3 (Fig. 5). The time-course of [^{125}I]T_3 uptake in the presence of 10 μM unlabeled T_3 was about the same in the three culture conditions (compare Fig.

5A, B, and C, lower curves). However, uptake of [^{125}I]T_3 without additions during incubation was significantly greater in cells that had been cultured under control conditions (Fig. 5A) than in cells that had been cultured in presence of 10 nM T_3 (Fig. 5B) or DITPA (Fig. 5C). Whereas the absolute reduction in T_3 uptake by 10 μM unlabeled T_3 after 24 h of incubation was 2.01% of the dose (42% inhibition; P<0.001) in the cells cultured under control conditions, this was only 0.44% and 0.47% of the

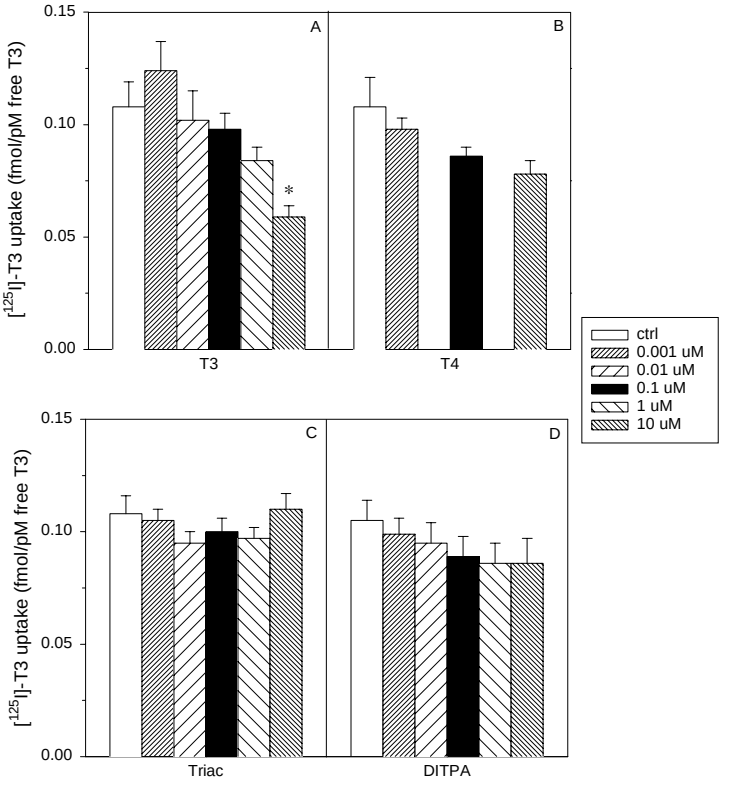

Figure 3 Competitive effects of unlabeled T_3, T_4, Triac and DITPA on the 15-min uptake of [^{125}I]T_3 in cardiomyocytes. The cells were cultured, preincubated and incubated with [^{125}I]T_3 as described in legend to Figure 1, with increasing concentrations (1 nM-10 μM) of the unlabeled compounds present during incubation. Data are expressed as fmol/pM free T_3. Bars represent mean ± SE of nine observations from three independent experiments.

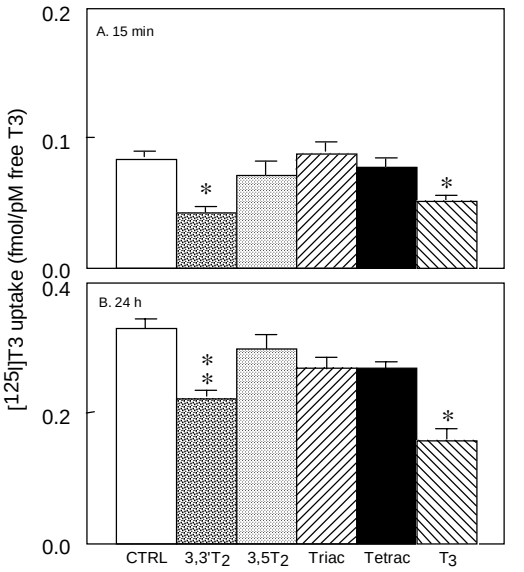

Figure 4 Effects of thyroid hormone metabolites on [^{125}I]T_3 uptake in cardiomyocytes at 15 min (A) and 24 h (B) of incubation. The cells were cultured, preincubated and incubated with [^{125}I]T_3 as described in legend to Figure 1, with 10 μM of the unlabeled metabolites present during incubation. Data are expressed as fmol/pM free T_3. Bars represent mean ± SE of nine observations from three independent experiments. **P*<0.001, and **:*P*<0.02 *vs*. controls (CTRL).

dose (12% inhibition; NS) in cells cultured in the presence of 10 nM T_3 or DITPA. The protein content per well under the different culture conditions was the same.

3.5 Discussion

In the present study, we explored further the uptake mechanism for T_3 in heart by comparing uptake of [^{125}I]T_3 with that of [^{125}I]Triac and by testing the effects of thyroid hormone metabolites and the analog DITPA on T_3 uptake in neonatal rat cardiomyocytes. Our previous studies showed that T_3 uptake in neonatal heart occurred by a temperature- and energy-dependent mechanism that was slightly dependent on

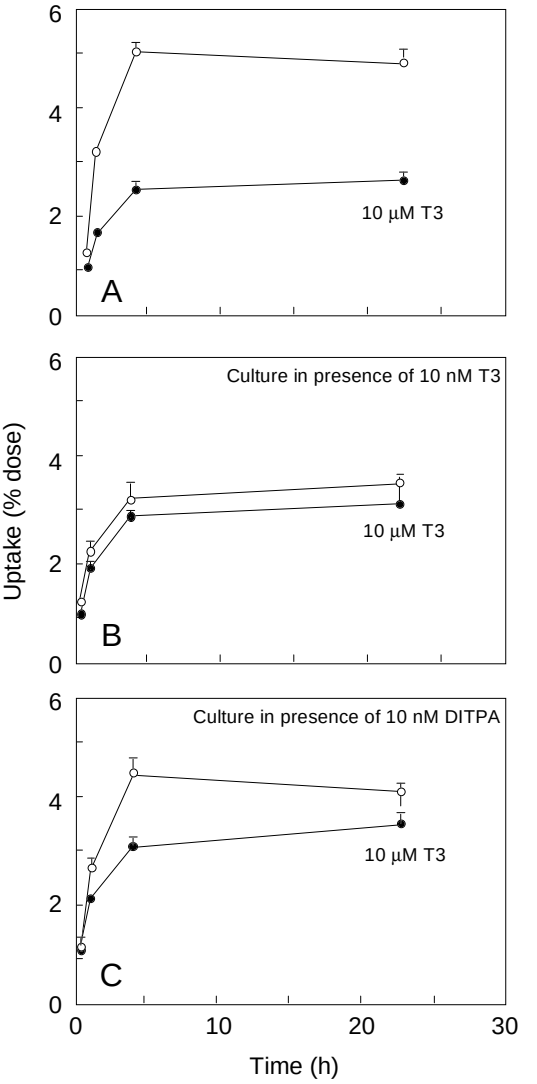

Figure 5 Time course of [^{125}I]T_3 uptake in absence (o) or presence (●) of 10 μM unlabeled T_3 in rat cardiomyocytes cultured without additions (A), with 10 nM T_3 present (B) or with 10 nM DITPA present (C). Data are expressed as % dose, and represent mean ± SE of six-twelve observations from two independent experiments.

the Na^+ gradient (Everts *et al.*, 1996b; van der Putten *et al.*, 2001), and sensitive to Ca^{2+} blockers (Verhoeven *et al.*, 2001), whereas results for T_4 uptake were less clear. The effects of the Ca^{2+} channel inhibitors were not secondary to inhibition of Ca^{2+} influx, suggesting interference with the putative T_3 carrier in the plasma membrane, rather than an energetic linkage to effects of T_3 on ion channels (Verhoeven *et al.*, 2001).

Comparison of the time-courses of T_3 and Triac uptake in neonatal rat cardiomyocytes showed that, when expressed as fmol/pM free hormone, Triac uptake was around 30% greater than that of T_3, but the difference was not as large as in cultured pituitary cells, in which an almost twofold difference was found (Everts *et al.*, 1994). When the 2-h cellular uptakes of T_3 and Triac were measured and expressed as percentages of the dose, a fivefold greater value for T_3 was observed, whereas the relative inhibition by 10 μM of the respective unlabeled hormone was roughly the same. Together, these findings indicate that Triac is taken up by cardiomyocytes, and bound to their nuclear receptors, as in pituitary cells (Everts *et al.*, 1994). In contrast to pituitary cells, in which T_3 uptake was inhibited by Triac and *vice versa* (Everts *et al.*, 1994), the 15-min T_3 uptake in cardiomyocytes was not significantly inhibited by increasing concentrations of Triac. Since T_3 and T_4 are scarcely metabolized by deiodination or conjugation in neonatal cultured cardiomyocytes within 24 h (Everts *et al.*, 1996b), we extended the incubation periods to test the various analogs up to 24 h. However, even after 24 h of incubation, we could not detect any inhibitory effect of 10 μM Triac, suggesting different transport mechanisms for T_3 and Triac in heart. Addition of Triac to the diet of rats induced an increase in oxygen consumption and heart rate, roughly to the same extent as addition of D-T_3 (Boyd & Oliver, 1960). In contrast, neither Triac nor D-T_3 acutely stimulated Na^+ currents in cardiomyocytes (Huang *et al.*, 1999). Tetrac 10 μM reduced neither the plasma membrane uptake of T_3 at 15 min, or its cellular uptake after 24 h. This is clearly at variance with the findings in pituitary cells, in which Triac and Tetrac inhibited [^{125}I]T_3 uptake over the range 15 min-4 h to the same extent as unlabeled T_3, and both suppressed thyrotropin releasing hormone (TRH)-stimulated TSH release (Everts *et al.*, 1994, 1995). Tetrac has also been reported to be more effective than T_4 in suppressing TSH secretion *in vivo* in hypothyroid rats (Lameloise *et al.*, 2001), whereas it was less effective in inducing cardiac hypertrophy. In our study, the inhibitory effect of T_4 (around 25%)

on T_3 uptake was, if anything, greater than that of Tetrac, albeit not significantly so.

Of the two other metabolites tested, 3,3'-T_2 significantly reduced T_3 uptake in cardiomyocytes, the effect after 15 min (52% inhibition) being twice as large as that after 24 h (26% inhibition), suggesting that 3,3'-T_2 primarily interacted at the level of the plasma membrane. The other diiodothyronine, 3,5-T_2, had no effect on T_3 uptake in heart cells between 15 min and 24 h of incubation. Interestingly, 3,5-T_2 has been shown to be more effective than 3,3'-T_2 in suppression of TRH-induced TSH release in pituitary cells (Everts *et al.*, 1995). It is also regarded as the important diiodothyronine with respect to (direct) stimulation of heat production in mitochondria (Goglia *et al.*, 1999), and is just as effective as T_3 in acutely stimulating Na^+ currents in neonatal rat cardiomyocytes (Huang *et al.*, 1999).

Because of its possible preferential effect on myocardial contractility, the analog DITPA has been used, with the purpose of improving heart function after infarction, but so far only in animal studies (Pennock *et al.*, 1993; Morkin *et al.*, 1996; Spooner *et al.*, 1999; Pennock *et al.*, 2001). It has been shown that DITPA binds to bacterially expressed thyroid hormone receptors (Morkin *et al.*, 1996). At the cellular level, DITPA induces sarcoplasmic reticulum Ca^{2+} transport and protein expression (Pennock *et al.*, 2001), but interpretation of data on myosin heavy-chain isoenzyme changes has been more complicated (Spooner *et al.*, 1999). From this point of view, there remains a lack of understanding of how DITPA works (Spooner *et al.*, 1999). In the present study, DITPA was tested for effects on T_3 uptake in the cardiomyocytes. Up to a concentration of 10 μM, DITPA had a less clear effect on the 15-min T_3 uptake (20-28%) as compared with 10 μM T_3 itself (46-52%). In contrast, the effect of 10 μM DITPA after 4 h of incubation was at least as great (56%) as that of unlabeled T_3 (42-63%)(compare Figs. 2 and 5A), suggesting that DITPA can bind to the cytosolic or nuclear T_3 binding sites of the cardiomyocytes. When DITPA was added during culture at a concentration of 10 nM, its effect was comparable to that of the same concentration T_3: the inhibitory effect of 10 μM T_3 on cellular T_3 uptake at 24 h was only 12%, whereas it was 42% in cells cultured under control conditions. As we have not tested whether DITPA interacts with the nuclear T_3 receptors, it is difficult to conclude whether DITPA primarily interferes with plasma membrane T_3 transport or with cytosolic or nuclear T_3 binding. To what extent the inhibitory effect of DITPA on T_3 uptake in the cardiomyocyte results in attenuation or stimulation of thyromimetic

effects, for example on the Na^+,K^+-ATPase isoform expression, is currently being investigated.

The aromatic amino acid tyrosine, the precursor of iodothyronines, and tryptophan (Trp) share the same transport system (Christensen, 1990). In two studies with cardiomyocytes, we reported that tryptophan in concentrations of 0.5 and 2 mM significantly reduced T_3 uptake (maximum response 30-45%; (Everts et al., 1996b; van der Putten et al., 2001). Moreover, the effects of tryptophan were additive to those of oligomycin, an inhibitor of oxidative phosphorylation, suggesting that at least two different transport systems may be responsible for T_3 uptake in neonatal heart (van der Putten *et al.*, 2001). One of those could be a transport system that is capable of transporting aromatic amino acids. Kragie (1994) and Hennemann *et al.* (2001) have reviewed the properties of thyroid hormone transport mechanisms in many cell types. We have previously compared uptake mechanisms for thyroid hormones in pituitary, and liver, and postulated that regulation of the transport in these two tissues was different (Everts *et al.*, 1996a). This difference could play a part in maintenance of the low serum T_3 and normal TSH concentrations in non-thyroidal illness and fasting (Everts *et al.*, 1996a; Hennemann *et al.*, 1998, 2001). When the present data are compared with the results obtained in pituitary cells (Everts *et al.*, 1994; Everts *et al.*, 1995), it seems that the transport mechanism for T_3 into the heart is different. Whether T_3 uptake in heart is similar or different as compared to that in liver (Everts *et al.*, 1996a) remains to be established. This question is of relevance in to the development of drugs with tissue-selective thyromimetic activity (Ichikawa *et al.*, 2000; Trost *et al.*, 2000).

Conclusion

3,3'-T_2, and to a lesser extent DITPA and T_4, inhibit plasma membrane T_3 uptake in the cardiomyocyte, while 3,5-T_2, Triac and Tetrac have no significant effects. In contrats, Triac itself is taken up by the cardiomyocyte, but probably through a transport mechanism other than that for T_3. Our current research plan includes investigation of whether exposure of cardiomyocytes to Triac and DITPA results in thyromimetic effects, and whether exposure to compounds that inhibit T_3 transport (in particular 3,3'-T_2) results in reduction of T_3-induced effects in the cardiomyocyte.

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3.7 References

1. Beck-Peccoz P, Sartorio A, De Medici C, Grugni G, Morabito F & Faglia G 1988 Dissociated thyrometric effects of 3,5,3'-triiodothyroaceticacid (TRIAC) at the pituitary and peripheral tissue levels. *J Endocrinol* **11**:113-118
2. Blondel B, Roijen I & Cheneval JP 1971 Heart cells in culture: a simple method for increasing the proportion of myoblasts. *Experientia* **27**:356-358
3. Boyd GS & Oliver MF 1960 Various effects of thyroxine analogues on the heart and serum cholesterol in the rat. *J Endocrinol* **21**:25-32
4. Camacho P & Dwarkanathan AA 1999 Sick euthyroid syndrome. What to do when thyroid function tests are abnormal in critically ill patients. *Postgraduate Medicine* **105**:215-219
5. Chowdhury D, Parnell VA, Ojamaa K, Boxer R, Cooper R & Klein I 1999 Usefulness of triiodothyronine (T_3) treatment after surgery for complex congenital heart disease in infants and children. *Am J Card* **84**:1107
6. Christensen HN 1990 Role of amino acid transport and counter transport in nutrition and metabolism. *Am J Cardiol* **84**:1107-1109
7. Clausen T, van Hardeveld C & Everts ME 1991 Significance of cation transport in control of energy metabolism and thermogenesis. *Physiol Rev* **71**:733-774
8. Davis PJ & Davis FB 1993 Acute cellular actions of thyroid hormone and myocardial function. *Ann Thorac Surg* **56**:S16-S23
9. Docter R, Krenning EP, Bernard HF & Hennemann G 1987 Active transport of iodothyronines into human cultured fibroblasts. *J Clin Endocrinol Metab* **65**:104-107
10. Docter R, Krenning EP, de Jong M & Hennemann G 1993 The sick euthyroid syndrome: changes in thyroid hormone serum parameters and hormone metabolism. *Clin Endocrinol* **39**:499-518
11. Downs TR & Wilfinger WW 1983 Fluorometric quantification of DNA in cells and

tissues. *Anal Biochem* **131**:538-547

12. Everts ME, Visser TJ, Moerings EPCM, Docter R, van Toor H, Tempelaars AMP, de Jong M, Krenning EP & Hennemann G 1994 Uptake of triiodothyroacetic acid and its effects on thyrotropin secretion in cultured anterior pituitary cells. *Endocrinology* **135**:2700-2707
13. Everts ME, Visser TJ, Moerings EPCM, Tempelaars AMP, van Toor H, Docter R, de Jong M, Krenning EP & Hennemann G 1995 Uptake of 3,3',5,5'-tetraiodothyroacetic acid and 3,3',5'-triiodothyronine in cultured rat anterior pituitary cells and their effects on thyrotropin secretion. *Endocrinology* **136**:4454-4461
14. Everts ME, de Jong M, Lim C, Docter R, Krenning EP, Visser TJ & Hennemann G 1996a Different regulation of thyroid hormone transport in liver and pituitary: its possible role in the maintenance of low T_3 production during nonthyroidal illness and fasting in man. *Thyroid* **6**:357-366
15. Everts ME, Verhoeven FA, Bezstarosti K, Moerings EPCM, Hennemann G, Visser TJ & Lamers JMJ 1996b Uptake of thyroid hormones in neonatal rat cardiac myocytes. *Endocrinology* **137**:4235-4242
16. Gammage M & Franklyn J 1997 Hypothyroidism, thyroxine treatment and the heart. *Heart* **77**:189-190
17. Goglia F, Moreno M & Lanni A 1999 Action of thyroid hormones at the cellular level: the mitochondrial target. *FEBS Lett* **452**:115-120
18. Hamilton MA 1993 Prevalence and clinical implications of abnormal thyroid hormone metabolism in advanced heart failure. *Ann Thorac Surg* **56**:s48-s53
19. Hamilton MA & Stevenson LW 1996 Thyroid hormone abnormalities in heart failure: possibilities for therapy. *Thyroid* **6**:527-529
20. Hennemann G, Everts ME, de Jong M, Lim C, Krenning EP & Docter R 1998 The significance of plasma membrane transport in the bioavailability of thyroid hormone. *Clin Endocrinol* **48**:1-8
21. Hennemann G, Docter R, Friesema ECH, de Jong M, Krenning EP & Visser TJ 2001 Plasma membrane transport of thyroid hormones and its role in thyroid hormone metabolism and bioavailability. *Endocrine Rev* **22**:451-476
22. van Heugten HAA, Bezstarosti K & Lamers JMJ 1994 Endothelin-1 and phenylephrine-induced activation of the phosphoinositide cycle increases cell injury of cultured cardiomyocytes exposed to hypoxia/reoxygenation. *J Mol Cell Cardiol* **26**:1513-1524
23. Holland FW, Brown PS, Weintraub BD & Clark RE 1991 Cardiopulmonary bypass and thyroid function: a “euthyroid sick syndrome”. *Ann Thorac Surg* **52**:46-50
24. Huang CJ, Geller HM, Green WL & Craelius W 1999 Acute effects of thyroid

hormone analogs on sodium currents in neonatal rat myocytes. *J Mol Cell Cardiol* **31**:881-893

25. Ichikawa K, Miyamoto T, Kakizawa T, Suzuki S, Kaneko A, Mori J, Hara M, Kumagai M, Takeda T & Hashizume K 2000 Mechanism of liver-selective thyromimetic activity of SK&F L-94901: evidence for the presence of a cell-type-specific nuclear iodothyronine transport process. *J Endocrinol* **165**:391-397
26. Klein I & Ojamaa K 2001 Thyroid hormone and the cardiovascular system. *N Engl J Med* **344**:501-509
27. Klemperer JD, Klein I, Gomez M, Helm RE, Ojamaa K, Thomas SJ, Isom W & Krieger K 1995 Thyroid hormone treatment after coronary-artery bypass surgery. *N Engl J Med* **333**:1522-1527
28. Kragie L 1994 Membrane iodothyronine transporters. Part I: Review of physiology. *Endocr Res* **40**:319-341
29. Lameloise N, Siegrist-Kaiser C, O'Connell M & Burger A 2001 Differences between the effects of thyroxine and tetraiodothyroacetic acid on TSH suppression and cardiac hypertrophy. *Eur J Endocrinol* **144**:145-154
30. Morkin E, Pennock GD, Raya TE, Bahl JJ & Goldman S 1996 Development of a thyroid hormone analogue for the treatment of congestive heart failure. *Thyroid* **6**:521-526
31. Moruzzi P, Doria E & Agostoni PG 1996 Medium-term effectiveness of L-thyroxine treatment in idiopathic dilated cardiomyopathy. *Am J Med* **101**:461-467
32. Mullis-Jansson SL, Argenziano M, Homma S, Weinberg AD, Williams M, Rose EA & Smith CR 1999 A randomized double-blind study of the effect of triiodothyronine on cardiac function and morbidity after coronary bypass surgery. *J Thorac Cardiovasc Surg* **117**:1128-1135
33. Ojamaa K, Klemperer JD & Klein I 1996 Acute effects of thyroid hormone on vascular smooth muscle. *Thyroid* **6**:505-512
34. Pennock GD, Raya TE, Bahl JJ, Goldman S & Morkin E 1993 Combination treatment with captopril and the thyroid hormone analogue 3,5-diiodothyropropionic acid. *Circulation* **88**:1289-1298
35. Pennock GD, Spooner PH, Summers CE & Litwin SE 2001 Prevention of abnormal sarcoplasmic reticulum calcium transport and protein expression in postinfarction heart failure using 3,5-diiodothyropropionic acid (DITPA). *J Mol Cell Cardiol* **32**:1939-1953
36. Peters A, Ehlers M, Blank B, Exler D, Falk C, Kohlmann T, Fruehwald-Schultes B, Wellhoener P, Kerner W & Fehm HL 2000 Excess triiodothyronine as a risk factor of coronary events. *Arch Intern Med* **160**:1993-1999

37. Polikar R, Burger MD, Scherrer U & Nicod P 1993 The thyroid and the heart. *Circulation* **87**:1435-1441

38. Pujol P, De Boisvilliers F, Bringer J & Jaffiol C 2000 Effects of thyrotropin suppression with tri-iodothyroacetic acid on radionuclide cardiovascular parameters. *Thyroid* **10**:1017-1018

39. van der Putten HHAGM, Joosten BJLJ, Klaren PHM & Everts ME 2001 Characterization of uptake and compartmentalization of 3,5,3'-triiodothyronine in cultured neonatal rat cardiomyocytes. *J Endocrinol* **171**:183-192

40. Rutgers M, Heusdens FA & Visser TJ 1989 Metabolism of triiodothyroacetic acid (TA_3) in rat liver. I. Deiodination of TA_3 and TA_3 sulfate by microsomes. *Endocrinology* **125**:424-432

41. Sherman SI & Ladenson PW 1992 Organ-specific effect of tiratricol: a thyroid hormone analog with hepatic, not pituitary, superagonist effects. *Clinical Endocrinology and Metabolism* **75**:901-905

42. Sherman SI, Ringel MD, Smith MJ, Kopelen HA, Zoghbi WA & Ladenson PW 1997 Augmented hepatic and skeletal thyromimetic effects of tiratricol in comparison with levothyroxine. *J Clin Endocrinol Metab* **82**:2153-2158

43. Spooner PH, Morkin E & Goldman S 1999 Thyroid hormone and thyroid hormone analogues in the treatment of heart failure. *Cor Art Disease* **10**:395-399

44. Staels B, van Tol A, Chan L, Will H, Verhoeven G & Auwerx J 1990 Alterations in thyroid status modulate apolipoprotein, hepatic triglyceride lipase and low densitiy lipoprotein receptor in rats. *Endocrinology* **127**:1144

45. Stephan ZF, Yurachek EC, Sharif R, Wasvary JM, Leonards KS, Hu C-W, Hintze TH & Steele RE 1996 Demonstration of potent lipid-lowering activity by a thyromimetic agent of cardiovascular and thermogenic effects. *Artherosclerosis* **126**:53-63

46. Sterling K & Brenner MA 1966 Free thyroxine in human serum: simplified measurement with the aid of magnesium precipitation. *J Clin Invest* **45**:153-163

47. Toft AD & Boon NA 2000 Thyroid disease and heart. *Heart* **84**:455-460

48. Trost SU, Swanson E, Gloss B, Wang-Iverson DB, Zhang H, Volodarsky T, Grover GJ, Baxter JD, Chiellini G, Scanlan TS & Dillmann WH 2000 The thyroid hormone receptor-β-selective antagonist GC-1 differentially affects plasma lipids and cardiac activity. *Endocrinology* **141**:3057-3064

49. Underwood AH, Emmet JC, Ellis D, Flynn SB, Leeson PD, Benson GM, Novelli R, Pearce NJ & Shah VP 1986 A thyromimetic that decreases plasma cholesterol levels without increasing cardiac activity. *Nature* **324**:425-429

50. Utiger RD 1995 Altered thyroid function in nonthyroidal illness and surgery. To treat or not to treat? *N Engl J Med* **333**:1562-1563

51. Verhoeven FA, Moerings EPCM, Lamers JMJ, Hennemann G, Visser TJ & Everts ME 2001 Inhibitory effects of calcium blockers on thyroid hormone uptake in neonatal rat cardiac myocytes. *Am J Physiol* **21**:H1985-H1991

CHAPTER 4

Uptake of T_3 and T_4 in myoblasts and myotubes of the embryonic heart cell line H9c2(2-1)

H.H.A.G.M. van der Putten, B.J.L.J. Joosten, P.H.M. Klaren and M.E. Everts.
J Endocrinol, accepted

4.1 Abstract

Uptake of T_3 was compared with that of T_4 in the embryonic heart cell line H9c2(2-1). These cells propagate as myoblasts and form differentiated myotubes upon reduction of the serum concentration, as indicated by a 31-fold increase in creatine kinase activity. Protein and DNA content per well were around 2-fold higher in myotubes than in myoblasts. T_3 and T_4 uptakes showed similar characteristics in myoblasts and myotubes, but both were 2.5-fold higher in myotubes, when expressed per well. At any time point, T_4 uptake was 2-fold higher than that of T_3, and both uptakes were energy-, but not Na^+ dependent. T_3 and T_4 uptake exhibited mutual inhibition in myoblasts and myotubes: 10 µM unlabeled T_3 reduced T_4 uptake by 51-60% ($P < 0.001$), while 10 µM T_4 inhibited T_3 uptake by 48-51% ($P < 0.001$). Furthermore, T_3 and T_4 uptake in myoblasts was dose-dependently inhibited by tryptophan (maximum inhibition around 70%; $P < 0.001$). Exposure of the cells to T_3 or T_4 during differentiation significantly increased the fusion index (35 and 40%; $P < 0.01$). Finally, both myoblasts and myotubes showed a small deiodinase (D) I activity, while DII activity was undetectable. In conclusion, T_3 and T_4 share a common energy-dependent transport system in H9c2(2-1) cells, that may be important for the availability of thyroid hormone during differentiation.

Keywords: H9c2(2-1) cell line – T_3 and T_4 uptake – myoblasts - myotubes - heart

4.2 Introduction

Thyroid hormones, thyroxine (T_4) and triiodothyronine (T_3), play a crucial role in normal growth and development of all organs including the heart. Early in the prenatal period, the fetus is supplied by maternal T_4 through transport across the placenta (Obrégon *et al.*, 1998). The fetal thyroid starts to produce thyroid hormones after 18-22 weeks of gestation in humans (Burrow *et al.*, 1994) and at embryonic day 18 in rats (Obrégon *et al.*, 1998). Both in humans and in rodents, the start of fetal thyroid activity is associated with a rise in cardiac contractility (Cluzeaut & Maurer-Schultze, 1986; Mayhew *et al.*, 1997). Canavan *et al.* (1994) showed that hypothyroidism significantly delayed this development of the heart, while hyperthyroidism led to the opposite result.

The mechanisms by which T_3 exerts its effects on the heart include binding of T_3 to specific nuclear receptors (Lazar, 1993; Muñoz & Bernal, 1997) as well as acute cellular actions via the plasma membrane (Davis & Davis, 1993; Huang *et al.*, 1999). T_4 action on the heart is less well characterized. It has been shown that T_4 stimulates differentiation of cardiomyocytes (Brik & Shainberg, 1990) and skeletal muscle cells (Nakashima *et al.*, 1998a, 1998b), although the mode of action is not clear. The affinity of T_4 for nuclear receptors is only one-tenth that of T_3 (Muñoz & Bernal, 1997), thereby making a nuclear pathway less likely. In some tissues, most of the T_3 associated with the nuclei is derived from the intracellular deiodination of T_4, catalyzed by deiodinase type II (DII) (Visser, 1996). Whether type II deiodinase is present in human or rodent heart is still a matter of debate (Nauman *et al.*, 1994; Croteau *et al.*, 1996; Sabatino *et al.*, 2000).

Rosic *et al.* (2001) examined the kinetics of T_3 and T_4 uptake in intact hearts and reported a saturable mechanism for the uptake of both T_3 and T_4. In our laboratory, uptake of T_3 and T_4 was investigated in cultures of neonatal rat cardiomyocytes (Everts *et al.*, 1996b; van der Putten *et al.*, 2001; Verhoeven *et al.*, 2001, 2002). Under conditions where the free T_3 and T_4 concentrations were in the picomolar range, evidence was provided for a specific energy- and temperature dependent mechanism for the uptake of T_3, while the results for T_4 were less equivocal. However, in two of these studies, it was found that unlabeled T_4 reduced the uptake of T_3 indicating that T_4 might enter the cardiomyocytes through a mechanism comparable to that of T_3

(van der Putten *et al.*, 2001; Verhoeven *et al.*, 2002). This idea is further explored in the present study.

While primary cultures of neonatal cardiomyocytes consist of a heterogeneous population of non-dividing and fully differentiated cells (Pinson, 1990), the myogenic cell line H9c2(2-1) is a homogenous population of cardiac cells derived from embryonic rat heart tissue (Kimes & Brandt, 1976). A major advantage of this cell line is its ability to differentiate from mononucleated myoblasts into myotubes upon reduction of the serum concentration (Hescheler *et al.*, 1991). Moreover, during the differentiation process, the cells retain several elements of the electrical and hormonal signaling pathway of cardiac cells (Kimes & Brandt, 1976) and have therefore become an accepted *in vitro* model to study the effects of ischaemia and diabetes on the heart (Eckel, 1996; Brostrom *et al.*, 2000; Wayman *et al.*, 2001). In addition, Gerrelli *et al.* (1994) showed that exposure of the H9c2(2-1) cells to thyroid hormone resulted in an increased expression of the cardiac spliceosome protein SmN, indicating that the cells are T_3 responsive.

In the present study, T_3 and T_4 uptake were characterized and compared in the H9c2(2-1) cells. To understand the role of thyroid hormone uptake mechanisms during cardiac differentiation, T_3 and T_4 uptake were compared in undifferentiated myoblasts and differentiated myotubes. In addition, we examined the effects of T_3 and T_4 on the fusion index as an indicator of the degree of differentiation.

4.3 Materials and Methods

Materials

Cell culture reagents were purchased from Life Technologies (Breda, The Netherlands). Bovine serum albumin (BSA) (fraction V) was obtained from Boehringer (Mannheim, Germany). Triiodothyronine (T_3), thyroxine (T_4), tryptophan (Trp), and oligomycin were purchased from Sigma (St. Louis, MO, USA), [^{125}I]T_3 (24.4 TBq/mmol), [^{125}I]T_4 (4.3 TBq/mmol) and [^{125}I]rT_3 (28.0 TBq/mmol) were from NEN (Boston, MA, USA). Prior to use [^{125}I]T_4 and [^{125}I]rT_3 were purified by Sephadex LH-20 column chromatography (Mol & Visser, 1985).

Cell Culture

The rat embryonic cardiac cell line H9c2(2-1) was obtained from the European Collection of Cell Cultures (Salisbury, Wiltshire). Cells were seeded into 24-well culture dishes at a density of 0.5×10^5 cells/well and propagated as myoblasts at 37 °C in a 5% CO_2 atmosphere in DMEM supplemented with 10% (v/v) FCS and 100 U/ml penicillin-streptomycin. At day 2 after seeding, half of the cultures was used for uptake experiments, while the other half was continued for 7 days in DMEM containing 1% (v/v) FCS and 100 U/ml penicillin-streptomycin to obtain fused myotubes. Medium was changed every 48 h. For the deiodinase assays, cells were cultured in 75 cm^2-culture flasks to a density of 2×10^6 cells/flask at day 2 under the same conditions as described above for cells seeded in 24-well culture dishes.

Cellular uptake studies

Uptake experiments were performed at day 2 and 9 after seeding, at which time the culture consisted of myoblasts and myotubes, respectively. After removal of the culture medium, cells were preincubated for 30 min at 37 °C in 0.5 ml Krebs-Ringer medium (139 mM NaCl, 4.2 mM KCl, 1.5 mM $CaCl_2$, 1.4 mM KH_2PO_4, 1.4 mM $MgSO_4$, 5.0 mM glucose; prepared in Tris at pH 7.4) supplemented with 0.1% BSA for determinations of T_4 uptake or 0.5% BSA for T_3 uptake. Incubation was initiated by quickly replacing the preincubation medium with 0.5 ml Krebs-Ringer buffer containing the additions described below and either 4×10^5 cpm/ml [^{125}I]T_4, 10 nM T_4 and 0.1% BSA, or 4×10^5 cpm/ml [^{125}I]T_3, 10 nM T_3 and 0.5% BSA. Incubations were performed at 37 °C, and the culture dishes were mildly agitated. Uptake was terminated by quick aspiration of the incubation medium, and cells were washed three times with 1 ml ice-cold saline. Cells were dissolved in 1 ml 0.1 M NaOH and analyzed for ^{125}I activity in a gamma-counter (Packard Cobra II, Packard Instruments Co., Meriden, CT, USA). The time-course of T_3 and T_4 uptake was assayed in the presence or absence of sodium. Sodium-free conditions were attained by replacing sodium with choline on a mol-for-mol basis in the preincubation and incubation media. When the effects of unlabeled T_3 and T_4 on 30-min uptake were examined, 10 µM of the unlabeled compound was added to the incubation medium. In all other experiments, the compounds were present during preincubation and incubation (10 µM oligomycin and 0.5 or 2 mM tryptophan (Trp).

The amount of [^{125}I]T_3 or [^{125}I]T_4 associated with the cells was expressed as fmol/pM free hormone (see free hormone fraction). Results were corrected for the amount of ^{125}I activity associated with the walls of the culture wells.

Light microscopy and determination of fusion index

At day 2, 7 and 9 after seeding, cultures were fixed subsequently with 70% (v/v) and 96% (v/v) ethanol and stained with 100% haematoxilin. Nuclei were counted using an inverted microscope (Nikon TMS-F, Bunnik, The Netherlands). Digital images were acquired using a Nikon Coolpix 990 camera. The fusion index was determined in ten microscopic fields and calculated by dividing the number of nuclei in the myotubes by the total number of nuclei. Myotubes were defined as cells containing three or more nuclei.

Creatine kinase (CK) activity measurements

After removal of the culture medium, the cells were lysed in 500 ìl 0.5% (v/v) Triton X-100 in PBS and mildly agitated for 10 minutes at room temperature. The cellular debris was scraped from the well using a rubber spatula and the lysate was collected in a tube. CK activity was determined with a NAC-Activated CK-kit (Sigma) according to the manufacturer's protocol.

Protein and DNA measurements

Cellular protein was measured according to the method of Lowry *et al.* (1951). DNA was quantified using a Hoechst fluorescence staining method (Downs & Wilfinger, 1983).

Free hormone fraction

Calculations of free T_3 and T_4 concentrations were based on the determination of the free hormone fraction by equilibrium dialysis (Sterling & Brenner, 1966). In Krebs-Ringer medium containing 4×10^5 cpm/ml [^{125}I]T_3, 10 nM T_3 and 0.5% BSA, the free T_3 fraction was 3.9 ± 0.3% (n=5). Addition of 10 µM T_3, 10 µM T_4, 10 µM oligomycin or a combination of oligomycin and T_3 (both at 10 µM) changed the free T_3 fraction to 4.6 ± 0.1% (n=3), 4.7 ± 0.0% (n=4), 3.8 ± 0.1% (n=4) and 4.9 ± 0.1% (n=4), respectively. The free T_4 fraction in Krebs-Ringer medium with 4×10^5 cpm/ml [^{125}I]T_4,

10 nM T_4 and 0.1% BSA was 3.3 ± 0.5% (n=3) and changed in the presence of 10 µM T_4, 10 µM T_3, 10 µM oligomycin or a combination of oligomycin and T_4 (both at 10 µM) to 5.1 ± 0.4% (n=3), 4.1 ± 0.3% (n=4), 3.5 ± 0.1% (n=4) and 5.4 ± 0.5% (n=4), respectively. The presence of 2 mM Trp did not alter the free fraction of T_3 or T_4.

Furthermore, the free T_3 and T_4 fractions, analyzed in DMEM supplemented with 1% FCS, were 4.4 ± 0.4% (n=3) and 3.0 ± 0.1% (n=3), respectively.

Deiodinase assays

DI and DII activities were determined in myoblasts and myotubes and compared to those of adult rat liver homogenate. DI and DII assays were performed as previously described (Sabatino *et al.*, 2000) with the following modifications. After removal of the culture medium, the cells were scraped from the culture flask using a rubber spatula and collected in 1 ml buffer consisting of 250 mM sucrose, 10 mM Hepes, 10 mM Tris pH 8, 1 mM DTT. Subsequently, the cells were homogenized using a Potter-Elvejhem device (Heidolph RZR 2020, Schwabach, Germany) equipped with a Teflon pestle. For measurement of DI activity, 100 µl aliquots of the homogenates (10 µg protein in a total assay volume of 200 µl) were incubated in triplicate for 15 min at 37 °C in 100 µl incubation medium (100 mM phosphate buffer, 2 mM EDTA, 10 mM DTT) containing 1×10^6 cpm/ml [^{125}I]rT_3 and 1 µM rT_3 in presence or absence of 100 µM PTU. The reaction was quenched by adding 100 µl 5% (w/v) ice-cold BSA followed by 500 µl 10% (v/v) TCA for deproteinization. Samples were centrifuged for 15 min at 4 °C and 1500 × g (Heraus labofuge 400R, Hanau, Germany) and 500 µl of the supernatant was acidified with 500 µl 1 M HCl. Liberated iodide was analyzed by Sephadex LH-20 column chromatography. The first three eluates were collected with three times 1 ml 0.1 M HCl and analyzed for ^{125}I activity in a ã-counter (Packard Cobra II). Specific DI activity was defined as the PTU-inhibitable part of iodide production. For measurement of DII activity, 100 µl aliquots of homogenates (10 µg protein in a total assay volume of 200 µl) were incubated in triplicate under the same conditions as for the DI assay, using 1×10^6 cpm/ml [^{125}I]T_4 and 0.5 µM T_4 with 20 mM DTT. PTU was omitted from the incubation medium.

Statistics

Data are presented as means ± SEM, unless stated otherwise. Statistical significance was evaluated by repeated measures ANOVA or Student's t-test, where appropriate. Statistical significance was accepted at $P < 0.05$.

4.4 Results

Characterization of the cell culture

In initial experiments, we followed the differentiation of the H9c2(2-1) myoblasts into myotubes. DNA and protein content both increased during the first days of culture (Figure 1A and C). At day 9, DNA content of the cells had reached a constant level, characteristic for non-dividing myotubes. Furthermore, DNA and protein contents of the myotubes (day 9) were around 1.8- and 1.9-fold higher, respectively, compared with myoblasts (day 2) ($P < 0.001$).

Myotubes are characterized by the expression of CK and the activity of this enzyme is markedly increased during differentiation (Kimes & Brandt, 1976). CK activity per well measured at day 2, 7 and 9 is shown in Figure 1B. CK activity increased 9-fold from day 2 to day 7 ($P < 0.001$) and 3.5-fold from day 7 to 9 (not significant). The total rise from day 2 to 9 was 31-fold ($P < 0.001$).

Myoblast differentiation into myotubes can be quantified by measuring the fusion index, which is the percentage of nuclei incorporated into myotubes relative to the total number. Figure 1D shows that the fusion index increased from 0% to 18 ± 4% at day 7 and further increased to 28 ± 3% at day 9, the only statistical significant increase being from day 2 to day 7 ($P < 0.01$).

In Figure 2 a light microscopic view of myoblasts (Fig. 2A) and myotubes (Fig. 2B) is shown. Myoblasts are large, flat, spindle-shaped, mononucleated cells which do not organize themselves into orderly arrays. In contrast, myotubes are fused multi-nucleated cells organized in linear parallel arrays.

Time-course of T_3 and T_4 uptake

Figure 3 shows the time-course of [^{125}I]T_3 uptake in myoblasts and myotubes. Both in myoblasts and myotubes, uptake leveled off after 90 min of incubation. When expressed per well, [^{125}I]T_3 uptake in myotubes was 2-fold higher than in myoblasts.

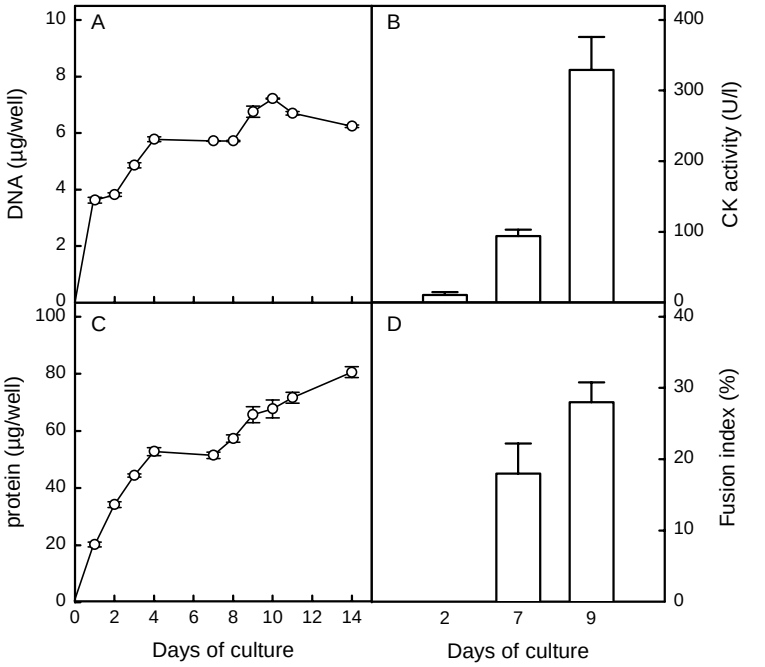

Figure 1 Characterization of H9c2(2-1) cell line during differentiation. Myoblasts were seeded at a density of 5×10^5 cells/well and cultured for two days in DMEM supplemented with 10% FCS. At day 2 the FCS in the medium was reduced to 1% to induce differentiation of myoblasts into myotubes. DNA (A)(n=3) and protein (C)(n=3) content of the cells were determined throughout the culture period. The creatine phosphokinase (CK) activity per well (B)(n=10) (C) and the degree of fusion in percent (D)(means ± SD, n=2) were determined at day 2, 7 and 9 of culture.

However, when the data were expressed relative to the protein content, $[^{125}I]T_3$ uptake in myotubes was around 1.3-fold higher compared to myoblasts ($P < 0.05$). $[^{125}I]T_3$ uptake in myoblasts and myotubes was not inhibited by replacement of Na^+ by choline in the medium, indicating that $[^{125}I]T_3$ uptake is Na^+ independent (Figure 3).

$[^{125}I]T_4$ uptake in myoblasts and myotubes as a function of incubation time leveled off after 1 h of incubation (Figure 4). $[^{125}I]T_4$ uptake per well was 2-fold higher in

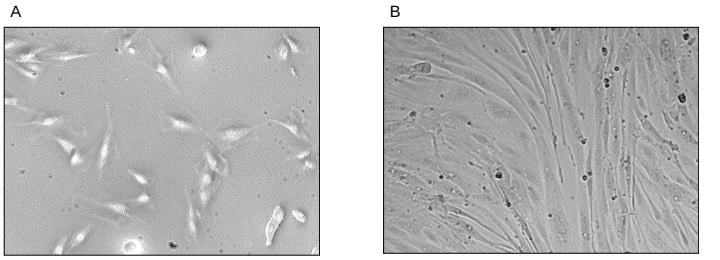

Figure 2 Phase-contrast pictures (10×) of H9c2(2-1) myoblasts (A) and myotubes (B). Myoblasts were seeded at a density of 5×10^5 cells/well and cultured for two days in DMEM supplemented with 10% FCS. At day 2 the FCS in the medium was reduced to 1% to induce differentiation of myoblasts into myotubes. The digital image of the myoblasts was taken at day 2, and that of the myotubes at day 9.

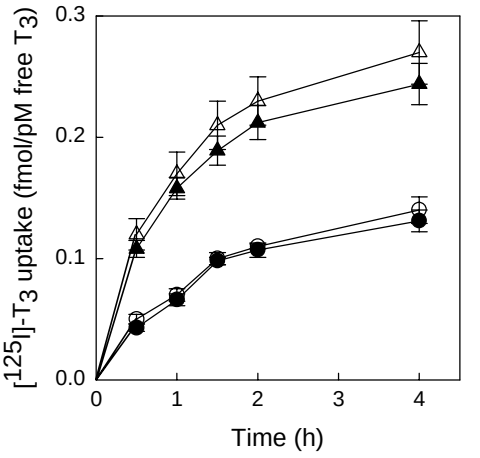

Figure 3 Time-course of $[^{125}I]T_3$ uptake in myoblasts (circles) and myotubes (triangles). H9c2(2-1) cells were cultured as described in the legend to Fig. 1. Uptake experiments were performed at day 2 with myoblasts and at day 9 with myotubes. The uptake of $[^{125}I]T_3$ was measured in Krebs-Ringer medium with Na^+ (closed symbols) or in Krebs-Ringer in which Na^+ was replaced by choline (open symbols), both supplemented with 0.5% BSA. Data show the means ± SEM of 7 - 13 observations of 5 experiments.

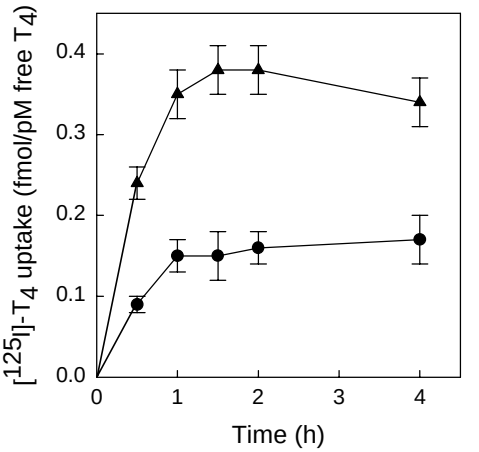

Figure 4 Time-course of $[^{125}I]T_4$ uptake in H9c2(2-1) myoblasts (circles) and myotubes (triangles). H9c2(2-1) cells were cultured as described in the legend to Fig. 1. Uptake experiments were performed at day 2 with myoblasts and at day 9 with myotubes. The uptake of $[^{125}I]T_4$ was measured in Krebs-Ringer medium with Na^+, supplemented with 0.1% BSA. Data show the means ± SEM of 6 observations from 2 experiments (myoblasts) and 9 observations from 3 experiments (myotubes).

myotubes than in myoblasts. When the uptake was expressed relative to the protein content of the wells, $[^{125}I]T_4$ uptake in myotubes was around 1.4-fold higher than in myoblasts ($P < 0.05$). This is comparable to the difference in $[^{125}I]T_3$ uptake between myotubes and myoblasts (see above). Furthermore, $[^{125}I]T_4$ uptake in myoblasts and myotubes was Na^+ independent (data not shown).

Comparison of $[^{125}I]T_3$ and $[^{125}I]T_4$ uptake

Figure 5 shows 30-min uptake of $[^{125}I]T_3$ (left panel) and $[^{125}I]T_4$ (right panel) in myoblasts. The control values for uptake of $[^{125}I]T_3$ and $[^{125}I]T_4$ were 0.040 ± 0.001 fmol/pM FT_3 and 0.072 ± 0.003 fmol/pM FT_4, respectively, a 1.8-fold difference ($P < 0.001$). $[^{125}I]T_3$ uptake was significantly inhibited by 10 µM unlabeled T_3 (73%; $P < 0.001$) and 10 µM unlabeled T_4 (48%; $P < 0.001$) (Figure 5, left panel). Oligomycin, added at a concentration of 10 µM, significantly inhibited $[^{125}I]T_3$ uptake by 20% (*P*

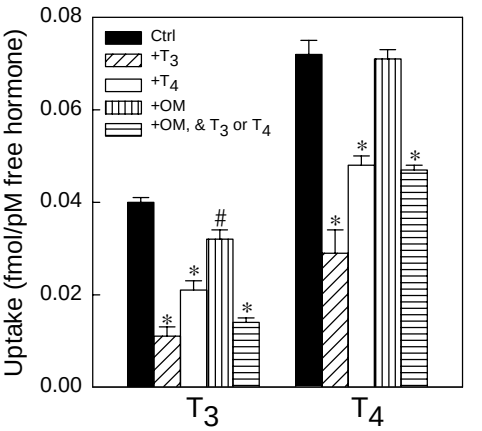

Figure 5 Effect of unlabeled T_3, T_4, oligomycin (OM) and combinations of these compounds on the 30-min [^{125}I]T_3 and [^{125}I]T_4 uptake in myoblasts. Unlabeled T_3 and T_4 (10 µM) were only present during incubation, whereas OM (10 µM) was present during preincubation (30 min) and incubation. The effect of the combination of OM with unlabeled hormone on T_3 and T_4 uptake was tested by incubations with OM and T_3 or OM and T_4, respectively. Data are presented as means ± SEM of 6 - 18 observations from three experiments. $*P <$ 0.001 *vs.* control; $\#P < 0.01$ *vs.* control.

< 0.01). Co-incubation with 10 µM unlabeled T_3 and 10 µM oligomycin resulted in a 65% ($P < 0.001$) reduction of T_3 uptake, comparable to the effect of unlabeled T_3 alone. Similar experiments were performed with [^{125}I]T_4 uptake in myoblasts (Figure 5, right panel). [^{125}I]T_4 uptake was significantly reduced by 10 µM unlabeled T_4 (33%; $P < 0.001$) and 10 µM unlabeled T_3 (60%; $P < 0.001$). [^{125}I]T_4 uptake in myoblasts was not inhibited when 10 µM oligomycin was added to the incubation medium. Co-incubation of 10 µM unlabeled T_4 and 10 µM oligomycin resulted in a comparable inhibition as the effect of T_4 alone (35%; $P < 0.001$).

Uptake of [^{125}I]T_3 and [^{125}I]T_4 was examined in parallel in myotubes (Figure 6) and showed the following control values: 0.088 ± 0.005 fmol/pM FT_3 and 0.180 ± 0.008 fmol/pM FT_4, respectively, a 2-fold difference ($P < 0.05$). Unlabeled T_3 (10 µM) inhibited [^{125}I]T_3 and [^{125}I]T_4 uptake by 56% ($P < 0.001$) and 51% ($P < 0.001$), respectively. Unlabeled T_4 inhibited [^{125}I]T_3 and [^{125}I]T_4 uptake by 51% ($P < 0.001$)

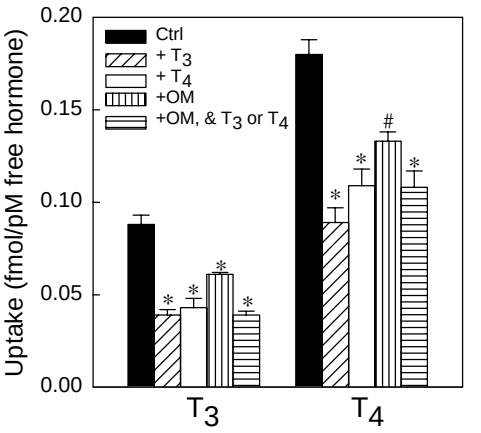

Figure 6 Effect of unlabeled T_3, T_4, oligomycin (OM) and combinations of these compounds on the 30-min [^{125}I]T_3 and [^{125}I]T_4 uptake in myotubes. Experiments were performed exactly as described for myoblasts (Fig. 5). Data are presented as means ± SEM of 6 - 18 observations from three experiments. ∗$P < 0.001$ *vs.* control; #$P < 0.01$ *vs.* control.

and 39% ($P < 0.001$), respectively. When myotubes were incubated with 10 µM oligomycin, [^{125}I]T_3 uptake was reduced by 31% ($P < 0.001$) (Figure 6, left panel) and [^{125}I]T_4 uptake was reduced by 26% ($P < 0.01$) (Figure 6, right panel). The effect of co-incubation of oligomycin and unlabeled T_3 on [^{125}I]T_3 uptake in myotubes was comparable to the effect of unlabeled hormone alone (56%; $P < 0.001$). This was also found for [^{125}I]T_4 uptake in presence of oligomycin and unlabeled T_4 (40%; $P < 0.001$)

Effect of Trp on [^{125}I]T_3 uptake

To test the possible involvement of amino acid transport system T in the uptake of [^{125}I]T_3 and [^{125}I]T_4, we preincubated and incubated (30 min) myoblasts and myotubes with 0.5 and 2 mM Trp. [^{125}I]T_3 and [^{125}I]T_4 uptake were dose-dependently inhibited by Trp, with a maximal effect of 69% and 71%, respectively ($P < 0.001$)(Table 1).

In myotubes, [^{125}I]T_3 uptake decreased from 0.063 ± 0.001 fmol/pM FT_3 to 0.032 ± 0.001 fmol/pM FT_3 in presence of 2 mM Trp (49%, $P < 0.001$).

Table 1. Effects of Trp on 30-min uptake of [^{125}I]T_3 and [^{125}I]T_4 in myoblasts.

	[^{125}I]T_3 uptake (fmol/pM FT_3)	**Effect** (%)	**[^{125}I]T_4 uptake** (fmol/pM FT_4)	**Effect** (%)
No additions	0.035 ± 0.001		0.112 ± 0.003	
0.5 mM Trp	0.014 ± 0.001∗	-60	0.077 ± 0.003∗	-31
2 mM Trp	0.011 ± 0.001∗	-69	0.032 ± 0.007∗	-71

H9c2(2-1) myoblasts were preincubated (30 min) and incubated in the absence or presence of Trp. Data show the mean ± SEM from two independent experiments. ∗$P < 0.001$, Trp *vs.* no additions.

Effect of T_3 and T_4 on differentiation of H9c2(2-1) cells

By comparing the fusion index of cultures exposed to 5 nM T_3 or 50 nM T_4 with that of control cultures, the effect of T_3 or T_4 treatment on differentiation could be examined. Under these conditions with 1% FCS in the medium, the free T_3 and T_4 concentrations were 0.2 and 1.5 nM, respectively. The fusion index was determined on day 2, 3, 7 and 9 (Figure 7). In general, fusion index increased from day 2 to 9, reflecting the morphological differentiation of the cells (see also Figure 1D). Only at day 9, exposure to T_3 and T_4 significantly stimulated fusion: T_3 treatment by 35 ± 2% ($P < 0.01$ *vs.* control at day 9), and T_4 by 40 ± 2% ($P < 0.001$ *vs.* control at day 9), as compared to 26 ± 1% in the control cultures. This effect was not accompanied by an increase in DNA and protein content nor in CK activity (data not shown). The control values of these parameters resembled those depicted in Figure 1.

Deiodinase activity

DI activity was 0.32 ± 0.05 fmol/µg protein/min in myoblasts (n=3), and 0.22 ± 0.07 fmol/µg protein/min in myotubes (n=3), respectively. The H9c2(2-1) cells did not exhibit DII activity (data not shown). In the same experiments, DI and DII activity in homogenates of adult rat liver amounted to around 30 fmol/µg protein/min and less than 1 fmol/µg protein/min, respectively.

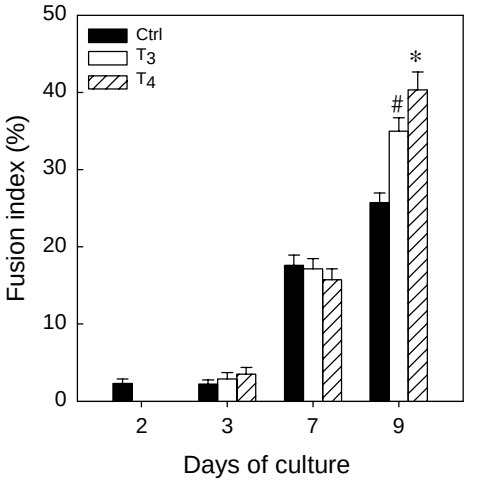

Figure 7 Effect of T_3 and T_4 on fusion index. Cells were cultured from day 2 to 9 in DMEM supplemented with 1% FCS and 5 nM T_3 (white bars) or 50 nM T_4 (hatched bars). Fusion index was determined at day 2, 3, 7 and 9 as described in Materials and Methods. Data are presented as means ± SEM of 2 independent experiments. #$P < 0.01$, and ∗$P < 0.001$ T_3 or T_4 *vs.* control at day 9.

4.5 Discussion

In the present study we examined T_3 and T_4 uptake in the H9c2(2-1) cell line derived from embryonic rat heart. Uptake of T_3 in these cells was inhibited by unlabeled T_3, T_4 and Trp. Incubation with oligomycin reduced T_3 uptake, indicating energy-dependence of the process. T_3 uptake was not Na^+ dependent. These observations provide evidence for a specific T_3 uptake mechanism in H9c2(2-1) cells. T_4 uptake at 30 min was around 2-fold higher than that of T_3, and the characteristics of uptake *e.g.* energy-dependence, Na^+-independence and ligand specificity were comparable to that of T_3. These observations support the idea that T_4 and T_3 share a common uptake mechanism in the H9c2(2-1) cells. Moreover, both T_3 and T_4 stimulated differentiation at concentrations (0.2 – 1.5 nM) in the range of that used in the uptake studies (both

around 0.3 nM).

We here present evidence that the H9c2(2-1) cells possess a specific mechanism for the uptake of T_4. Whereas carrier-mediated uptake of T_3 has been demonstrated in various tissues (Kragie, 1994; Hennemann *et al.*, 2001), evidence for an active transport mechanism for T_4 is not as abundant. T_4 uptake has been explored in hepatocytes (Krenning *et al.*, 1981), anterior pituitary cells (Everts *et al.*, 1994), neuroblastoma cells (Lakshmanan *et al.*, 1990), and skeletal muscle (van Hardeveld & Kassenaar, 1978). T_4 uptake in these cell types is mediated by a saturable and energy-dependent mechanism. The characteristics of T_3 and T_4 uptake were comparable in myotubes and myoblasts. First, T_3 and T_4 uptake were inhibited roughly to the same extent by oligomycin (most clearly in myotubes), and thus, T_3 and T_4 uptake depend on the energy status of the cell. Second, T_3 and T_4 uptake did not depend on the Na^+ gradient. Third, the uptakes exhibited mutual inhibition, but the effect of unlabeled T_3 on T_4 uptake was somewhat larger than that of unlabeled T_4, probably because of the higher free T_3 concentration in medium with 0.1% BSA. Together these findings are consistent with the idea that T_3 and T_4 share the same transporter in cardiac cells. Studies with cultured anterior pituitary cells also suggested a common carrier for uptake of T_3 and T_4 (Everts *et al.*, 1994). This is different from the situation in the liver, where separate carriers presumably are involved in uptake of T_3 and T_4 (Krenning *et al.*, 1981). These differences suggest that thyroid hormone uptake is regulated in a tissue-specific manner, which may significantly influence total thyroid hormone bioactivity (Everts *et al.*, 1996a; Hennemann *et al.*, 1998, 2001).

The H9c2(2-1) cell line is frequently used as a model for cardiac myocytes (Eckel, 1996; Brostrom *et al.*, 2000; Wayman *et al.*, 2001). However, the ability of the cells to form multinucleated tubes is a skeletal muscle rather than a cardiac muscle characteristic (Kimes & Brandt, 1976; Hescheler *et al.*, 1991). Nevertheless, the morphological characteristics and several elements of the electrical and hormonal signaling pathway are similar to those of embryonic and adult cardiomyocytes, respectively (Hescheler *et al.*, 1991). An active transport mechanism has also been described for the uptake of T_3 in intact rat skeletal muscle (van Hardeveld & Kassenaar, 1978; Pontecorvi & Robbins, 1986) and in a skeletal muscle cell line (Pontecorvi *et al.*, 1987); a mechanism that is dependent on the Na^+ gradient (Centanni & Robbins, 1987). In agreement with our study in cardiomyocytes where Na^+ dependency was

also evaluated by substituting Na^+ with choline (van der Putten *et al.*, 2001), T_3 uptake in H9c2(2-1) cells was Na^+ independent. Thus, based on Na^+-independency, the differentiated H9c2(2-1) cells exhibit a cardiac like T_3 transport mechanism.

Previously, we showed that Trp inhibited T_3 uptake and suggested that amino acid transport system T may be an accessory transport system in the uptake of thyroid hormones in heart (Everts *et al.*, 1996b; van der Putten *et al.*, 2001). In H9c2(2-1) cells, Trp also inhibited the uptake of T_3, supporting this idea. Moreover, the effect of Trp on T_3 uptake was comparable to the effect of unlabeled T_3: 69% and 73% in myoblasts, 49% and 60% in myotubes, respectively. In general, incubations with excess unlabeled T_3 distinguish membrane-associated T_3 from that translocated across the plasma membrane by a specific mechanism (Cheng, 1983; Pontecorvi & Robbins, 1986; van der Putten *et al.*, 2001). Thus, it seems that Trp inhibits specific T_3 uptake in H9c2(2-1) cells. Trp also inhibited the uptake of T_4 to the same extent as that of T_3 (71% and 69%, respectively). When compared to the inhibition by unlabeled T_4 (33%), the effect of Trp was higher (71%), but comparable to the inhibitory effect of unlabeled T_3 on T_4 uptake (60%). These findings further support the idea that T_4 and T_3 share a common mechanism, in which amino acid transport system T may play a role. Recently, a novel member of this system, called TAT1, has been identified (Kim *et al.*, 2001). When expressed in *Xenopus laevis* oocytes, TAT1 did not induce uptake of T_3 or T_4 (Kim *et al.*, 2001). In addition to TAT1, a number of transporters belonging to different families have been identified as potential thyroid hormone transporters (Hennemann *et al.*, 2001). It is possible that some of these transporters are involved in thyroid hormone transport into the heart.

T_3 and T_4 significantly increased the fusion index at day 9, indicating that the T_3 and T_4 uptake mechanisms are important for the availability of thyroid hormones during differentiation. Although actions of T_4 on differentiation have been described (Brik & Shainberg, 1990; Nakashima *et al.*, 1998a, 1998b), it is generally thought that T_3 and not T_4 is the main regulator of such processes. The affinity of T_4 for the nuclear T_3 receptor is one-tenth that of T_3 (Muñoz & Bernal, 1997). The effects on differentiation of the H9c2(2-1) cells were examined under conditions in which the free T_4 concentration was 7-fold higher than that of T_3 *i.e.* 1.5 and 0.2 nM, respectively. Thus, theoretically T_4 may have stimulated differentiation of the H9c2(2-1) cells by association with the nuclear T_3 receptor. If T_4 not by itself induces differentiation, it

should be deiodinated to T_3 to allow binding to the nuclear T_3 receptor. We found a small DI activity in myoblasts and myotubes of the H9c2(2-1) cells, that was around 1% of the activity in rat liver. This is consistent with the findings of Mori *et al.* (1991), who reported DI activity in neonatal rat cardiomyocytes. In a recent study of Sabatino *et al.* (2000) DI activity in rat and human heart was measured. The values were comparable, and amounted to around 1% of DI activity in rat liver. Although DI converts T_4 into T_3, it is supposed to play a minor role in the local production of T_3 (Visser, 1996). Local T_3 production is primarily mediated by DII (Visser, 1996), but we could not detect DII activity in H9c2(2-1) myoblasts. Studies on the presence of DII have only been performed in adult heart. DII mRNA was found in human, but not in rat heart (Croteau *et al.*, 1996). While Naumann *et al.* (1994) could not detect DII activity in rat heart, Sabatino *et al.* (2000) found a low DII activity in human and rat heart.

In summary, the H9c2(2-1) cell line provides a useful model system for examination of thyroid hormone uptake in cardiac cells. Uptake of T_3 and T_4 is regulated by a specific and energy-dependent mechanism, and it appears that T_3 and T_4 share a common transport mechanism in H9c2(2-1) cells. Furthermore, the ability of both T_3 and T_4 to induce differentiation suggests that the thyroid hormone uptake mechanisms are important for the availability of thyroid hormones during further development. Another potential application of our study is the therapeutic approach to target the heart with T_3, T_4 or analogs (Klein & Ojamaa, 2001; Verhoeven *et al.*, 2002), since a failing heart recapitulates the fetal profile of gene expression (Klein & Ojamaa, 2001; Sussman, 2001).

4.6 Acknowledgements

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4.7 References

1. Brik H & Shainberg A 1990 Thyroxine induces transition of red towards white muscle in cultured heart cells. *Basic Res Cardiol* **85**:237-246
2. Brostrom MA, Reilly BA, Wilson FJ & Brostrom CO 2000 Vasopressin-induced hypertrophy in H9c2 heart-derived myocytes. *Int J Biochem Cell Biol* **32**:993-1006
3. Burrow GN, Fisher DA & Larsen PR 1994 Maternal and fetal thyroid function. *N Engl J Med* **331**:1072-1078
4. Canavan JP, Holt J & Goldspink DF 1994 The influence of thyroid hormones on the growth of the atria and ventricles of the heart in immature rats. *J Endocrinol* **142**:171-179
5. Centanni M & Robbins J 1987 Role of sodium in thyroid hormone uptake by rat skeletal muscle. *J Clin Invest* **80**:1068-1072
6. Cheng SY 1983 Characterization of binding and uptake of 3,3',5-L-triiodothyronine in cultured mouse fibroblasts. *Endocrinology* **112**:1754-1762
7. Cluzeaut F & Maurer-Schultze B 1986 Proliferation of cardiomyocytes and interstitial cells in the cardiac muscle of the mouse during pre- and postnatal development. *Cell Tissue Kinet* **19**:267-274
8. Croteau W, Davey JC, Galton VA & Germain DLS 1996 Cloning of the mammalian type II iodothyronine deiodinase. A selenoprotein differentially expressed and regulated in human and rat brain and other tissues. *J Clin Invest* **98**:405-417
9. Davis PJ & Davis FB 1993 Acute cellular actions of thyroid hormone and myocardial function. *Ann Thorac Surg* **56**:S16-S23
10. Downs TR & Wilfinger WW 1983 Fluorometric quantification of DNA in cells and tissues. *Anal Biochem* **131**:538-547
11. Eckel J 1996 Direct effects of glimepiride on protein expression of cardiac glucose transporters. *Horm Metab Res* **28**:508-11
12. Everts ME, Docter R, Moerings EPCM, van Koetsveld PM, Visser TJ, de Jong M, Krenning EP & Hennemann G 1994 Uptake of thyroxine in cultured anterior pituitary cells of euthyroid rats. *Endocrinology* **134**:2490-2497
13. Everts ME, de Jong M, Lim C, Docter R, Krenning EP, Visser TJ & Hennemann G 1996a Different regulation of thyroid hormone transport in liver and pituitary: its possible role in the maintenance of low T_3 production during nonthyroidal illness and fasting in man. *Thyroid* **6**:357-366
14. Everts ME, Verhoeven FA, Bezstarosti K, Moerings EPCM, Hennemann G, Visser

TJ & Lamers JMJ 1996b Uptake of thyroid hormones in neonatal rat cardiac myocytes. *Endocrinology* **137**:4235-4242

15. Gerrelli D, Huntriss JD & Latchman DS 1994 Antagonistic effects of retinoic acid and thyroid hormone on the expression of the tissue-specific splicing protein SmN in a clonal cell line derived from rat heart. *J Mol Cell Cardiol* **26**:713-9
16. van Hardeveld C & Kassenaar AAH 1978 Thyroid hormone uptake and T_4 derived T_3 formation in different skeletal muscle types of normal and hypothyroid rats. *Acta Endocrinol Copenh* **88**:306-320
17. Hennemann G, Everts ME, de Jong M, Lim C, Krenning EP & Docter R 1998 The significance of plasma membrane transport in the bioavailability of thyroid hormone. *Clin Endocrinol* **48**:1-8
18. Hennemann G, Docter R, Friesema ECH, de Jong M, Krenning EP & Visser TJ 2001 Plasma membrane transport of thyroid hormones and its role in thyroid hormone metabolism and bioavailability. *Endocrine Rev* **22**:451-476
19. Hescheler J, Meyer R, Plant S, Krautwurst D, Rosenthal W & Schultz G 1991 Morphological, biochemical, and electrophysiological characterization of a clonal cell (H9c2) line from rat heart. *Circ Res* **69**:1476-86
20. Huang CJ, Geller HM, Green WL & Craelius W 1999 Acute effects of thyroid hormone analogs on sodium currents in neonatal rat myocytes. *J Mol Cell Cardiol* **31**:881-893
21. Kim DK, Kanai Y, Chairoungdhua A, Matsuo H, Cha SH & Endou H 2001 Expression cloning of a Na^+-independent aromatic amino acid transporter with structural similarity to H^+/monocarboxylaat transporters. *J Biol Chem* **276**:17221-17228
22. Kimes BW & Brandt L 1976 Properties of a clonal muscle cell line from rat heart. *Exp Cell Res* **98**:367-381
23. Klein I & Ojamaa K 2001 Thyroid hormone - Targeting the heart. *Endocrinology* **142**:11-12
24. Kragie L 1994 Membrane iodothyronine transporters. Part I: Review of physiology. *Endocr Res* **40**:319-341
25. Krenning EP, Docter R, Bernard B, Visser TJ & Hennemann G 1981 Characteristics of active transport of thyroid hormone into rat hepatocytes. *Biochem Biophys Acta* **676**:314-320
26. Lakshmanan M, Gonçalves E, Lessly G, Foti D & Robbins J 1990 The transport of thyroxine into mouse neuroblastoma cells, NB41A3: the effect of L-system amino acids. *Endocrinology* **126**:3245-3250
27. Lazar MA 1993 Thyroid hormone receptors: multiple forms, multiple possibilities. *Endocrine Rev* **14**:184-193
28. Lowry OH, Rosebrough NJ, Farr AL & Randall RJ 1951 Protein measurement with

the Folin phenol reagent. *J Biol Chem* **193**:265-275

29. Mayhew TM, Pharaoh A, Austin A & Fagan DG 1997 Stereological estimates of nuclear number in human ventricular cardiomyocytes before and after birth obtained using physical disectors. *J Anat* **191**:107-15
30. Mol JA & Visser TJ 1985 Synthesis and some properties of sulfate esters and sulfamates of iodothyronines. *Endocrinology* **117**:1-7
31. Mori Y, Nishikawa M, Toyoda N, Yonemoto T, Matsubara H & Inada M 1991 Iodothyronine 5'-deiodinase activity in cultured rat myocardial cells: characteristics and effects of triiodothyronine and angiotensine II. *Endocrinology* **128**:3105-3112
32. Muñoz A & Bernal J 1997 Biological activities of thyroid hormone receptors. *Eur J Endocrinol* **137**:433-445
33. Nakashima K, Ohtsuka A & Hayashi K 1998a Comparison of the effects of thyroxine and triiodothyronine on protein turnover and apoptosis in primary chick muscle cell cultures. *Biochem Biophys Res Comm* **251**:442-448
34. Nakashima K, Ohtsuka A & Hayashi K 1998b Effects of thyroid hormones on myofibrillar proteolysis and activities of calpain, proteasome, and cathepsin in primary cultured chick muscle cells. *J Nutr Sci Vitaminol* **44**:799-807
35. Nauman A, Nauman J, Porta S & Grzesniuk W 1994 Iodothyronine-5'-deiodinase in rat heart, regulatory action of catecholamines. In: Braverman LE, Eber O & Langsteger W (eds) Heart and thyroid. Blackwell Scientific Publications Inc., Cambridge, MA, pp. 196-172
36. Obrégon MJ, Calvo RM, Escobar del Rey F & Morreale de Escobar G 1998 Thyroid hormones and fetal development. In: Pinchera A, Mann K & Hostalek U (eds) The thyroid and age. Schattauer, Stuttgart, pp. 49-73
37. Pinson 1990 Neonatal rat heart muscle cells. In: Piper HM (ed) Cell culture techniques in heart and vessel research. Springer, Berlin, pp. 20-35
38. Pontecorvi A & Robbins J 1986 Energy-dependent uptake of 3,5,3'-triiodo-L-thyronine in rat skeletal muscle. *Endocrinology* **119**:2755-2761
39. Pontecorvi A, Lakshmanan M & Robbins J 1987 Intracellular transport of 3,5,3'-triiodo-L-thyronine in rat skeletal myoblasts. *Endocrinology* **121**:2145-2152
40. van der Putten HHAGM, Joosten BJLJ, Klaren PHM & Everts ME 2001 Characterization of uptake and compartmentalization of 3,5,3'-triiodothyronine in cultured neonatal rat cardiomyocytes. *J Endocrinol* **171**:183-192
41. Rosic MA, Pantovic SB, Lucic AP, Ribarac-Stepic N & Andjelkovic I 2001 Kinetics of thyroxine (T_4) and triiodothyronine (T_3) transport in the isolated rat heart. *Exp Physiol* **86**:13-18
42. Sabatino L, Iervasi G, Ferrazzi P, Francesconi D & Chopra IJ 2000 A study of

iodothyronine 5'-monodeiodinase activities in normal and pathological tissues in man and their comparison with activities in rat tissues. *Life Sci* **68**:191-202

43. Sterling K & Brenner MA 1966 Free thyroxine in human serum: simplified measurement with the aid of magnesium precipitation. *J Clin Invest* **45**:153-163
44. Sussman MA 2001 When the thyroid speaks, the heart listens. *Circ Res* **89**:557-559
45. Verhoeven FA, Moerings EPCM, Lamers JMJ, Hennemann G, Visser TJ & Everts ME 2001 Inhibitory effects of calcium blockers on thyroid hormone uptake in neonatal rat cardiac myocytes. *Am J Physiol* **21**:H1985-H1991
46. Verhoeven FA, van der Putten HHAGM, Hennemann G, Lamers JMJ, Visser TJ & Everts ME 2002 Uptake of triiodothyronine and triiodothyroacetic acid in neonatal rat cardiomyocytes: effect of metabolites and analogs. *J Endocrinol* **173**:247-255
47. Visser TJ 1996 Pathways of thyroid hormone metabolism. *Acta Med Austriaca* **23**:10-6
48. Wayman N, McDonald MC, Thompson AS, Threadgill MD & Thiemermann C 2001 5-aminoisoquinelinone, a potent inhibitor of poly (adenosine 5'-diphosphate ribose) polymerase, reduces myocardial infarct size. *Eur J Pharmacol* **430**:93-100

Expression Cloning as a Strategy for Characterization of the Putative Thyroid Hormone Transport Protein in Rat Heart

H.H.A.G.M. van der Putten, E.C.H. Friesema, R. Docter, M.E. Everts and T.J. Visser.

5.1 Abstract

In heart and other organs, thyroid hormones cross the plasma membrane by a specific energy-dependent transport system, referred to as thyroid hormone transport protein. The structure of this protein has not yet been characterized. We micro-injected *Xenopus laevis* oocytes with mRNA isolated from neonatal and adult rat heart to induce expression of the putative cardiac thyroid hormone transport protein and subsequently, to examine thyroid hormone uptake. In parallel, oocytes were injected with rat liver mRNA and tested for T_3 uptake. Injection of neonatal or adult heart mRNA did not result in stimulation of T_3 uptake, whereas oocytes injected with liver mRNA showed a 1.2-fold induction of T_3 uptake. The presence of 0.5% BSA did not result in a higher induction of T_3 uptake by the mRNAs, but total T_3 uptake relative to the free T_3 concentration was increased by BSA. Size-fractionation seemed to improve the results, but the induction observed after injection with size-fractionated neonatal heart mRNA was low (1.1-fold). Moreover, some fractions reduced T_3 uptake compared to uninjected and water-injected oocytes. Finally, the use of an alternative ligand, T_3 sulfamate (T_3NS), diminished the background, but there was no detectable stimulation of T_3NS uptake in oocytes injected with heart mRNA. In contrast, injection of liver mRNA resulted in a 6- to 7-fold induction of 10 and 100 nM T_3NS uptake, respectively.

In conclusion, injection of oocytes with mRNA isolated from neonatal or adult rat heart did not result in stimulation of T_3 or T_3NS uptake. The results obtained with injection of liver mRNA were comparable to those previously reported. We conclude that this expression cloning method using *Xenopus laevis* oocytes can be used for the characterization of a liver thyroid hormone transport protein, but is less attractive for characterization of such a protein in heart.

Key words: thyroid hormone transport, oocytes, expression cloning, mRNA

5.2 Introduction

Heart function is regulated by hemodynamic (Klein *et al.*, 1992; Ojamaa *et al.*, 1996) and humoral factors, of which thyroid hormones are most extensively studied (Dillmann, 1990; Polikar *et al.*, 1993; Klein & Ojamaa, 2001). Thyroxine (T_4) is synthesized and produced by the thyroid gland. T_4 is relatively inactive and is converted to T_3, the biologically active hormone (Visser, 1988). This conversion mainly takes place in the liver. T_3 mediates, via specific nuclear receptors, transcription of mRNAs encoding cardiac specific proteins *e.g.* myosin heavy chain isoforms (Ojamaa & Klein, 1993) and sarcoplasmic reticulum Ca^{2+}-ATPase (Muller *et al.*, 1997). Furthermore, T_3 induces, without nuclear receptor involvement, uptake of fatty acids (Blennemann *et al.*, 1992), glucose (Segal, 1989; Segal & Ingbar, 1990) and calcium (Warnick *et al.*, 1988), thereby improving cardiac efficiency and contractile function (Liu *et al.*, 1998). The intracellular actions of T_3 require transport across the plasma membrane. Studies in cultured neonatal cardiomyocytes suggested that T_3 uptake occurs via an energy-dependent carrier-mediated mechanism (Everts *et al.*, 1996; van der Putten *et al.*, 2001; Verhoeven *et al.*, 2001). Such a mechanism has also been described for other cell types *e.g.* hepatocytes (Rao *et al.*, 1976; Krenning *et al.*, 1979; Pardridge & Mietus, 1980; Blondeau *et al.*, 1988; Weisiger *et al.*, 1992; de Jong *et al.*, 1994; Kragie, 1994; Hennemann *et al.*, 2001). However, the recent review by Hennemann *et al.* (2001) provided information about the possible molecular characteristics of these plasma membrane transport mechanisms.

For a number of uptake mechanisms, functional transporters have been cloned with an expression cloning strategy using *Xenopus laevis* oocytes. These include transporters for amino acids (Palacín *et al.*, 1990), and organic anions (Wolkoff, 1996), substrates of which the molecular structure is comparable to that of T_3. Therefore, Docter *et al.* (1997) adopted this technique to identify the thyroid hormone transport protein in liver. Injection of oocytes with a 0.8-2.1 kb fraction of liver mRNA resulted in a rapid stimulation of saturable, Na^+-dependent T_3 transport, indicating the presence of a T_3 transporter. In addition, they showed that stimulation of the uptake of T_3 sulfamate (T_3NS), a less lipophilic hormone, was comparable to that of T_3. Moreover, the background in these experiments was low due to a negligible uptake of T_3NS in native oocytes (Friesema *et al.*, 1999). Based on similarities between the results of

cell physiological studies with hepatocytes and cardiomyocytes, we decided to further investigate the transport of T_3 in heart using the expression cloning strategy. We injected oocytes with neonatal or adult rat heart mRNA or size-fractionated neonatal mRNA and examined uptake of T_3 and T_3NS. For validation of the method and comparison with previous studies of Docter *et al.* (1997), oocytes were also injected with rat liver mRNA, and uptake of T_3 and T_3NS was measured.

5.3 Materials and methods

Animals

Male adult Wistar rats, body weight 300 g, and 3-day old Wistar rats, of both sexes, were obtained from laboratory stock (Utrecht University, The Netherlands). Adult rats were killed by cervical dislocation, 3-day old rats were killed by decapitation. Hearts and livers were quickly dissected and frozen in liquid nitrogen. Two to 3-yr old *Xenopus laevis* were obtained from Amrep (Breda, The Netherlands) and maintained as described previously (Docter *et al.*, 1997; Friesema *et al.*, 1998). Under MS-222 anesthesia (Sigma, St. Louis, MO, USA; 1 g/l 3-aminobenzoic acid ethyl ester, in tap water) and hypothermia, ovarian fragments were removed for isolation of oocytes (Docter *et al.*, 1997).

Materials

Unlabeled T_3 was obtained from Henning Berlin (Berlin, Germany), bovine serum albumin (BSA) fraction V and 1 g/l MS-222 from Sigma. [^{125}I]T_3 (74 TBq/mmol) was purchased from Amersham (Amersham, UK). [^{125}I]T_3NS and T_3NS were prepared and purified as described previously (Mol & Visser, 1985).

mRNA preparation

We used a commercial mRNA isolation kit (Stratagene, La Jolla, CA, USA) for isolation of mRNA from heart and liver tissue. To isolate mRNA from hearts, 1 g deep-frozen material was pulverized in a mortar, disrupted with an ultra-turrax blender (IKA-Werke, Staufen, Germany) in guanidinium isothiocyanate buffer supplemented with β-mercaptoethanol and finally homogenized in the same buffer using a Potter-Elvejhem device (Heidolph RZR 2020, Schwabach, Germany) equipped with a glass

pestle. Livers were directly homogenized in the guanidinium isothiocyanate buffer supplemented with β-mercaptoethanol. After homogenization, the protein precipitate was removed by centrifugation, and mRNA was bound to oligo(deoxythymidine)-cellulose (oligodT). After rinsing the mRNA-oligodT mixture, mRNA was eluted with elution buffer at 65 °C. For size-fractionation of neonatal heart mRNA, 75 µg mRNA in water was heated to 65 °C for 5 min and then loaded on a linear 6-20% (wt/vol) sucrose gradient containing 15 mM piperazine-N,N´[2-ethanesulfonic acid] (PIPES) (pH 6.4), 5 mM Na_2-EDTA, and 0.25% (wt/vol) Sarkosyl. The gradient was centrifuged for 19 h at 4 °C at 80,000 × *g* in a Beckman SW 41 rotor (Beckman, Palo Alto, CA, USA). Subsequently, 0.7 ml fractions were collected from the bottom of the tube. Total and size-fractionated mRNA was precipitated with 0.3 M sodium acetate pH 5.2 in ethanol (Sambrook *et al.*, 1989), and resuspended in sterile water. The concentration of mRNA was measured spectrophotometrically (wavelength 260 nm). mRNA was stored at –80 °C at a concentration of 1 µg/µl. The integrity of the mRNA was checked by electrophoresis of 1 µg mRNA on a 1.0 % agarose gel, stained with ethidium bromide, which was also used for estimation of size-fractions of neonatal rat heart mRNA. Each fraction contains a maximum concentration of one size of mRNA ($mRNA_{max}$) with gradually lower concentrations of smaller and larger species of mRNA, extending about 0.6 kb on each side of the $mRNA_{max}$.

Oocyte isolation and mRNA injection

Oocytes were prepared as described previously (Docter *et al.*, 1997). After a 3-h collagenase B (2 mg/ml; Roche, Mannheim, Germany) treatment, oocytes were selected manually using morphological criteria, such as size, pigmentation, and absence of follicular layer debris. Healthy-looking stage V-VI oocytes (Dumont, 1972) were transferred to six-well tissue culture plates and incubated overnight at 18 °C in modified Barth's solution (88 mM NaCl, 1 mM KCl, 0.82 mM $MgSO_4$, 0.4 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, 2.4 mM $NaHCO_3$, 10 mM HEPES (pH 7.4)), containing 20 IU/ml penicillin, and 20 µg/ml streptomycin. The day after isolation, oocytes were injected with 23 ng mRNA in 23 nl water or with water alone, using the Nanoject system (Drummond, Broomall, PA, USA). Injected and uninjected (native) oocytes were kept at 18 °C for 4 days in modified Barth's solution, with a daily change of medium.

Uptake assays in oocytes

Experiments were performed as described previously (Docter *et al.*, 1997). Ten oocytes were incubated for 1 h at 25 °C in 100 µl incubation medium (100 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 10 mM Tris, pH 7.5) supplemented with 0.5% BSA (as indicated in figure legends), and to which 10 - 100 nM T_3 and 4×10^5 cpm [^{125}I]T_3, or 10 - 100 nM T_3NS and 4×10^5 cpm [^{125}I]T_3NS was added. We assessed Na^+-dependency of the uptake by substituting NaCl by choline chloride on a mole-for-mole basis in the incubation medium. After 1 h, incubation was terminated by aspiration of the incubation medium, followed by washing four times with 2.5 ml ice-cold Na^+-containing incubation medium supplemented with 0.1% BSA. Oocytes were transferred to new tubes and analyzed individually in a γ-counter (Nuclear Enterprise, Edinburgh, UK). The amount of [^{125}I]T_3 and [^{125}I]T_3NS associated with the oocytes was expressed as fmol/oocyte/h.

Statistics

Data are presented as means ± SEM. Statistical significance was evaluated by one-way ANOVA, followed by Student's *t*-test, or paired *t*-test where appropriate. Statistical significance was accepted at $P < 0.05$.

5.4 Results

Uptake experiments

According to Docter *et al.* (1997), incubations were performed in medium without BSA. Fig. 1 shows the results of T_3 uptake (100 nM) by uninjected oocytes, water-injected oocytes and oocytes injected with mRNA isolated from liver, adult heart (AH) and neonatal heart (NH). Oocytes injected with liver mRNA (L) showed an 1.2-fold increase in T_3 uptake compared to water-injected controls ($P < 0.05$). T_3 uptake was not induced after injection with adult heart mRNA or neonatal heart mRNA. Uptake of T_3 was not altered by replacement of Na^+ in the medium with choline (results not shown). Note that there is a significant amount of T_3 uptake in uninjected oocytes: 65.4 fmol/oocyte/h.

In our previous studies with cultured neonatal cardiomyocytes, T_3 uptake was examined in the presence of 0.5% BSA to mimic physiological conditions (Everts *et*

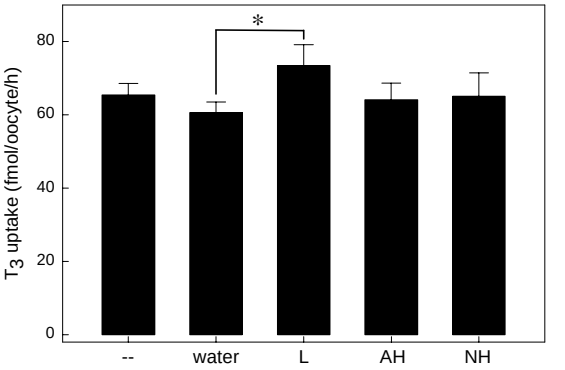

Figure 1 Uptake of T_3 by uninjected oocytes (—) and oocytes injected with water, or 23 ng liver (L), adult heart (AH) or neonatal (NH) mRNA. Four days after injection, oocytes were incubated for 1 h at 25 °C in medium supplemented with 100 nM [^{125}I]T_3. Data are presented as means ± SEM of 4 to 5 experiments. *: $P < 0.05$ liver mRNA *vs.* water-injected oocytes.

al., 1996; van der Putten *et al.*, 2001; Verhoeven *et al.*, 2001). Fig. 2 shows the results of T_3 uptake by uninjected and injected oocytes incubated in medium supplemented with 0.5% BSA. In the presence of 0.5% BSA, uptake of T_3 by oocytes injected with adult (AH) or neonatal heart mRNA (NH) was not increased. On the other hand, parallel incubations with oocytes injected with liver mRNA (L) resulted in an increase from 5.5 ± 0.4 fmol/oocyte/h in uninjected oocytes and 5.3 ± 0.3 fmol/oocyte/h in water-injected oocytes to 6.2 ± 0.5 fmol/oocyte/h. Actually, incubations in the presence of 0.5% BSA resulted in around 10-fold lower total T_3 uptake values in uninjected, water-injected and mRNA injected oocytes. However, when the data were analyzed relative to the free T_3 concentration, incubations with 0.5% BSA resulted in 2.8 to 3.6-fold higher uptake values in all oocytes examined independent of injected RNA.

Previous studies showed that size-fractionation of liver mRNA on a 6-20% sucrose gradient resulted in enrichment of mRNAs inducing T_3 uptake (Docter *et al.*, 1997). In Fig. 3 the results of size-fractionation of neonatal heart mRNA are shown. After fractionation, mRNA with a size of 0.8 – 2.0 kb (fraction c) showed an 1.1-fold stimulation of T_3 uptake, compared to uninjected oocytes and water-injected oocytes.

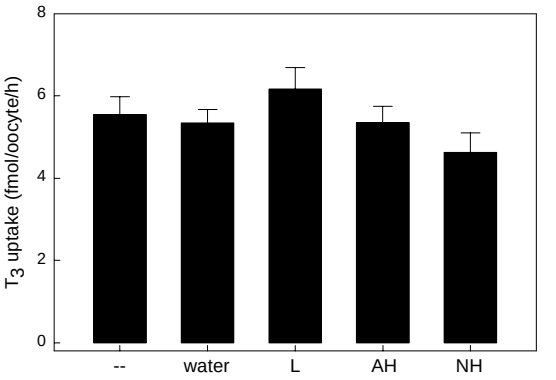

Figure 2 Uptake of T_3 in presence of 0.5% BSA by uninjected oocytes (—) and oocytes injected with water, or 23 ng liver (L), adult heart (AH) or neonatal (NH) mRNA. Four days after injection, oocytes were incubated for 1 h at 25 °C in medium supplemented with 100 nM [^{125}I]T_3 and 0.5% BSA. Data are presented as means ± SEM of 2 to 3 experiments.

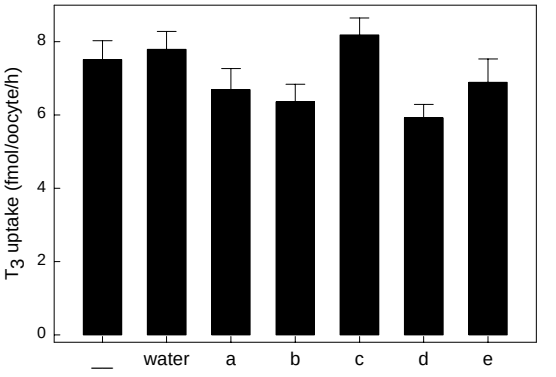

Figure 3 Uptake of T_3 by oocytes injected with size-fractionated neonatal heart mRNA. Four days after injection, injected oocytes were incubated for 1 h at 25 °C in medium supplemented with 10 nM [^{125}I]T_3. In parallel incubations, uptakes in uninjected- and water injected-oocytes were examined. Data are presented as means ± SEM of 10 observations from a representative experiment. Size ranges of mRNA fractions: a, 2.3-3.5 kb; b, 1.6-2.8 kb; c, 0.8-2.0 kb; d, 0.5-1.7 kb; e, 0.3-1.5 kb.

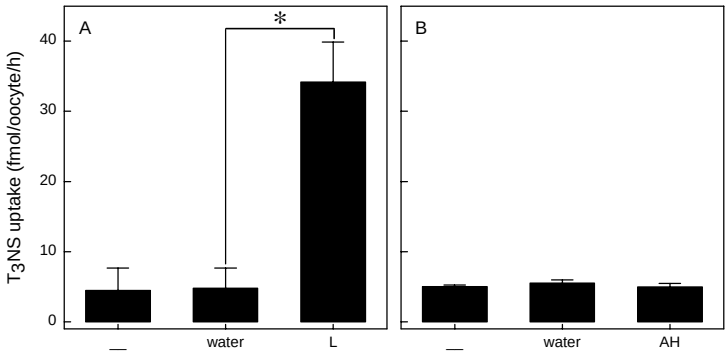

Figure 4 Uptake of T_3NS by uninjected oocytes (—) and oocytes injected with water or 23 ng liver (L) mRNA (A) or adult heart mRNA (B). Four days after injection, oocytes were incubated for 1 h at 25 °C in medium containing 100 nM [^{125}I]T_3NS. Data are presented as means ± SEM from 7 and 3 experiments, respectively. *: $P < 0.001$ liver mRNA *vs.* water-injected oocytes.

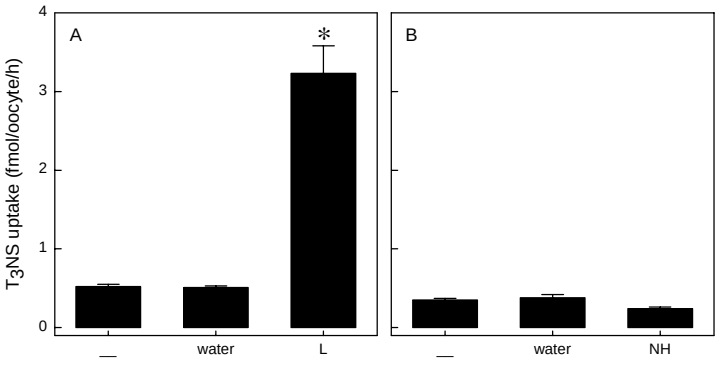

Figure 5 T_3NS uptake in uninjected oocytes and oocytes injected with water or 23 ng liver mRNA (A) or neonatal heart mRNA (B). Four days after injection, oocytes were incubated for 1 h at 25 °C in medium containing 10 nM [^{125}I]T_3NS. Data are presented as means ± SEM of 10 observations from a single experiment. *: $P < 0.001$ liver mRNA *vs.* uninjected and water-injected oocytes.

The other four mRNA size-fractions a, b, d and e showed lower uptake values compared to uninjected and water-injected oocytes.

We examined T_3NS uptake (100 nM) in the absence of BSA in oocytes injected with liver (L) (Fig. 4a) or adult heart mRNA (AH) (Fig. 4b). T_3NS uptake by uninjected or water-injected oocytes was 14.5 and 12.6-fold lower, respectively, compared to the uptake of T_3 in these oocytes (see Fig. 1). T_3NS uptake was increased from 4.5 ± 0.5 fmol/oocyte/h in uninjected oocytes and 4.8 ± 0.6 fmol/oocyte/h in water-injected oocytes up to 34.2 ± 7.8 fmol/oocyte/h ($P < 0.001$, n=7) after injection of liver mRNA (L) (Fig. 4A), whereas no increase of T_3NS uptake was found after injection of adult heart mRNA (5.0 ± 0.3 in uninjected oocytes, 5.5 ± 0.5 fmol/oocyte/h in water-injected oocytes and 5.0 ± 0.5 fmol/oocyte/h in adult heart mRNA-injected oocytes) (n=3, Fig. 4B). Comparable experiments were performed with a 10-fold lower concentration of T_3NS (Fig. 5). Again, oocytes injected with liver mRNA showed a marked rise in T_3NS uptake (6.2-fold compared to uninjected and water-injected oocytes, $P < 0.001$, n=10 observations from a single experiment) (Fig. 5A). Furthermore, the results of injection with neonatal heart mRNA are shown in Fig. 5B, and were not different from uptake observed in uninjected and water-injected oocytes.

5.5 Discussion

The present study was conducted to characterize the molecular structure of a putative cardiac thyroid hormone transport protein. We adopted an expression cloning strategy to express this protein in *Xenopus laevis* oocytes using neonatal, adult rat heart mRNA and fractions of neonatal heart mRNA, followed by examination of T_3 and T_3NS uptake. Injection of neonatal or adult heart mRNA in oocytes did not result in a stimulation of T_3 or T_3NS uptake. In contrast, after injection of rat liver mRNA, both T_3 and T_3NS uptake were significantly increased.

Absence of induction of T_3 uptake after injection of neonatal or adult heart mRNA was not expected. First, our previous studies clearly suggest the presence of a specific carrier-mediated mechanism for T_3 uptake in neonatal heart (Everts *et al.*, 1996; van der Putten *et al.*, 2001; Verhoeven *et al.*, 2001), which was also established in other cell types including hepatocytes (Kragie, 1994; Hennemann *et al.*, 2001). Incubation of rat hepatocytes with a monoclonal antibody raised against a plasma membrane

protein with a M_r of 55 kDa inhibited T_3 and T_4 uptake in rat liver cells (Mol *et al.*, 1986). In addition, this coincides remarkably with the observation that two membrane associated proteins with a Mr of 53 and 55 kDa, respectively, were sensitive to N-bromoacetyl[^{125}I]T_3 affinity labeling in cultured mouse fibroblast (Cheng, 1983b), a cell type shown to exhibit carrier-mediated T_3 uptake (Cheng, 1983a). Thus, it was concluded that liver expresses a protein involved in thyroid hormone transport. The next step involved characterization of the mRNA encoding this protein by expression of liver mRNA in *Xenopus laevis* oocytes and examination of T_3 and T_3NS uptake (Docter *et al.*, 1997; Friesema *et al.*, 1999). This procedure was repeated in the present study and we obtained comparable values for uptake after injection of liver mRNA. In addition, these experiments proved the validity of the method. Second, in a recent study of Vasilets *et al.* (2001) oocytes were injected with RNA isolated from rat heart leading to functional expression of other cardiac proteins, *i.e.* the Na^+,K^+-ATPase from the plasma membrane. This again shows that the *Xenopus* expression system provides a valuable tool for identification of mRNAs and their products in particular for transport proteins.

In our cell physiological studies, T_3 uptake was examined in medium containing 0.5% BSA to mimic the *in vivo* situation in which almost all of the hormone is associated with protein (Bartalena, 1990). In addition, it has been suggested that albumin facilitates the uptake of thyroid hormones (Krenning *et al.*, 1979; Pardridge & Mietus, 1980). Overall, total T_3 uptake in medium with albumin was lower than that observed in medium without albumin, confirming the general agreement that only the unbound T_3 determines the rate of uptake (Docter & Krenning, 1990). However, when the data were analyzed as function of the free T_3 fraction, which is 2.5% in medium with 0.5% BSA (van der Putten *et al.*, 2001), T_3 uptake in presence of albumin was higher compared to incubations without albumin, confirming the suggested facilitating role of albumin. On the other hand, albumin prevents a significant fraction of thyroid hormone from absorbing to assay tubes and pipette walls (Abumrad *et al.*, 1998). Despite these advantages, incubations of oocytes injected with neonatal or adult heart mRNA in medium supplemented with 0.5% BSA did not improve the results and thus, again no induction of T_3 uptake was observed.

Docter *et al.* (1997) showed that size-fractionation of total liver mRNA resulted in a larger increase in T_3 uptake compared with injection of unfractionated liver mRNA.

Furthermore, information on the size-range of mRNA stimulating T_3 uptake may lead to the characterization of the mRNA encoding the putative thyroid hormone transport protein. When compared with the controls, size-fractionation of neonatal heart mRNA resulted in a little or no increase of T_3 uptake with mRNA fraction ranging from 0.8 to 2.0 kb, a size in the range of the fraction of liver mRNA that most clearly stimulated T_3 uptake (Docter *et al.*, 1997). Finally, injection of smaller and larger fractions of neonatal mRNA resulted in a decrease of T_3 uptake, of which the functional implication remains to be established.

We (Docter *et al.*, 1997; Friesema *et al.*, 1999) observed a high amount of T_3 uptake by uninjected and water-injected oocytes, which is a major drawback, because it masks the uptake expected after injection of mRNAs encoding exogenous transporter proteins. The high background implicates that oocytes themselves exhibit thyroid hormone transport mechanism. The structure of this mechanism has not yet been identified, and attempts to find specific inhibitors for this mechanism were not yet successful (Friesema *et al.*, 1999). Because T_3 is very hydrophobic and binds non-specifically to any available surface, this will also mask uptake performed by a specific mechanism (Lai *et al.*, 1985). In a previous report, we showed that a high amount of T_3 (62%) was associated with the plasma membrane of isolated rat cardiomyocytes (van der Putten *et al.*, 2001). Similar findings were reported by others (Yusta *et al.*, 1988; Chehín *et al.*, 1999). Water-solubility of lipophilic substances can be increased by sulfate conjugation (Visser, 1988, 1994), which results in decreased non-specific binding. Friesema *et al.* (1999) showed that uptake of sulfonated iodothyronines *e.g.* T_3NS resembles uptake of T_3 by oocytes injected with liver mRNA and suggested the involvement of a common transporter. Thus, examination of T_3NS uptake seemed to be an attractive approach. Our results show that uptake of T_3NS is negligible in native oocytes, but high in oocytes injected with liver mRNA. From this we can conclude that mRNA is intact and translated to functional proteins. Unfortunately, no induction of T_3NS uptake was observed after injection of adult heart mRNA. Incubations of oocytes with a lower T_3NS concentration resulted in a similar picture: uptake was induced after injection of liver mRNA, but not after injection of neonatal heart mRNA. Together with the finding that we did not observe uptake of T_3NS in neonatal rat cardiomyocytes (unpublished results), we conclude that neonatal rat heart does not express a transport protein for T_3NS and thus, for our purpose T_3NS is not a suitable

alternative ligand in contrast to what seems the case for identification of liver thyroid hormone transporters.

Finally, in this study we isolated mRNA from total rat heart and thus from all the cell types present in heart. On a protein basis cardiomyocytes cover around 90% of the heart. However, measured by cell numbers the cardiac fibroblast is the most abundant cell type, covering two-third of the total cell population (Eghbali, 1992). This suggests that the pool of mRNAs in our preparation is mainly derived from cardiac fibroblasts. In addition, we have shown in a previous study that both cell types transport T_3, but the uptake of T_3 expressed per µg protein is more than 10-fold higher in cardiomyocytes than in cardiac fibroblasts (Verhoeven *et al.*, 2001). Thus, in total cardiac mRNA the abundance of the mRNA encoding the thyroid hormone transport protein of the cardiomyocyte may be too low to induce significant uptake in oocytes. Therefore, it would be interesting to examine thyroid hormone transport activity of oocytes injected with mRNA isolated from cultured cardiomyocytes.

In summary, in this report we show that injection of oocytes with neonatal and adult heart mRNA did not result in stimulation of T_3 uptake, examined under various conditions. This cannot be explained by failure of the method itself, because in parallel incubations, oocytes injected with liver mRNA show a significant induction of T_3 uptake. Expression cloning in *Xenopus laevis* has also been applied for further identification of cDNAs encoding *e.g.* amino acid transporters (Palacín *et al.*, 1998), one of the mechanisms we suggested in our previous studies to be involved in uptake of thyroid hormones in heart (Everts *et al.*, 1996; van der Putten *et al.*, 2001). Other interesting types of transporters which may be involved in thyroid hormone uptake in heart, are the fatty acid transporters. Their role in cardiac thyroid hormone uptake is subject of our current study.

5.6 Acknowledgements

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5.7 References

1. Abumrad NA, Harmon C & Ibrahimi A 1998 Membrane transport of long-chain fatty acids: evidence for a facilitated process. *J Lipid Res* **39**:2309-2318
2. Bartalena L 1990 Recent achievements in studies on thyroid hormone-binding proteins. *Endocrine Rev* **11**:47-64
3. Blennemann B, Moon YK & Freake HC 1992 Tissue-specific regulation of fatty acid synthesis by thyroid hormone. *Endocrinology* **130**:637-643
4. Blondeau JP, Osty J & Francon J 1988 Characterization of the thyroid hormone transport system of isolated hepatocytes. *J Biol Chem* **263**:2685-2692
5. Chehín RN, Issé BG, Rintoul MR & Farías RN 1999 Differential transmembrane diffusion of triiodothyronine and thyroxine in liposomes: Regulation by lipid composition. *J Membrane Biol* **167**:251-256
6. Cheng SY 1983a Characterization of binding and uptake of 3,3',5-L-triiodothyronine in cultured mouse fibroblasts. *Endocrinology* **112**:1754-1762
7. Cheng SY 1983b Structural similarities in the plasmamembrane 3,3',5-triiodo-L-thyronine receptors from human, rat and mouse cultured cells. Analysis by affinity labeling. *Endocrinology* **113**:1155-1157
8. Dillmann WH 1990 Biochemical basis of thyroid hormone action in the heart. *Am J Med* **88**:626-630
9. Docter R & Krenning EP 1990 Role of cellular transport systems in the regulation of thyroid hormone bioactivity. In: Greer MA (ed) The Thyroid Gland. Raven Press, New York, pp. 233-254
10. Docter R, Friesema ECH, van Stralen PGJ, Krenning EP, Everts ME, Visser TJ & Hennemann G 1997 Expression of rat liver cell membrane transporters for thyroid hormone in *Xenopus laevis* oocytes. *Endocrinology* **138**:1841-1846
11. Dumont JN 1972 Oogenesis in *Xenopus laevis* (Daudin) I. Stages of oocyte development in laboratory maintained animals. *J Morphol* **136**:153-180
12. Eghbali M 1992 Cardiac fibroblasts: function, regulation of gene expression, and phenotypic modulation. *Basic Res Cardiol* **87**:183-189
13. Everts ME, Verhoeven FA, Bezstarosti K, Moerings EPCM, Hennemann G, Visser TJ & Lamers JMJ 1996 Uptake of thyroid hormones in neonatal rat cardiac myocytes. *Endocrinology* **137**:4235-4242
14. Friesema ECH, Docter R, Krenning EP, Everts ME, Hennemann G & Visser TJ 1998 Rapid sulfation of 3,3',5'-triiodothyronine in native *Xenopus laevis* oocytes. *Endocrinology* **139**:596-600

15. Friesema ECH, Docter R, Moerings EPCM, Stieger B, Hagenbuch B, Meier PJ, Krenning EP, Hennemann G & Visser TJ 1999 Identification of thyroid hormone transporters. *Biochem Biophys Res Comm* **254**:497-501
16. Hennemann G, Docter R, Friesema ECH, de Jong M, Krenning EP & Visser TJ 2001 Plasma membrane transport of thyroid hormones and its role in thyroid hormone metabolism and bioavailability. *Endocrine Rev* **22**:451-476
17. de Jong M, Docter R, van der Hoek HJ, Krenning EP, van der Heide D, Quero C, Plaisier P, Vos RA & Hennemann G 1994 Different effects of amiodarone on transport of T4 and T3 into perfused rat liver. *Am J Physiol* **266**:E44-49
18. Klein I, Ojamaa K, Samarel AM, Welikson R & Hong C 1992 Hemodynamic regulation of myosin heavy chain gene expression. Studies in the transplanted rat heart. *J Clin Invest* **89**:68-73
19. Klein I & Ojamaa K 2001 Thyroid hormone and the cardiovascular system. *N Engl J Med* **344**:501-509
20. Kragie L 1994 Membrane iodothyronine transporters. Part I: Review of physiology. *Endocr Res* **40**:319-341
21. Krenning EP, Docter R, Bernard HF, Visser TJ & Hennemann G 1979 The essential role of albumin in the active transport of thyroid hormones into primary cultured rat hepatocytes. *FEBS Lett* **107**:227-230
22. Lai CS, Korytowski W, Niu CH & Cheng SY 1985 Transverse motion of spin-labeled 3,3',5-triiodo-L-thyronine in phospholipid bilayers. *Biochem Biophys Res Comm* **131**:408-412
23. Liu Q, Clanachan AS & Lopaschuk GD 1998 Acute effects of triiodothyronine on glucose and fatty acid metabolism during reperfusion of ischemic rat hearts. *Am J Physiol* **275**:E392-E399
24. Mol JA & Visser TJ 1985 Synthesis and some properties of sulfate esters and sulfamates of iodothyronines. *Endocrinology* **117**:1-7
25. Mol JA, Krenning EP, Docter R, Rozing J & Hennemann G 1986 Inhibition of iodothyronine transport into rat liver cells by a monoclonal antibody. *J Biol Chem* **261**:7640-7643
26. Muller A, Zuidwijk MJ, Simonides WS & van Hardeveld C 1997 Modulation of SERCA2 expression by thyroid hormone and norepinephrine in cardiocytes: role of contractility. *Am J Physiol* **272**:H1876-H1885
27. Ojamaa K & Klein I 1993 *In vivo* regulation of recombinant cardiac myosin heavy chain gene expression by thyroid hormone. *Endocrinology* **132**:1002-1006
28. Ojamaa K, Klemperer JD, MacGilvray SS, Klein I & Samarel AM 1996 Thyroid hormone and hemodynamic regulation of beta-myosin heavy chain promoter in the

heart. *Endocrinology* **137**:802-808

29. Palacín M, Werner A, Dittmer J, Murer H & Biber J 1990 Expression of rat liver Na^+/L-alanine co-transport in *Xenopus laevis* oocytes. Effect of glucagon *in vivo*. *Biochem J* **270**:189-195
30. Palacín M, Estévez R, Bertran J & Zorzano A 1998 Molecular biology of mammalian plasma membrane amino acid transporters. *Physiol Rev* **78**:969-1054
31. Pardridge WM & Mietus LJ 1980 Influx of thyroid hormones into rat liver *in vivo*. Differential availability of thyroxine and triiodothyronine bound by plasma proteins. *J Clin Invest* **66**:367-374
32. Polikar R, Burger MD, Scherrer U & Nicod P 1993 The thyroid and the heart. *Circulation* **87**:1435-1441
33. van der Putten HHAGM, Joosten BJLJ, Klaren PHM & Everts ME 2001 Characterization of uptake and compartmentalization of 3,5,3'-triiodothyronine in cultured neonatal rat cardiomyocytes. *J Endocrinol* **171**:183-192
34. Rao GS, Eckel J, Rao ML & Breuer H 1976 Uptake of thyroid hormone by isolated liver cells. *Biochem Biophys Res Comm* **73**:98-104
35. Sambrook J, Fritsch EF & Maniatis T 1989 Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory Press, United States of America
36. Segal J 1989 A rapid, extranuclear effect of 3,5,3'-triiodothyronine on sugar uptake by several tissues in the rat *in vivo*. Evidence for a physiological role for the thyroid hormone action at the level of the plasma membrane. *Endocrinology* **24**:2755-2764
37. Segal J & Ingbar SH 1990 3,5,3'-Tri-iodothyronine enhances sugar transport in rat thymocytes by increasing the intrinsic activity of the plasma membrane sugar transporter. *J Endocrinol* **124**:133-140
38. Vasilets LA, Darmer D, Isenberg G & Schwarz W 2001 RNA from heart of young and old rats leads to the expression of protein(s) in *Xenopus* oocytes that alter the transport activity of rat Na^+,K^+-ATPases differently. *Pflügers Arch* **443**:84-91
39. Verhoeven FA, Moerings EPCM, Lamers JMJ, Hennemann G, Visser TJ & Everts ME 2001 Inhibitory effects of calcium blockers on thyroid hormone uptake in neonatal rat cardiac myocytes. *Am J Physiol* **21**:H1985-H1991
40. Visser TJ 1988 Metabolism of thyroid hormone. In: Cooke BA, King RBJ & van der Molen HJ (eds) Hormones and Their Actions Part 1. Elsevier Science Publishers BV, Amsterdam, pp. 81-103
41. Visser TJ 1994 Sulfation and glucuronidation pathways of thyroid hormone metabolism. In: Wu SY & Visser TJ (eds) Thyroid hormone metabolism: molecular biology and alternate pathways. CRC Press, Boca Raton, pp. 85-117
42. Warnick PR, Davis FB, Mylotte KM, Davis PJ, Dube MP & Blas SD 1988 Calcium

channel blocker inhibition of the calmodulin-dependent effects of thyroid hormone and milrinone on rabbit myocardial membrane Ca^{2+}-ATPase activity. *Biochem Pharmacol* **37**:2619-2623

43. Weisiger RA, Luxon BA & Cavalieri RR 1992 Hepatic uptake of 3,5,3'-triiodothyronine: electrochemical driving forces. *Am J Physiol* **262**:G1104-G1112
44. Wolkoff AW 1996 Hepatocellular sinusoidal membrane organic anion transport and transporters. *Sem Liver Dis* **16**:121-127
45. Yusta B, Ortiz-Caro J, Pascual A & Aranda A 1988 Mechanism of L-triiodothyronine (T_3) uptake by glial C6 cells: regulation by butyrate. *Mol Cell Endocrinol* **58**:191-198

CHAPTER 6

T_3 transport by rFAT

Thyroid Hormone Transport by the Rat Fatty Acid Translocase

H.H.A.G.M. van der Putten, E.C.H. Friesema, N.A. Abumrad,
M.E. Everts and T.J. Visser.
Submitted

6.1 Abstract

We examined the hypothesis that rFAT, rat fatty acid translocase, mediates cellular uptake of T_3 and other iodothyronines. Uninjected *Xenopus laevis* oocytes and oocytes injected 4 days previously with rFAT cRNA were incubated for 60 min at 25 C in medium containing 0.01-10 µM [^{125}I]T_3 and 0.1% BSA, or 1-100 µM [^{3}H]oleic acid and 0.5% BSA. Injection of rFAT cRNA resulted in an 1.9-fold increase in uptake of T_3 (10 nM) and an 1.4-fold increase in uptake of oleic acid (100 µM). Total T_3 uptake was lower in the presence than in the absence of BSA, but relative to the free T_3 concentration uptake was increased by BSA. The fold induction of T_3 upake by rFAT was not influenced by BSA. By analyzing uptake as function of the ligand concentration, we estimated a K_m value of 3.6 µM for (total) T_3 and 56 µM for (total) oleic acid. In addition to T_3, rFAT also mediates uptake of T_4, rT_3, 3,3'-diiodothyronine and T_3 sulfate. Injection of human type III deiodinase cRNA with or without rFAT cRNA resulted in the complete deiodination of T_3 taken up by the oocytes, indicating that it is indeed transported to the cytoplasm. In conclusion, our results demonstrate transport of T_3 and other iodothyronines by the rat fatty acid translocase.

Key words: expression cloning, iodothyronines, oleic acid, oocytes, uptake

6.2 Introduction

Thyroid hormones, most importantly triiodothyronine (T_3), induce a rise in cardiac contractility and frequency (Polikar *et al.*, 1993; Klein & Ojamaa, 2001). To meet the energy requirements necessary for its work, the heart depends on uptake of fatty acids (Neely & Morgan, 1974) for supplying around 70% of the energy demand, while oxidation of carbohydrates such as glucose and lactate accounts for the remaining 30%. The contribution of fatty acids to cardiac energy production may change depending on the subject's activity or under pathological conditions. During hyperthyroidism, fatty acid oxidation and the expression of uncoupling proteins (UCPs) in the heart is enhanced (Paradies *et al.*, 1996; Djouadi *et al.*, 1997; Wrutniak-Cabello *et al.*, 2001). Van der Lee *et al.* (2000) showed that UCP expression in the heart is regulated by thyroid hormones, though only in the presence of fatty acids. Fatty acid synthesis in heart and other organs is also regulated by thyroid hormones (Blennemann *et al.*, 1995). Regulation of fatty acid oxidation and synthesis is initiated by binding of T_3 to the nuclear T_3 receptors (Djouadi *et al.*, 1997; Wrutniak-Cabello *et al.*, 2001). Yamamoto *et al.* (2001) showed that the binding of T_3 to its receptors in nuclei isolated from heart tissue is inhibited by fatty acids.

The above findings suggest multiple interactions between thyroid hormones and fatty acids in the heart. The intracellular action and utilization of thyroid hormones and fatty acids in the heart require the transport of these compounds across the plasma membrane of the cardiomyocyte. For uptake of fatty acids, three types of transport proteins have been identified: fatty acid transport protein (FATP), plasma membrane fatty acid binding protein (FABPpm) and fatty acid translocase (FAT) (Glatz & van der Vusse, 1996; Abumrad *et al.*, 1999; Frohnert & Bernlohr, 2000). FAT expression is abundant in adipose tissue, skeletal muscle and heart (Abumrad *et al.*, 1999; van Nieuwenhoven *et al.*, 1999; Pelsers *et al.*, 1999), and in the latter tissue FAT was documented *in vivo* to facilitate a major fraction of fatty acid uptake (Coburn *et al.*, 2000; Yoshizumi *et al.*, 2000). FAT mRNA is upregulated when fatty acid utilization increases (Greenwalt *et al.*, 1995). Furthermore, muscle contraction is acutely associated with translocation of FAT from intracellular sites to the plasma membrane (Bonen *et al.*, 2000).

FAT is an 88 kDa integral membrane protein, which presumably has two membrane-

spanning regions. In humans, FAT is identical to the cell surface antigen CD36 (Abumrad *et al.*, 1993). In addition to fatty acid uptake, FAT/CD36 has been implicated in the binding of plasma lipoproteins and anionic phospholipids (Abumrad *et al.*, 1999). For thyroid hormones, no cardiac transport protein has yet been identified, although we (Everts *et al.*, 1996; van der Putten *et al.*, 2001a; Verhoeven *et al.*, 2001) and the group of Rosic *et al.* (2001) have shown that cardiomyocytes exhibit a specific mechanism for the uptake of T_3. Other groups reported on the existence of such mechanisms in other cell types, including liver cells (Kragie, 1994; Hennemann *et al.*, 2001). Interestingly, in liver cells, non-esterified fatty acids inhibit thyroid hormone uptake (Lim *et al.*, 1993). In addition, FAT antisense mRNA expression in rat preadipocytes is associated with reduced FAT protein levels, decreased fatty acid uptake and, moreover, inhibition of the effect of T_3 on the differentiation of these cells to adipocytes (Sfeir *et al.*, 1999). These observations and the high abundance of FAT in heart prompted us to test the hypothesis that FAT mediates the uptake of T_3. This appears an attractive hypothesis if it is considered that FAT transports (anionic) fatty acids and that iodothyronines are ligands for different organic anion transporters (Abumrad *et al.*, 1999; Hennemann *et al.*, 2001).

Xenopus laevis oocytes have been used successfully for the functional cloning of cDNAs encoding several plasma membrane transporters (Hediger *et al.*, 1987; Meier, 1996; Wolkoff, 1996; Palacín *et al.*, 1998). We used this system to express rat FAT (rFAT) cRNA to examine uptake of T_3 and other iodothyronines, and compare this with the uptake of oleic acid, a preferred fatty acid ligand for FAT.

6.3 Materials and methods

Animals

Two to 3-yr old *Xenopus laevis* were obtained from Amrep (Breda, The Netherlands) and maintained as described previously (Docter *et al.*, 1997; Friesema *et al.*, 1998). Under MS-222 anesthesia (Sigma, St. Louis, MO; 1 g/l 3-aminobenzoic acetic ethyl ester, in tap water) and hypothermia, ovarian fragments were removed for the isolation of oocytes (Docter *et al.*, 1997).

Materials

Iodothyronines were obtained from Henning (Berlin, Germany), oleic acid and bovine serum albumin (BSA) (fraction V) from Sigma, and L-tryptophan (Trp) from Fluka (Buchs, Switzerland). [^{125}I]T_3 (81.4 TBq/mmol) was purchased from NEN (Boston, MA), and [^{125}I]T_4 (35.6 TBq/mmol), [9,10(n)-^{3}H]oleic acid (296 GBq/mmol), and [^{3}H]Trp (1.11 TBq/mmol) from Amersham (Amersham, UK). All other ^{125}I-labeled iodothyronines were prepared as described previously (Mol & Visser, 1985).

RNA preparation

A 1.5 kb rFAT cDNA fragment was isolated from pSG5-rFAT (Greenwalt *et al.*, 1995) using *Eco*RI and *Xba*I (Promega, Leiden, The Netherlands), and subcloned into the multiple cloning region of pGEM3Z, located in between the 5'- and 3'-untranslated regions (including poly-A tail) of the *Xenopus laevis* β-globin gene (Patient *et al.*, 1983). Capped rFAT cRNA and human type III deiodinase (hD3) (Salvatore *et al.*, 1995) cRNA were prepared from the cDNA clones linearized with *Nhe*I and *Xba*I (Promega), respectively, using the T7 RNA transcription kit (Epicentre, Madison, WI). For capping the m7G[5']ppp[5']G cap analog was used (Epicentre). cRNAs were stored in water at –80 C.

Oocyte isolation and RNA injection

Oocytes were prepared as described previously (Docter *et al.*, 1997). After a 3-h collagenase B (2 mg/ml; Roche, Mannheim, Germany) treatment, the isolated oocytes were selected manually using morphological criteria such as size, pigmentation, and absence of follicular debris. Healthy looking stage V-VI oocytes (Dumont, 1972) were transferred to six-well tissue culture plates and incubated overnight at 18 C in modified Barth's solution (88 mM NaCl, 1 mM KCl, 0.82 mM $MgSO_4$, 0.4 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, 2.4 mM $NaHCO_3$, 10 mM HEPES, pH 7.4), containing 20 IU/ml penicillin, and 20 μg/ml streptomycin. The next day, oocytes were injected with 23 nl water containing 4.6 ng rFAT cRNA and/or 2.3 ng hD3 cRNA using the Nanoject system (Drummond, Broomall, PA). In experiments in which the uptake was examined as a function of rFAT cRNA concentration, oocytes were injected with 23 nl water containing 1.2 to 9.2 ng cRNA. Uninjected oocytes were used as controls, but similar results were obtained using water-injected oocytes (data not shown).

Injected and uninjected oocytes were maintained for 3 to 4 days at 18 C in modified Barth's solution, with a daily change of medium.

Uptake

Uptake assays were performed as described previously (Docter *et al.*, 1997). Four days after injection, 8 to 10 oocytes were incubated in 100 µl incubation medium (100 mM NaCl or 100 mM choline chloride, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES and 10 mM Tris, pH 7.5), supplemented with 4×10^5 cpm [^{125}I]T_3, 0.01 to 100 µM unlabeled T_3 and 0.1% BSA, or 4×10^5 cpm [^{3}H]oleic acid, 1 to 100 µM unlabeled oleic acid and 0.5% BSA. Similarly, uptake of 10 nM ^{125}I-labeled 3,3'-diiodothyronine (3,3'-T_2), T_4 and rT_3 was examined in medium with 0.1% BSA, and that of 10 nM [^{125}I]T3 sulfate (T_3S) and 10 µM [^{3}H]Trp in medium without BSA. The ligand and BSA concentrations were adjusted using 5-fold concentrated stock solutions of iodothyronine and oleic acid in medium with 0.5 or 2.5% (wt/vol) BSA, respectively. To test the effect of albumin on T_3 uptake, uninjected and injected oocytes were incubated with 10 nM T_3 with 0, 0.1 or 0.5% BSA. The free T_3 fraction under these conditions is 8% in the presence of 0.1% BSA, and 2.5% in the presence of 0.5% BSA, as determined by equilibrium dialysis (van der Putten *et al.*, 2001a). The fraction of free or unbound fatty acid was calculated using a computer routine based on the association constants for oleate:BSA binding determined by Richieri *et al.* (1993).

After 1 h, the incubation was terminated by aspiration of the incubation medium, followed by washing of the oocytes 4 times with 2.5 ml ice-cold Na^+-containing incubation medium supplemented with 0.1% BSA. Oocytes were transferred to new tubes and analyzed individually for ^{125}I-activity in a γ-counter (Nuclear Enterprise, Edinburgh, UK). For analysis of ^{3}H-activity associated with the oocytes, individual oocytes were transferred to scintillation vials and lysed in 0.2 ml 2% (wt/vol) SDS. Subsequently, 4 ml scintillation fluid was added (Pico-fluor, Packard, Groningen, The Netherlands), and the radioactivity was determined in a Tri-carb 2100TR liquid scintillation analyzer (Packard).

Metabolism

Three days after injection, groups of 10 oocytes were incubated for 60 min at 25 C in medium containing 10 nM (1.5×10^6 cpm) [^{125}I]T_3 and 0.1% BSA, as described above.

Prior to preparation for HPLC analysis, groups of oocytes were counted for radioactivity, providing uptake values comparable with individually analyzed oocytes. For HPLC analysis, the oocytes were homogenized in 100 µl ice-cold methanol. After centrifugation (15 min, 2500 × *g*, 4 C), 75 µl of the supernatant was mixed with 50 µl 0.02 M ammonium acetate (pH 4.0). 100 µl of the mixture was applied to a 4.6 × 250 mm Symmetry C18 column connected to an Alliance HPLC system (Waters, Etten-Leur, The Netherlands), and eluted with a gradient of acetonitrile in 0.02 M ammonium acetate (pH 4.0) at a flow of 1.2 ml/min. The proportion of acetonitrile was increased in 15 min from 28% to 42%. The radioactivity in the eluate was determined using a Radiomatic A-500 flow scintillation detector (Packard, Meriden, CT).

Statistics and calculations

Data are presented as means ± SEM. Statistical significance was evaluated by repeated measures ANOVA or *t*-test, where appropriate. Statistical significance was accepted at $P < 0.05$.

6.4 Results

Initial experiments were carried out to confirm that rFAT, in our expression system, induced uptake of oleic acid as reported by others (Abumrad *et al.*, 1993; Ibrahimi *et al.*, 1996; Sfeir *et al.*, 1999). Injection of rFAT cRNA increased uptake of oleic acid (100 µM) in the presence of 0.5% BSA) by 1.4-fold compared with uninjected oocytes ($P < 0.05$) (Fig. 1). Oleic acid uptake by uninjected and rFAT cRNA-injected oocytes was not inhibited by replacement of Na^+ in the medium with choline (data not shown), indicating that oleic acid uptake is Na^+ independent. Figure 1 also shows the uptake of 10 nM T_3 in presence of 0.1% BSA by uninjected and rFAT cRNA-injected oocytes. rFAT cRNA-injected oocytes show an 1.9-fold induction of T_3 uptake ($P < 0.01$), which was also Na^+ independent (data not shown). Note that, there is significant uptake of T_3 and oleic acid in uninjected oocytes.

Figure 2 shows that the uptake of 10 nM T_3 in medium containing 0.1% BSA, corrected for uptake in uninjected oocytes, was stimulated by injection of increasing amounts of rFAT cRNA, reaching a near-maximum level after injection of 4.6 ng

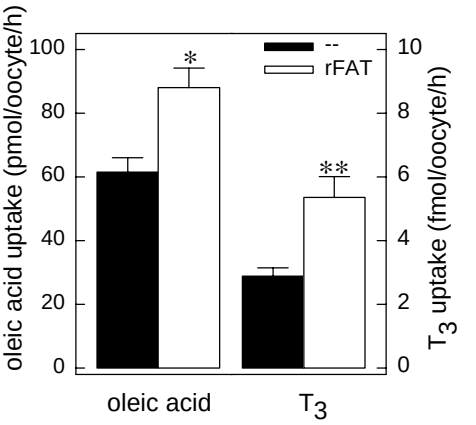

Figure 1 Oleic acid uptake and T_3 uptake in uninjected oocytes (black bars) and in oocytes injected with 4.6 ng rFAT cRNA (white bars). Oocytes were incubated for 60 min at 25 C in medium supplemented with 100 µM [^{3}H]oleic acid and 0.5% BSA or 10 nM [^{125}I]T_3 and 0.1% BSA. Data are presented as means ± SEM of 4 or 9 experiments, respectively. *) $P < 0.05$, *vs.* uninjected oocytes; **) $P < 0.01$ *vs.* uninjected oocytes.

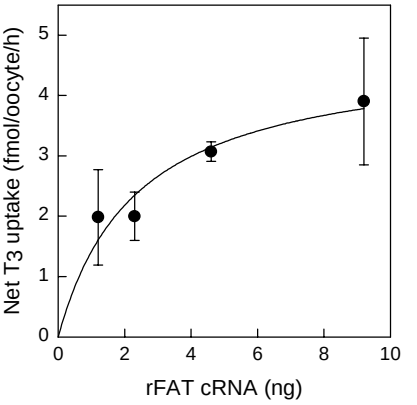

Figure 2 Induction of 10 nM [^{125}I]T_3 uptake by oocytes after injection with 1.2 – 9.2 ng rFAT cRNA. Oocytes were incubated for 60 min at 25 C in medium supplemented with 0.1% BSA. Data are presented as means ± SEM of 2-3 experiments and were corrected for uptake in uninjected oocytes.

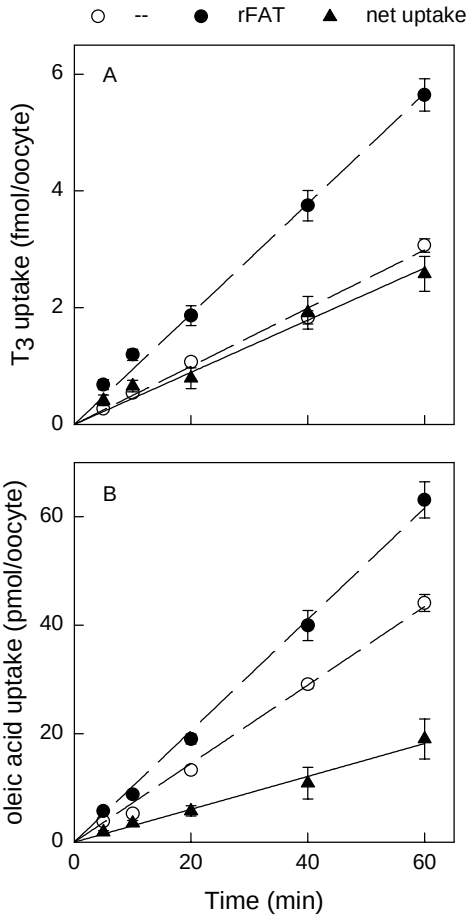

Figure 3 Time-course of uptake of T_3 (A) and oleic acid (B) by uninjected oocytes (open circles) or oocytes injected with 4.6 ng rFAT cRNA (closed circles). Oocytes were incubated for 5–60 min at 25 C in medium containing 10 nM [^{125}I]T_3 and 0.1% BSA or 75 µM [^{3}H]oleic acid and 0.5% BSA. Uptake induced by expression of rFAT cRNA was calculated by subtraction of uptake in uninjected oocytes from that observed in injected oocytes (closed triangles). The plotted lines show the result of a regression analysis of the data points using the linear function $y=ax$. Data are the means ± SEM of 10 oocytes in a representative experiment (out of 3).

cRNA (3.1 ± 0.2 fmol/oocyte/h). Injection of 2.3 or 4.6 ng rFAT cRNA induced uptake of oleic acid (75 µM in the presence of 0.5% BSA) from 44.1 ± 0.9 (uninjected) to 56.3 ± 4.3 and 68.5 ± 9.2 pmol/oocyte/h, respectively (n=2, data not shown). In all further experiments 4.6 ng rFAT cRNA was injected per oocyte.

Figure 3 shows the uptake of 10 nM T_3 in the presence of 0.1% BSA (A) and of 75 µM oleic acid in presence of 0.5% BSA (B) as a function of incubation time. Net uptake was calculated by subtracting uptake values measured in uninjected oocytes from that in rFAT cRNA-injected oocytes. Total and net uptake of T_3 and oleic acid was linear with incubation time for at least 60 min.

From Fig. 3, we chose an incubation-time of 60 min to measure unidirectional uptake rates of T_3 (Fig. 4A) and of oleic acid (Fig. 4B). T_3 and oleic acid uptake through endogenous transporter(s) was determined in parallel incubations with uninjected oocytes. Uptake mediated by rFAT (white bars) was determined by subtraction of the uptake in uninjected oocytes (black bars) from that in oocytes injected with rFAT cRNA (hatched bars). rFAT-induced T_3 uptake was saturable, with almost complete inhibition of fractional T_3 uptake at a ligand concentration of 10 µM. Endogenous T_3 uptake showed only partial saturation at 10 µM T_3 (Fig. 4A). rFAT-mediated oleic acid transport was also saturable, showing a 46% inhibition of fractional oleic acid uptake at the highest ligand concentration tested (100 µM) (Fig. 4B). By Lineweaver-Burk analysis of the rFAT-mediated T_3 uptake as a function of the total T_3 concentration, we estimated an apparent K_m value of 3.6 µM. In terms of the free T_3 concentration, the apparent K_m value amounted to 0.28 µM. For (total) oleic acid, the estimated K_m value was 56 µM. In the presence of 0.5% (75 µM) BSA, this corresponds to a K_m value of 5 nM for unbound oleic acid. This value is in close agreement with that determined for oleate uptake by several cell types (Abumrad *et al.*, 1999), by isolated rat heart giant sarcolemmal vesicles (Luiken *et al.*, 1999) and by CD36-transfected cells (Ibrahimi *et al.*, 1996).

To test the effects of albumin on T_3 uptake induced by rFAT, we incubated uninjected oocytes and rFAT cRNA-injected oocytes with T_3 in incubation medium with 0, 0.1 or 0.5% BSA. Figure 5A shows that increasing concentrations of BSA resulted in lower T_3 uptake values in both uninjected and rFAT cRNA-injected oocytes. However, when uptake rates were expressed relative to the free T_3 concentration (Fig. 5B), incubations with 0.1 or 0.5% BSA resulted in 4- to 5-fold higher uptake values in

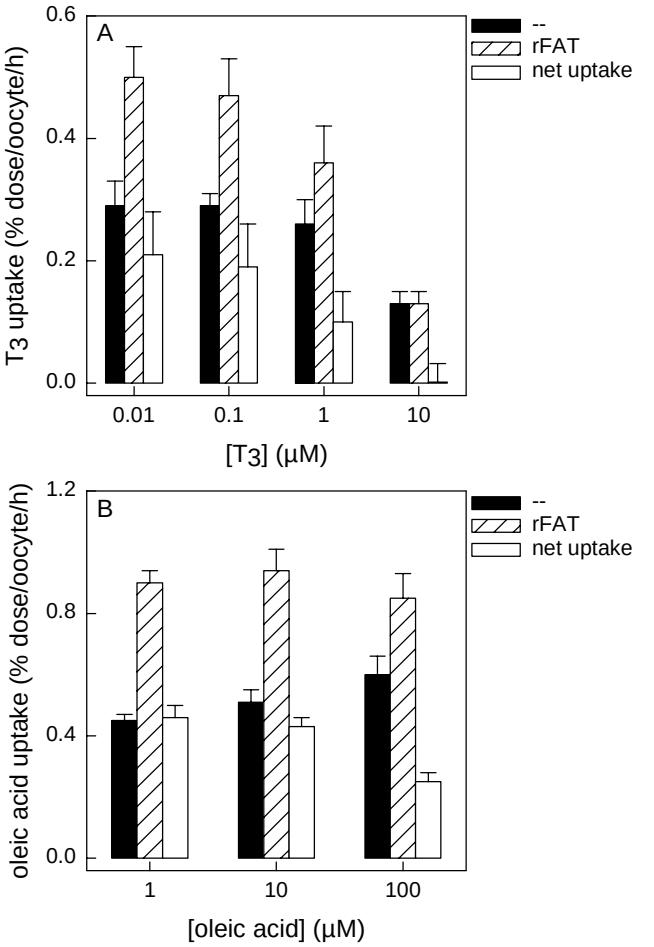

Figure 4 Ligand concentration-dependent uptake of [^{125}I]T_3 (A) and [^{3}H]oleic acid (B) by uninjected oocytes (black bars) and rFAT cRNA (4.6 ng) injected oocytes (hatched bars). Oocytes were incubated for 60 min at 25 C in medium containing 0.01–10 µM [^{125}I]T_3 and 0.1% BSA, or 1–100 µM [^{3}H]oleic acid and 0.5% BSA. Uptake induced by expression of rFAT cRNA was calculated by subtraction of uptake in uninjected oocytes from that observed in injected oocytes (white bars). Data are expressed in % dose per oocyte/h and presented as means ± SEM of 3 experiments.

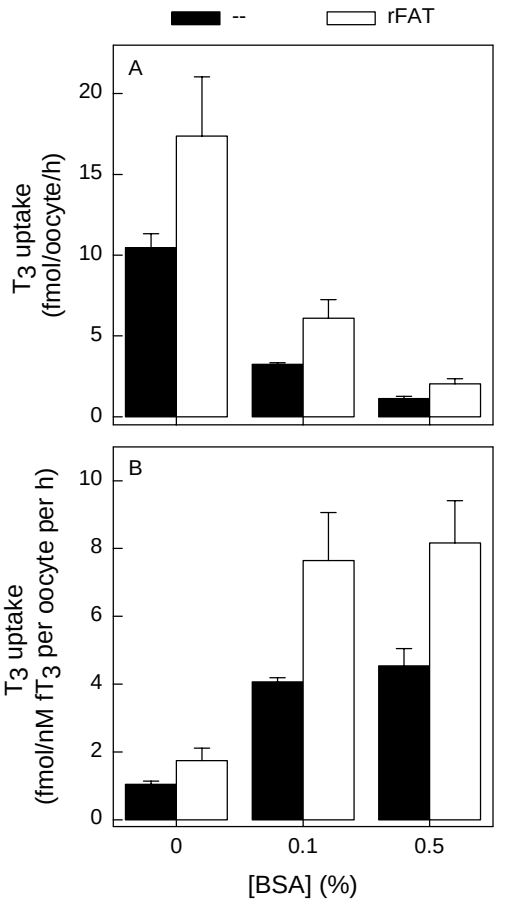

Figure 5 (A) Effects of BSA on [^{125}I]T_3 uptake by uninjected oocytes (black bars) and oocytes injected with 4.6 ng rFAT cRNA (white bars) expressed in femtomoles per oocyte/h (A) or femtomoles per nM free T_3 per oocyte/h (B). Oocytes were incubated for 60 min at 25 C in medium containing 10 nM [^{125}I]T_3 without or with 0.1 or 0.5% BSA. Data are presented as means ± SEM of 4 experiments.

both uninjected and rFAT cRNA-injected oocytes. Uptake of T_3 in the presence of 0, 0.1 or 0.5% BSA was induced 1.7-, 1.8- and 1.8-fold, respectively, after injection of rFAT cRNA.

To examine intracellular metabolism of T_3, uninjected oocytes and oocytes injected with rFAT cRNA and/or hD3 cRNA were incubated for 1 h at 25 C with 10 nM T_3 and 0.1% BSA. Oocytes injected with hD3 cRNA alone showed similar T_3 uptake as uninjected oocytes (3.7 ± 0.3 *vs.* 3.5 ± 0.2 fmol/oocyte/h). Also, oocytes injected with rFAT cRNA plus hD3 cRNA showed similar T_3 uptake as oocytes injected with rFAT cRNA alone (4.5 ± 0.3 *vs.* 4.7 ± 0.2 fmol/oocyte/h) (data not shown). Metabolism of

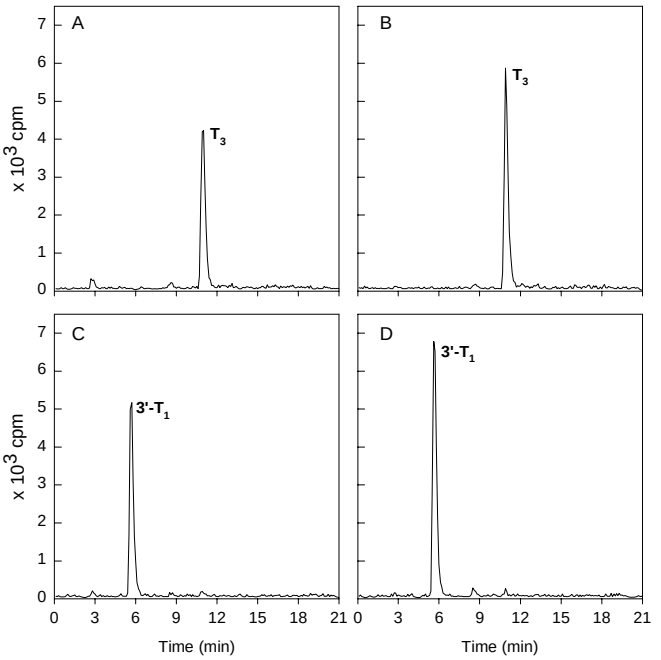

Figure 6 HPLC chromatograms of oocyte lysate obtained from pools of 10 uninjected oocytes (A) or, pools of 10 oocytes injected with 4.6 ng rFAT cRNA (B), 2.3 ng hD3 cRNA (C) or the combination of rFAT cRNA and hD3 cRNA (D) after incubation for 60 min at 25 C with 10 nM [^{125}I]T_3 and 0.1% BSA. Retention times are 5.6, 8.8, and 10.9 min for 3'-T_1, 3,3'-T_2, and T_3, respectively.

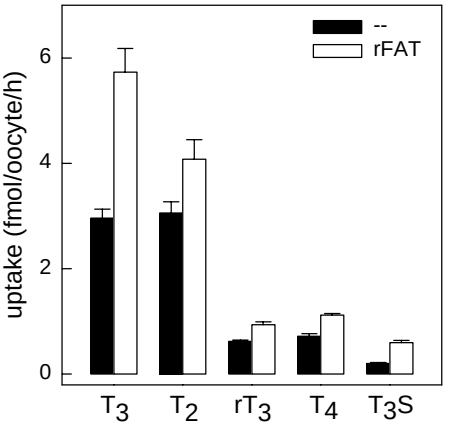

Figure 7 Uptake of 10 nM ^{125}I-labeled iodothyronines by uninjected oocytes (black bars) and oocytes injected with 4.6 ng rFAT cRNA (white bars). Oocytes were incubated for 60 min at 25 C with 10 nM [^{125}I]T_3, [^{125}I]3,3'-T_2, [^{125}I]rT_3 or [^{125}I]T_4 and 0.1% BSA, or 10 nM [^{125}I]T_3S without BSA. Data are the means ± SEM of 9-10 oocytes in a representative experiment (out of 2).

T_3 taken up by uninjected and injected oocytes was analyzed by HPLC, and the results are presented in Fig. 6. In oocytes injected with hD3 cRNA alone (Fig. 6C) or with hD3 cRNA plus rFAT cRNA (Fig. 6D), all T_3 taken up by these oocytes was converted to 3'-T_1, whereas in uninjected oocytes (Fig. 6A) and in oocytes injected with rFAT cRNA alone (Fig. 6B), T_3 was not metabolized.

Finally, uptake of T_3, 3,3'-T_2, rT_3, T_4 (in the presence of 0.1% BSA) and T_3S (in the absence of BSA) was determined in uninjected and rFAT cRNA-injected oocytes (Fig. 7). Injection of rFAT cRNA resulted in an increased uptake of all iodothyronine derivatives, which decreased in the order $T_3 > 3,3'\text{-}T_2 > T_4 \approx T_3S \approx rT_3$. Uptake of Trp was not increased by injection of rFAT cRNA (not shown), indicating that the rFAT-induced increase in iodothyronine and fatty acid uptake was not due to a non-specific effect on the oocyte cell membrane.

6.5 Discussion

This is the first report showing that a fatty acid transporter, rFAT, transports T_3 and other iodothyronines. Uptake of T_3 is Na^+-independent and shows saturation at increasing ligand concentrations, with an estimated K_m value of 3.6 µM total T_3 and 0.3 µM free T_3. These values are well within the range of K_m values (1.5-7 µM) for other transporters mediating T_3 uptake after expression in oocytes (see below), although these studies were carried out in the absence of BSA (Abe *et al.*, 1996, 1999; Friesema *et al.*, 2001b; Hennemann *et al.*, 2001). In addition, we confirmed that rFAT, when expressed in *Xenopus laevis* oocytes, exhibits Na^+-independent oleic acid transport, as reported previously in other cell expression systems (Ibrahimi *et al.*, 1996).

In addition to T_3, rFAT also transports other iodothyronines, showing a substrate preference for $T_3 > 3,3'\text{-}T_2 > T_4 \approx rT_3$ as tested at 10 nM substrate concentration in the presence of 0.1% BSA. Tested in the absence of BSA, T_3S was also found to be transported by rFAT. The free iodothyronine fractions in these experiments may be approximated as 100% for T_3S, 8% for T_3 and 3,3'-T_2, 3% for rT_3, and 0.8% for T_4 (this study; Ref. (Everts *et al.*, 1995). If the rFAT-mediated transport is considered relative to the approximate free hormone concentration, the substrate preference decreases in the order $T_4 \approx T_3 > 3,3'\text{-}T_2 \approx rT_3 > T_3S$.

In addition to rFAT, various other transporters were shown to exhibit thyroid hormone transport activity. These include 1) the Na^+-dependent organic anion transporter (NTCP), 2) members of the multi-specific Na^+-independent organic anion transporter (OATP) family, and 3) the L type heterodimeric amino acid transporter, comprised of the human 4F2 heavy chain (*h*4F2hc) and the LAT1 or LAT2 light chains (Hennemann *et al.*, 2001). In agreement with our previous study in neonatal rat cardiomyocytes showing that T_3 uptake is Na^+-independent (van der Putten *et al.*, 2001a), we show here that T_3 transport mediated by rFAT is Na^+-independent. Since NTCP is a Na^+-dependent transporter that is expressed exclusively in the liver, it does not play a role in T_3 uptake in the heart (Hagenbuch *et al.*, 1991). Recently, Fujiwara *et al.* (2001) cloned a novel human organic anion transporter OATP-E, which mediates Na^+-independent uptake of T_3. The mRNA encoding OATP-E is present in heart and various other tissues. The physiological relevance of thyroid hormone transport in heart by OATP-E remains to be established. On the other hand, in one of our previous

studies (van der Putten *et al.*, 2001a), we showed that T_3 uptake by neonatal cardiomyocytes is not inhibited by sulfobromophthalein (BSP), which is a ligand for OATP-E (Fujiwara *et al.*, 2001).

Ritchie *et al.* (1999) and Friesema *et al.* (2001b) showed induction of T_3 uptake by the heterodimeric system L amino acid transporter (LAT) expressed in *Xenopus laevis* oocytes. The presence of its subunits in heart tissue (Carsten *et al.*, 2001) suggests that this transporter plays a role in T_3 uptake in heart. However, 2-amino-bicyclo[2,2,1]-heptanecarboxylic acid (BCH), the specific system L ligand, has no effect on uptake of T_3 in neonatal rat cardiomyocytes. Therefore, we concluded that the system L amino acid transporter is not involved in T_3 uptake in neonatal heart (van der Putten *et al.*, 2001a). Because, T_3 uptake in neonatal cardiomyocytes was partly reduced by the aromatic amino acids, tryptophan and tyrosine (Everts *et al.*, 1996; van der Putten *et al.*, 2001a), we proposed that amino acid transport system T could be an additional uptake mechanism for T_3 in these cells. Recently, a Na^+-independent aromatic amino acid transporter, TAT1, has been characterized which mediates uptake of tryptophan, tyrosine and phenylalanine but not of T_4 and T_3 (Kim *et al.*, 2001).

Thus, a number of transporters have been identified in heart, which are capable of thyroid hormone transport in a Na^+-independent manner. Results from our *in vitro* cell studies suggest that LAT and rOATP-E are not responsible for T_3 uptake in the neonatal heart, but we cannot exclude that the results will be different in myocytes from adult rat heart. Furthermore, yet unidentified members of these transporter families may be present in heart and exhibit T_3 transport. The 1.9-fold increase in T_3 uptake after injection of oocytes with rFAT cRNA is somewhat higher than the increases observed with other transporters characterized under the same conditions in our laboratory, *i.e.* 1.7-fold for rNTCP (Friesema *et al.*, 1999), 1.7-fold for rOATP1 (Friesema *et al.*, 1999), and 1.5-fold for human LAT (Friesema *et al.*, 2001b). Together with the abundant expression of rFAT in rat heart (van Nieuwenhoven *et al.*, 1999; Pelsers *et al.*, 1999), these results suggest that rFAT is an important transporter for T_3 uptake in the heart. However, the physiological relevance *in vivo* of rFAT and other transporters for cardiac T_3 uptake remains to be established. For rFAT, this question may be addressed in animal models, in which FAT expression is impaired *e.g.* CD36/FAT null mice (Coburn *et al.*, 2000), and spontaneously hypertensive rats (SHR) (Hajri *et al.*, 2001). These models showed defective myocardial fatty acids uptake,

whereas fatty acid uptake was increased in a transgenic mouse model with muscle-targeted overexpression of FAT (Ibrahimi *et al.*, 1999). In addition, CD36 deficiency in humans may underlie defective myocardial fatty acids uptake and some cases of heart disease (Yoshizumi *et al.*, 2000). In addition to these models, the primary culture of neonatal rat cardiomyocytes has proven to be an useful model in the examination of thyroid hormone transport (Everts *et al.*, 1996; van der Putten *et al.*, 2001a; Verhoeven *et al.*, 2001). We will continue to examine the physiological relevance of thyroid hormone uptake by fatty acid transport mechanisms in this model and in the embryonic heart cell line H9c2 (Kimes & Brandt, 1976; van der Putten *et al.*, 2001b).

We observed marked oleic acid uptake in uninjected oocytes which is explained by the presence of endogenous fatty acid transporters (Pelsers *et al.*, 1999). Similar results were found for thyroid hormone uptake by native oocytes in the present and earlier studies (Docter *et al.*, 1997; Friesema *et al.*, 1998). Attempts to find specific inhibitors of these endogenous transporters to diminish the background were not successful. Use of the water-soluble synthetic ligand, T_3 sulfamate (T_3NS), seemed to be attractive, because it combines a low uptake in uninjected oocytes with a similarly high induction as T_3 itself after injection of rat liver mRNA (Friesema *et al.*, 2001a). Unfortunately, neonatal cardiomyocytes showed no uptake of this ligand (unpublished results). Interestingly, also rFAT failed to transport T_3NS (not shown). Therefore, we decided to continue our studies with T_3.

Albumin is one of the thyroid hormone-binding proteins in plasma (Bartalena, 1990). It has been shown that BSA facilitates uptake of thyroid hormones in isolated rat hepatocytes (Krenning *et al.*, 1979; Pardridge & Mietus, 1980). Our results show no difference in fold-stimulation of T_3 uptake induced by rFAT in the presence or absence of 0.1 or 0.5% BSA. Furthermore, the results showed that total T_3 uptake decreased with increasing BSA, in agreement with the general notion that the unbound hormone determines the rate of uptake (Docter & Krenning, 1990; Abumrad *et al.*, 1998). However, when we analyzed the data relative to the free hormone concentration, which decreases with increasing concentration of BSA, T_3 uptake was higher in incubations with BSA than in incubations without BSA. These findings may be explained as follows: with BSA present in the water-layer around the oocyte, the free T_3 concentration is buffered, providing a constant pool of T_3 available for uptake. Furthermore, addition of BSA prevents the loss of thyroid hormones and fatty acids

by adsorption to assay tubes and pipette walls (Abumrad *et al.*, 1998).

Finally, T_3 uptake mediated by rFAT is linear during the first 60 min, suggesting that uptake represents more than just binding of T_3 to the plasma membrane. Metabolism of T_3 by an intracellular enzyme would represent unequivocal proof that T_3 enters the oocytes by rFAT expression on the cell membrane. T_3 is not metabolized by native oocytes and, thus, hD3 cRNA was injected to induce expression of a T_3-metabolizing enzyme in the oocyte. hD3 converts T_3 initially to 3,3'-T_2 and subsequently to 3'-T_1 (Visser, 1988). The results show that in oocytes injected with rFAT plus hD3 cRNA or with hD3 cRNA alone, T_3 is completely converted to 3'-T_1, whereas in oocytes injected with rFAT cRNA alone no conversion was observed. Together with the finding that an increase in T_3 uptake was only observed in oocytes injected with rFAT cRNA, this indicates that T_3 transport mediated by rFAT is independent of the metabolic capacity of the oocyte and is rate- limiting for entry and metabolism of T_3.

In summary, this report shows that rFAT exhibits Na^+-independent T_3 uptake with an estimated K_m of 3.6 µM total T_3 and 0.28 µM free T_3, which was further evidenced by the complete conversion of T_3 into 3'-T_1. Furthermore, rFAT transports other iodothyronines such as 3,3'-T_2, T_4, T_3S and rT_3. We also confirmed the function of rFAT as a fatty acid transporter by inducing uptake of oleic acid. We are currently examining the physiological implications of T_3 uptake in heart via fatty acid transport mechanisms in neonatal rat cardiomyocytes and H9c2 cells.

6.6 Acknowledgements

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6.7 References

1. Abe T, Kakyo M, Sakagamii H, Tokui T, Nishio T, Tanemoto M, Nomura H, Hebert SC, Matsuno S, Kondo H & Yawo H 1996 Molecular characterization and tissue distribution of a new organic anion transporter subtype (oatp3) that transports thyroid hormones and taurocholate and comparison with oatp2. *J Biol Chem* **273**:22395-22401
2. Abe T, Kakyo M, Tokui T, Nakagomi R, Nishio T, Nakai D, Nomura H, Unno M, Suzuki M, Naitoh T, Matsuno S & Yawo H 1999 Identification of a novel gene family encoding human liver-specific organic anion transporter LST-1. *J Biol Chem* **274**:17159-17163
3. Abumrad NA, Raafat El-Maghrabi M, Amri E, Lopez E & Grimaldi PA 1993 Cloning of a rat adipocyte membrane protein implicated in binding or transport of long-chain fatty acids that is induced during preadipocyte differentiation. Homology with human CD36. *J Biol Chem* **268**:17665-17668
4. Abumrad NA, Harmon C & Ibrahimi A 1998 Membrane transport of long-chain fatty acids: evidence for a facilitated process. *J Lipid Res* **39**:2309-2318
5. Abumrad NA, Coburn CT & Ibrahimi A 1999 Membrane proteins implicated in long-chain fatty acid uptake by mammalian cells: CD36, FATP and FABPm. *Biochim Biophys Acta* **1441**:4-13
6. Bartalena L 1990 Recent achievements in studies on thyroid hormone-binding proteins. *Endocrine Rev* **11**:47-64
7. Blennemann B, Leahy P, Kim T & Freake HC 1995 Tissue-specific regulation of lipogenic mRNAs by thyroid hormone. *Mol Cell Endocrinol* **110**:1-8
8. Bonen A, Luiken JJFP, Arumugan Y, Glatz JFC & Tandon NN 2000 Acute regulation of fatty acid uptake involves the cellular redistribution of fatty acid translocase. *J Biol Chem* **275**:14501-14508
9. Carsten W, Lang F & Bröer S 2001 Function and structure of heterodimeric amino acid transporters. *Am J Physiol Cell Physiol* **281**:C1077-C1093
10. Coburn CT, Knapp Jr. FF, Febbraio M, Beets AL, Silverstein RL & Abumrad NA 2000 Defective uptake and utilization of long chain fatty acids in muscle and adipose tissue of CD36 knockout mice. *J Biol Chem* **275**:32523-32529
11. Djouadi F, Riveau B, Merlot-Benichou C & Bastin J 1997 Tissue-specific regulation of medium-chain acyl-CoA dehydrogenase gene by thyroid hormones in the developing rat. *Biochem J* **324**:289-294
12. Docter R & Krenning EP 1990 Role of cellular transport systems in the regulation of thyroid hormone bioactivity. In: Greer MA (ed) The Thyroid Gland. Raven Press,

New York, pp. 233-254

13. Docter R, Friesema ECH, van Stralen PGJ, Krenning EP, Everts ME, Visser TJ & Hennemann G 1997 Expression of rat liver cell membrane transporters for thyroid hormone in *Xenopus laevis* oocytes. *Endocrinology* **138**:1841-1846
14. Dumont JN 1972 Oogenesis in *Xenopus laevis* (Daudin) I. Stages of oocyte development in laboratory maintained animals. *J Morphol* **136**:153-180
15. Everts ME, Visser TJ, Moerings EPCM, Tempelaars AMP, van Toor H, Docter R, de Jong M, Krenning EP & Hennemann G 1995 Uptake of 3,3',5,5'-tetraiodothyroacetic acid and 3,3',5'-triiodothyronine in cultured rat anterior pituitary cells and their effects on thyrotropin secretion. *Endocrinology* **136**:4454-4461
16. Everts ME, Verhoeven FA, Bezstarosti K, Moerings EPCM, Hennemann G, Visser TJ & Lamers JMJ 1996 Uptake of thyroid hormones in neonatal rat cardiac myocytes. *Endocrinology* **137**:4235-4242
17. Friesema ECH, Docter R, Krenning EP, Everts ME, Hennemann G & Visser TJ 1998 Rapid sulfation of 3,3',5'-triiodothyronine in native *Xenopus laevis* oocytes. *Endocrinology* **139**:596-600
18. Friesema ECH, Docter R, Moerings EPCM, Stieger B, Hagenbuch B, Meier PJ, Krenning EP, Hennemann G & Visser TJ 1999 Identification of thyroid hormone transporters. *Biochem Biophys Res Comm* **254**:497-501
19. Friesema ECH, Docter R, Krenning EP, Hennemann G & Visser TJ Role of organic anion transporters in hepatic uptake of thyroid hormone. Proc 73t^{h} Annual Meeting of the American Thyroid Association,, Washington DC, 2001a, p 219
20. Friesema ECH, Docter R, Moerings EPCM, Verrey F, Krenning EP, Hennemann G & Visser TJ 2001b Thyroid hormone transport by the heterodimeric human system L amino acid transporter. *Endocrinology* **142**:4339-4348
21. Frohnert BI & Bernlohr DA 2000 Regulation of fatty acid transporters in mammalian cells. *Prog Lipid Res* **39**:83-107
22. Fujiwara K, Adachi H, Nishio T, Unno M, Tokui T, Okabe M, Onogawa T, Suzuki T, Asano N, Tanemoto M, Seki M, Shiiba K, Suzuki M, Kondo Y, Nunoki K, Shimosegawa T, Ionuma K, Ito S, Matsuno S & Abe T 2001 Identification of thyroid hormone transporters in humans: different molecules are involved in a tissue-specific manner. *Endocrinology* **142**:2005-2012
23. Glatz JFC & van der Vusse GJ 1996 Cellular fatty acid-binding proteins: their function and physiological significance. *Prog Lipid Res* **35**:243-282
24. Greenwalt DE, Scheck SH & Rhinehart-Jones T 1995 Heart CD36 expression is increased in murine models of diabetes and in mice fed a high fat diet. *J Clin Invest* **96**:1382-1388

25. Hagenbuch B, Stieger B, Foguet M, Lübbert H & Meier PJ 1991 Functional expression cloning and characterization of the hepatocyte Na^+/bile acid cotransport system. *Proc Natl Acad Sci USA* **88**:10629-10633
26. Hajri T, Ibrahimi A, Coburn CT, Knapp FFJ, Kurtz T, Pravened M & Abumrad NA 2001 Defective fatty acid uptake in the spontaneously hypertensive rat is a predeterminant of altered glucose metabolism, hyperinsulinemia, and myocardial hypertrophy. *J Biol Chem* **276**:23661-23666
27. Hediger MA, Coady MJ, Ikeda TS & Wright EM 1987 Expression cloning and cDNA sequencing of the Na^+/glucose co-transporter. *Nature* **330**:379-381
28. Hennemann G, Docter R, Friesema ECH, de Jong M, Krenning EP & Visser TJ 2001 Plasma membrane transport of thyroid hormones and its role in thyroid hormone metabolism and bioavailability. *Endocrine Rev* **22**:451-476
29. Ibrahimi A, Sfeir Z, Magharaie H, Amri E, Grimaldi PA & Abumrad NA 1996 Expression of the CD36 homolog (FAT) in fibroblast cells: Effects on fatty acid transport. *Proc Natl Acad Sci USA* **93**:2646-2651
30. Ibrahimi A, Bonen A, Blinn WD, Hajri T, Li X, Zhong K, Cameron R & Abumrad NA 1999 Muscle-specific overexpression of FAT/CD36 enhances fatty acid oxidation by contracting muscle, reduces plasma triglycerides and fatty acids, and increases plasma glucose and insulin. *J Biol Chem* **274**:26761-26766
31. Kim DK, Kanai Y, Chairoungdhua A, Matsuo H, Cha SH & Endou H 2001 Expression cloning of a Na^+-independent aromatic amino acid transporter with structural similarity to H^+/monocarboxylaat transporters. *J Biol Chem* **276**:17221-17228
32. Kimes BW & Brandt L 1976 Properties of a clonal muscle cell line from rat heart. *Exp Cell Res* **98**:367-381
33. Klein I & Ojamaa K 2001 Thyroid hormone and the cardiovascular system. *N Engl J Med* **344**:501-509
34. Kragie L 1994 Membrane iodothyronine transporters. Part I: Review of physiology. *Endocr Res* **40**:319-341
35. Krenning EP, Docter R, Bernard HF, Visser TJ & Hennemann G 1979 The essential role of albumin in the active transport of thyroid hormones into primary cultured rat hepatocytes. *FEBS Lett* **107**:227-230
36. van der Lee KAJM, Willemsen PHM, van der Vusse GJ & van Bilsen M 2000 Effects of fatty acids on uncoupling protein-2 expression in the rat heart. *FASEB J* **14**:495-502
37. Lim CF, Docter R, Visser TJ, Krenning EP, Bernard B, van Toor H, de Jong M & Hennemann G 1993 Inhibition of thyroxine transport into cultured rat hepatocytes by serum of nonuremic critically ill patients: effects of bilirubin and nonesterified

fatty acids. *J Clin Endocrinol Metab* **76**:1165-1172

38. Luiken JJFP, Turcotte LP & Bonen A 1999 Protein-mediated palmitate uptake and expression of fatty acid transport proteins in heart giant vesicles. *J Lipid Res* **40**:1007-1016
39. Meier PJ 1996 Hepatocellular transport systems: from carrier identification in membrane vesicles to cloned proteins. *J Hepatol* **24**:29-35
40. Mol JA & Visser TJ 1985 Synthesis and some properties of sulfate esters and sulfamates of iodothyronines. *Endocrinology* **117**:1-7
41. Neely JH & Morgan HE 1974 Relationship between carbohydrate and lipid metabolism and the energy balance of heart muscle. *Annu Rev Physiol* **36**:413-459
42. van Nieuwenhoven FA, Willemsen PHM, van der Vusse GJ & Glatz JF 1999 Co-expression in rat heart and skeletal muscle of four genes coding for proteins implicated in long-chain fatty acid uptake. *Int J Biochem Cell Biol* **31**:489-498
43. Palacín M, Estévez R, Bertran J & Zorzano A 1998 Molecular biology of mammalian plasma membrane amino acid transporters. *Physiol Rev* **78**:969-1054
44. Paradies G, Ruggiero FM, Petrosillo G & Quagliariello E 1996 Stimulation of carnitine acylcarnitine translocase activity in heart mitochondria from hypothyroid rats. *FEBS Lett* **397**:260-262
45. Pardridge WM & Mietus LJ 1980 Influx of thyroid hormones into rat liver *in vivo*. Differential availability of thyroxine and triiodothyronine bound by plasma proteins. *J Clin Invest* **66**:367-374
46. Patient RK, Harris RA, Walmsley ME & Williams JG 1983 The complete nucleotide sequence of the major adult beta globin gene of *Xenopus laevis*. *J Biol Chem* **258**:8521-8523
47. Pelsers MMAL, Lutgerink JT, van Nieuwenhoven FA, Tandon NN, van der Vusse GJ, Arends JW, Hoogenboom HR & Glatz JFC 1999 A sensitive immunoassay for rat fatty acid translocase (CD36) using phage antibodies selected on cell transfectants: abundant presence of fatty acid translocase/CD36 in cardiac and red skeletal muscle and up-regulation in diabetes. *Biochem J* **337**:407-414
48. Polikar R, Burger MD, Scherrer U & Nicod P 1993 The thyroid and the heart. *Circulation* **87**:1435-1441
49. van der Putten HHAGM, Joosten BJLJ, Klaren PHM & Everts ME 2001a Characterization of uptake and compartmentalization of 3,5,3'-triiodothyronine in cultured neonatal rat cardiomyocytes. *J Endocrinol* **171**:183-192
50. van der Putten HHAGM, Joosten BJLJ, Klaren PHM & Everts ME 2001b Comparison of [^{125}I]T_3 and [^{125}I]T_4 uptake in the embryonic heart cell line H9c2. *J Endocrinol Invest* **24**:105 (abstract)

51. Richieri GV, Anel A & Kleinfeld AM 1993 Interactions of long-chain fatty acids and albumin: determination of free Fatty acid levels using the fluorescent probe ADIFAB. *Biochemistry* **32**:7574-7580

52. Ritchie JW, Peter GJ, Shi YB & Taylor PM 1999 Thyroid hormone transport by 4F2hc-IU12 heterodimers expressed in *Xenopus* oocytes. *J Endocrinol* **163**:R5-R9

53. Rosic MA, Pantovic SB, Lucic AP, Ribarac-Stepic N & Andjelkovic I 2001 Kinetics of thyroxine (T_4) and triiodothyronine (T_3) transport in the isolated rat heart. *Exp Physiol* **86**:13-18

54. Salvatore D, Low SC, Berry M, Maia AL, Harney JW, Croteau W, St. Germain DL & Larsen PR 1995 Type 3 iodothyronine deiodinase: cloning, *in vitro* expression, and functional analysis of the placental selenoenzyme. *J Clin Invest* **96**:2421-2430

55. Sfeir Z, Ibrahimi A, Amri E, Grimaldi PA & Abumrad NA 1999 CD36 antisense expression in 3T3-F442A preadipocytes. *Mol Cell Biochem* **192**:3-8

56. Verhoeven FA, Moerings EPCM, Lamers JMJ, Hennemann G, Visser TJ & Everts ME 2001 Inhibitory effects of calcium blockers on thyroid hormone uptake in neonatal rat cardiac myocytes. *Am J Physiol* **21**:H1985-H1991

57. Visser TJ 1988 Metabolism of thyroid hormone. In: Cooke BA, King RBJ & van der Molen HJ (eds) Hormones and Their Actions Part 1. Elsevier Science Publishers BV, Amsterdam, pp. 81-103

58. Wolkoff AW 1996 Hepatocellular sinusoidal membrane organic anion transport and transporters. *Sem Liver Dis* **16**:121-127

59. Wrutniak-Cabello C, Casas F & Cabello G 2001 Thyroid hormone action in mitochondria. *J Mol Endocrinol* **26**:67-77

60. Yamamoto N, Li QL, Mita S, Morisawa S & Inoue A 2001 Inhibition of thyroid hormone binding to the nuclear receptor by mobilization of free fatty acids. *Horm Metab Res* **33**:131-137

61. Yoshizumi T, Nozaki S, Fukuchi K, Yamasaki K, Fukuchi T, Maruyama T, Tomiyama Y, Yamashita S, Nishimura T & Matsuzawa Y 2000 Pharmacokinetics and metabolism of ^{123}I-BMIPP fatty acid analog in healthy and CD36-deficient subjects. *J Nucl Med* **41**:1134-1138

CHAPTER 7

General Discussion

This thesis describes cell physiological and molecular studies on the characterization of thyroid hormone uptake in heart. In the preceding chapters the following issues have been addressed: a) the characterization of thyroid hormone uptake, b) the effects of T_3, T_4 and analogs on T_3 uptake in embryonic and neonatal heart cells, c) the functional expression of the putative cardiac thyroid hormone transport protein in *Xenopus laevis* oocytes, and finally, d) examination of thyroid hormone transporting properties of the fatty acid translocator (FAT).

7.1 T_3 uptake

Similar to pituitary and liver cells (Kragie, 1994; Hennemann *et al.*, 2001), T_3 uptake in neonatal cardiomyocytes is mediated by a temperature- and energy-dependent mechanism (*Chapter 2*). In contrast to pituitary (Everts *et al.*, 1993) and liver cells (Krenning *et al.*, 1981; de Jong *et al.*, 1993), T_3 uptake in neonatal cardiomyocytes does not depend on the Na^+ gradient over the plasma membrane. Furthermore, the effects of the T_3 metabolite Triac on T_3 uptake in the cardiomyocytes were different compared to those in pituitary cells; Triac inhibited T_3 uptake in pituitary cells (Everts *et al.*, 1994b), but not in cardiomyocytes (*Chapter 3*).

Finally, we examined T_3 uptake in the embryonic heart derived cell line H9c2(2-1) (*Chapter 4*) and showed that the characteristics of T_3 uptake resemble those of the neonatal cardiomyocytes. When T_3 was added to a similar free concentration as used in the uptake studies, T_3 stimulated fusion of the myoblasts, which is an indicator of the degree of differentiation. Moreover, T_3 uptake in myotubes was higher than in myoblasts. This led us to conclude that T_3 may play a role in the differentiation of the embryonic heart cells.

7.2 T_4 uptake

Studies in several other cell types report the presence of an energy-dependent T_4 uptake mechanism (Hennemann *et al.*, 2001). However, this mechanism has not been as extensively characterized as that for T_3. In *Chapter 2*, we show that transport of T_4 across the plasma membrane of the cardiomyocytes increases with increasing incubation temperature. In addition, T_4 uptake was at least as high as that of T_3. In

Chapter 4, we extended our research to T_4 uptake in the H9c2(2-1) cell line. T_4 uptake was energy-dependent, but not Na^+ dependent. Furthermore, unlabeled T_3 inhibited T_4 uptake to the same extent as unlabeled T_4. Thus, cardiac cells exhibit a carrier-mediated mechanism for the uptake of T_4 that could be similar to that of T_3. Inhibition of T_4 uptake by the amino acid tryptophan, indicates that amino acid system T may be involved in cardiac T_4 uptake. Finally, we hypothesized that T_4 could play a role in differentiation. Two observations support this hypothesis 1) T_4 uptake was higher in myotubes compared with myoblasts and 2) T_4, when applied at a 7-fold higher free concentration than T_3, stimulated the fusion of the H9c2(2-1) cells to the same extent as T_3 (*Chapter 4*). It is generally thought that T_3 and not T_4 is the main regulator of such processes. The affinity of T_4 for the T_3 nuclear receptor is one-tenth that of T_3 (Muñoz & Bernal, 1997). Thus, T_4 should first be deiodinated to T_3 to allow binding to the nucleus and regulate gene transcription. This hypothesis is supported by the following finding: myoblasts and myotubes exhibit a small deiodinase type I activity, which was around 1% that in adult rat liver (*Chapter 4*)(Mori *et al.*, 1991; Sabatino *et al.*, 2000). In conclusion, our studies indicate that the embryonic and neonatal heart exhibits a T_4 transport system, which may play an important role during differentiation. Furthermore, we believe it is important to further characterize the T_4 uptake mechanism in other tissues and compare its properties with those found in heart.

7.3 Tissue-selective thyroid hormone uptake

Theoretical Model

In neonatal cardiomyocytes, T_3 uptake is inhibited by T_4, suggesting that the T_3 uptake mechanism accepts T_4 as a ligand (*Chapter 2*). In the H9c2(2-1) cells, T_3 uptake is inhibited by T_4 and *vice versa* (*Chapter 4*). Furthermore, T_3 and T_4 uptake showed a similar dependency on the energy status in these cells. Finally, the group of Rosic *et al.* (2001) compared T_3 and T_4 uptake in intact rat hearts and showed that the affinities of the transport mechanisms for T_3 and T_4 were comparable. Thus, we conclude that T_3 and T_4 uptake in heart cells is mediated by one carrier. In pituitary also one carrier is present for the uptake of T_3 and T_4 (Yan & Hinkle, 1993; Everts *et al.*, 1994a), whereas in liver one mechanism for the transport of T_3 in addition to a separate one for the transport of T_4 is found (Krenning *et al.*, 1981). As suggested before (Everts *et*

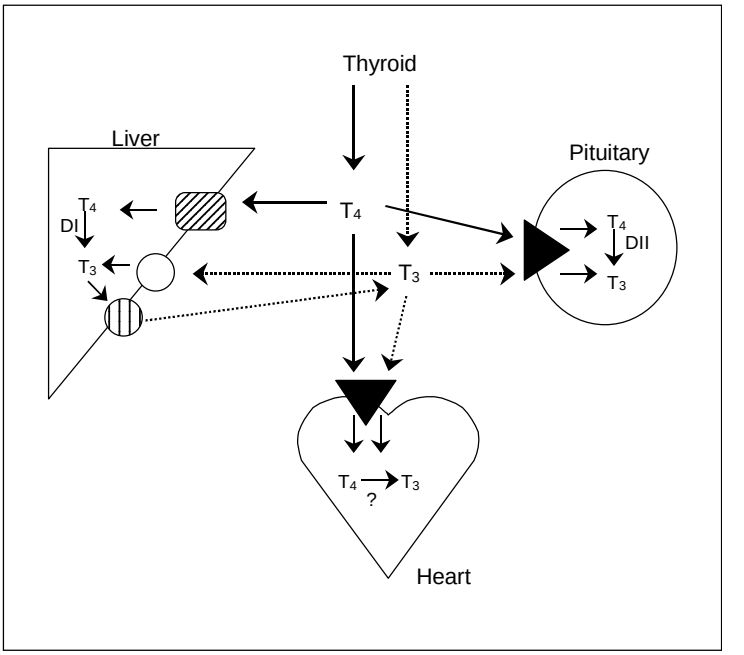

Figure 1 Simplified diagram showing transport mechanisms in three tissues. Transporters are indicated by different symbols. DI: deiodinase type I and, DII: deiodinase type II.

al., 1996; Hennemann *et al.*, 2001), these observations support the idea that thyroid hormone uptake is regulated in a tissue-specific manner (Figure 1), which allows tissue-specific regulation of intracellular availability of thyroid hormones. Figure 1 also represents tissue-specific deiodinase activity. It is still a matter of debate whether the heart is able to convert T_4 into T_3 (Mori *et al.*, 1991; Nauman *et al.*, 1994; Croteau *et al.*, 1996; Sabatino *et al.*, 2000). We are currently exploring the presence of deiodinases in the embryonic and neonatal cardiomyocytes.

Implications for treatment

Patients with heart disease may show profound changes in thyroid hormone metabolism, characteristic for the low T_3 syndrome or non-thyroidal illness (NTI)

(Hamilton & Stevenson, 1996; Gomberg-Maitland & Frishman, 1998; Klein & Ojamaa, 2001). During NTI, the serum T_3 concentration is reduced, while serum T_4 is usually normal and TSH levels do not rise (Docter *et al.*, 1993). Docter and Krenning (1990) suggested that inhibition of T_4 uptake into the liver is responsible for the diminished T_3 production. Studies in which the effect of incubations with serum from NTI patients on T_4 uptake in hepatocytes was examined, supported this hypothesis (Lim *et al.*, 1994). Furthermore, it was shown that T_4 uptake into the pituitary is unaffected by serum of NTI patients. This could explain the absence of a stimulatory effect on TSH secretion in NTI patients (Everts *et al.*, 1995). Whether thyroid hormone uptake in the heart in patients with NTI or cardiac disease is altered is not known. From Figure 1, it may be concluded that in patients with heart disease or NTI no changes occur with respect to cardiac thyroid hormone uptake similar to the situation in the pituitary. However, the comparison made in this figure is based on the physiological properties of the uptake systems and not on their molecular structures. Therefore, studies concerning the functional cloning of genes encoding the thyroid hormone transporters become even more relevant.

Tissue-specificity of iodothyronine uptake may provide the possibility to treat patients with heart failure in such a way that cardiac contractile function is affected. On the other hand, it has been suggested that the low T_3 concentration of NTI is an adaptive mechanism to save energy and to protect organ function (Docter & Krenning, 1990; Docter *et al.*, 1993; Everts *et al.*, 1996). Thus, it seems favorable to maintain the low T_3 concentration, until the patient has recovered from his disease. This is further supported by the following observation: prolonged thyroid hormone supplementation after coronary artery bypass surgery caused chronotropic and probably deleterious effects (Spooner *et al.*, 1999; Peters *et al.*, 2000). This raises the question whether thyroid hormone therapy should be recommended for patients with heart failure (Spooner *et al.*, 1999; Peters *et al.*, 2000). In addition, thyroid hormone administration after coronary artery bypass surgery did not reduce the need for treatment with inotropic agents and the use of mechanical devices like a pacemaker (Gomberg-Maitland & Frishman, 1998). On the other hand, in the early post-operative period, T_3 and T_4 administration did improve cardiac function (Moruzzi *et al.*, 1996; Chowdhury *et al.*, 1999; Mullis-Jansson *et al.*, 1999). This may be ascribed to the inotropic properties of T_3 and T_4. Therefore, research has focused on the development

of thyroid hormone analogs with such properties *e.g.* DITPA, the propionic acid analog of 3,5-T_2 (Spooner *et al.*, 1999). DITPA has been used to improve contractility of the heart after infarction, up to now only in animals. We tested DITPA for its effect on plasma membrane uptake of T_3, and found that DITPA could inhibit T_3 uptake in neonatal cardiomyocytes. When DITPA was present during culture of the cells, its effect was comparable to that of T_3, suggesting a down-regulation of the T_3 uptake mechanism (*Chapter 3*). Unfortunately, the studies describing the effects of DITPA in animals do not include effects on the liver or pituitary (Spooner *et al.*, 1999). Therefore, we cannot draw a conclusion with respect to tissue-specific regulation of T_3 uptake by DITPA. While DITPA has been developed as an analog with inotropic properties, another analog, GC-1, has been developed based on its selective thyroid hormone receptor binding properties (Trost *et al.*, 2000). In addition to its to its T_3 receptor subtype selectivity, the authors suggested that the tissue-selective thyromimetic effects effects of GC-1 may arise from a tissue-selective uptake (Trost *et al.*, 2000).

Finally, Triac is transported into the heart by a mechanism distinct from that of T_3 (*Chapter 3*). This observation is similar to what has been reported for the pituitary (Everts *et al.*, 1994b). Thus, the heart and pituitary may have a similar mechanism for the uptake of Triac. Although, Triac did not inhibit T_3 uptake in the heart (*Chapter 3*), it reduced T_3 uptake in the pituitary (Everts *et al.*, 1994b). This indicates that this metabolite, which is used in the treatment of patients with thyroid cancer (Beck-Peccoz *et al.*, 1988; Sherman & Ladenson, 1992; Bracco *et al.*, 1993; Sherman *et al.*, 1997), shows selectivity for thyroid hormone transport mechanisms. In addition, two studies report on the marked differences between the effect of Triac and T_3 on heart to body weight ratio, Ca^{2+}-ATPase and ß-myosin heavy chain (Liang *et al.*, 1997; Lameloise *et al.*, 2001), the effect on liver parameters was similar. Discrepancies with respect to the distinct affinity of Triac for the various nuclear receptor isoforms and the presence of these receptors in heart and liver, assumes that the *in vivo* action of Triac may also be the result from differences in cellular uptake (Lameloise *et al.*, 2001). In conclusion, these findings provide further support for the idea that compounds with selective effects on plasma membrane thyroid hormone uptake can be generated, and may have their application in clinical practice.

7.4 Molecular basis for the cardiac thyroid hormone transporter

The model depicted in Figure 1 is based on the physiological properties of uptake. The final proof of this model is the identification of the molecular structure of these transporters. *Chapter 5* describes the attempts we have made to characterize the molecular structure of the cardiac thyroid hormone transporter by means of expression cloning in *Xenopus laevis* oocytes. Unfortunately, injection of mRNA isolated from rat heart in oocytes and subsequent examination of thyroid hormone uptake did not result in increased thyroid hormone uptake as compared with native oocytes. In contrast, comparable studies with tissue from liver did result in identification of messengers inducing thyroid hormone transport (Docter *et al.*, 1997). Moreover, the group of Vasilets *et al.* (2001) showed that injection of cardiac mRNA results in functional expression of proteins, suggesting that our negative results are not explained by the improper expression of the mRNA. As discussed in C*hapter 5*, we cannot exclude that amongst the pool of cardiac mRNAs the abundance of those encoding the thyroid hormone transporter is too low to detect a sufficient stimulation of T_3 transport.

Since only a sufficient mRNA-induced increase in T_3 uptake allows construction and screening of a cDNA library, we choose to focus on the investigation of the role of other transporters in cardiac thyroid hormone uptake. In addition, recent studies on thyroid hormone uptake in liver revealed that amino acid transporters and organic anion transporters may be responsible for part of the uptake of thyroid hormones into the liver (Friesema *et al.*, 1999, 2001a, 2001b). For reasons discussed in *Chapter 6*, we conclude that these transporters are probably not involved in thyroid hormone uptake in the heart. However, the idea that other multispecific transporters may play a role in cardiac thyroid hormone transport is attractive. FAT is a multi-specific fatty acid transporter, responsible for the major part of fatty acid uptake in heart (Coburn *et al.*, 2000; Yoshizumi *et al.*, 2000). In *Chapter 6*, we examined the hypothesis that FAT mediates the uptake of thyroid hormones and show that FAT induces Na^+-independent T_3 uptake. The physiological relevance of FAT as a thyroid hormone transporter remains to be established. This may be addressed in our primary cell culture model of the neonatal rat cardiomyocytes. Animal models in which FAT expression is impaired (Coburn *et al.*, 2000) or induced (Ibrahimi *et al.*, 1999) may

also address this issue. Finally, it would be interesting to examine whether thyroid hormone uptake is impaired in patients and animals with FAT deficiency (Yoshizumi *et al.*, 2000).

7.5 Future studies

While we were able to characterize many aspects of thyroid hormone uptake in heart, some major questions remain unanswered. For example, is thyroid hormone uptake altered in patients with heart disease? Apart from the changes in thyroid hormone metabolism discussed above, it is suggested that alterations at the level of gene expression may take place in patients with heart failure (Kinugawa *et al.*, 2001). An important topic at this moment is whether a failing heart recapitulates the fetal program of gene expression (Sussman, 2001). Therefore, elucidation of the molecular structure of the cardiac thyroid hormone transporter should be elaborated to address two questions: does the fetal heart express a different gene encoding the thyroid hormone transporter as compared with the adult heart? And if so, is the transcription and expression of these genes altered in a failing heart? In addition, the two types of cell culture provide good models to study cell physiological properties of thyroid hormone uptake. This is relevant for the examination of alterations in thyroid hormone uptake in a failing heart such as the effect of incubations with serum from patients with heart disease on uptake.

Another question left unanswered is that of the physiological importance of FAT in cardiac thyroid hormone uptake. In addition to *in vivo* studies in available animal and human models described before, we aim to extend our research described in *Chapter 6*. This includes further characterization of the affinity and capacity of the system expressed in *Xenopus laevis* oocytes as well as mutual inhibition of fatty acid and thyroid hormone uptake by their unlabeled ligands. Also, the neonatal cardiomyocytes and the H9c2(2-1) cell line will be useful in these studies. Fatty acid uptake has been explored in neonatal cardiomyocytes by Stremmel *et al.* (1988). Together with our knowledge about thyroid hormone uptake in these cells, we will investigate the interaction between fatty acid and thyroid hormone uptake. In addition, the effects of specific inhibitors on uptake of both fatty acids and T_3 or T_4 can be tested. Finally, H9c2(2-1) cells transfected with FAT (van Nieuwenhoven *et al.*, 1998)

may provide a suitable model system to examine the interaction between thyroid hormones and fatty acid uptake in heart.

7.6 Concluding remarks

This thesis provides evidence for the presence of a specific mechanism for thyroid hormone transport in embryonic and neonatal heart. Part of the T_3 and T_4 uptake into the cardiac cell may be mediated by FAT. The T_3 and T_4 transport mechanism may play an important role in the intracellular availability of thyroid hormones during development and may provide a tool for tissue-specific treatment of heart and thyroid diseases.

7.7 References

1. Beck-Peccoz P, Sartorio A, De Medici C, Grugni G, Morabito F & Faglia G 1988 Dissociated thyrometric effects of 3,5,3'-triiodothyroaceticacid (TRIAC) at the pituitary and peripheral tissue levels. *J Endocrinol* **11**:113-118
2. Bracco D, Morin O, Schutz Y, Liang H, Jéquier E & Burger AG 1993 Comparison of the metabolic and endocrine effects of 3,5,3'-triiodothyracetic acid and thyroxine. *J Clin Endocrinol Metab* **77**:221-228
3. Chowdhury D, Parnell VA, Ojamaa K, Boxer R, Cooper R & Klein I 1999 Usefulness of triiodothyronine (T_3) treatment after surgery for complex congenital heart disease in infants and children. *Am J Card* **84**:1107
4. Coburn CT, Knapp Jr. FF, Febbraio M, Beets AL, Silverstein RL & Abumrad NA 2000 Defective uptake and utilization of long chain fatty acids in muscle and adipose tissue of CD36 knockout mice. *J Biol Chem* **275**:32523-32529
5. Croteau W, Davey JC, Galton VA & Germain DLS 1996 Cloning of the mammalian type II iodothyronine deiodinase. A selenoprotein differentially expressed and regulated in human and rat brain and other tissues. *J Clin Invest* **98**:405-417
6. Docter R & Krenning EP 1990 Role of cellular transport systems in the regulation of thyroid hormone bioactivity. In: Greer MA (ed) The Thyroid Gland. Raven Press, New York, pp. 233-254
7. Docter R, Krenning EP, de Jong M & Hennemann G 1993 The sick euthyroid syndrome: changes in thyroid hormone serum parameters and hormone metabolism. *Clin Endocrinol* **39**:499-518

8. Docter R, Friesema ECH, van Stralen PGJ, Krenning EP, Everts ME, Visser TJ & Hennemann G 1997 Expression of rat liver cell membrane transporters for thyroid hormone in *Xenopus laevis* oocytes. *Endocrinology* **138**:1841-1846
9. Everts ME, Docter R, van Buuren JCJ, van Koetsveld PM, Hofland LJ, de Jong M, Krenning EP & Hennemann G 1993 Evidence for carrier-mediated uptake of triiodothyronine in cultured anterior pituitary cells of euthyroid rats. *Endocrinology* **132**:1278-1285
10. Everts ME, Docter R, Moerings EPCM, van Koetsveld PM, Visser TJ, de Jong M, Krenning EP & Hennemann G 1994a Uptake of thyroxine in cultured anterior pituitary cells of euthyroid rats. *Endocrinology* **134**:2490-2497
11. Everts ME, Visser TJ, Moerings EPCM, Docter R, van Toor H, Tempelaars AMP, de Jong M, Krenning EP & Hennemann G 1994b Uptake of triiodothyroacetic acid and its effects on thyrotropin secretion in cultured anterior pituitary cells. *Endocrinology* **135**:2700-2707
12. Everts ME, Lim C, Moerings EPCM, Docter R, Visser TJ, de Jong M, Krenning EP & Hennemann G 1995 Effects of a furan fatty acid and indoxyl sulfate on thyroid hormone uptake in cultured anterior pituitary cells. *Am J Physiol* **268**:E974-E979
13. Everts ME, de Jong M, Lim C, Docter R, Krenning EP, Visser TJ & Hennemann G 1996 Different regulation of thyroid hormone transport in liver and pituitary: its possible role in the maintenance of low T_3 production during nonthyroidal illness and fasting in man. *Thyroid* **6**:357-366
14. Friesema ECH, Docter R, Moerings EPCM, Stieger B, Hagenbuch B, Meier PJ, Krenning EP, Hennemann G & Visser TJ 1999 Identification of thyroid hormone transporters. *Biochem Biophys Res Comm* **254**:497-501
15. Friesema ECH, Docter R, Krenning EP, Hennemann G & Visser TJ Role of organic anion transporters in hepatic uptake of thyroid hormone. Proc 73t^{h} Annual Meeting of the American Thyroid Association,, Washington DC, 2001a, p 219
16. Friesema ECH, Docter R, Moerings EPCM, Verrey F, Krenning EP, Hennemann G & Visser TJ 2001b Thyroid hormone transport by the heterodimeric human system L amino acid transporter. *Endocrinology* **142**:4339-4348
17. Gomberg-Maitland M & Frishman WH 1998 Thyroid hormone and cardiovascular disease. *Am Heart J* **135**:187-196
18. Hamilton MA & Stevenson LW 1996 Thyroid hormone abnormalities in heart failure: possibilities for therapy. *Thyroid* **6**:527-529
19. Hennemann G, Docter R, Friesema ECH, de Jong M, Krenning EP & Visser TJ 2001 Plasma membrane transport of thyroid hormones and its role in thyroid hormone metabolism and bioavailability. *Endocrine Rev* **22**:451-476

20. Ibrahimi A, Bonen A, Blinn WD, Hajri T, Li X, Zhong K, Cameron R & Abumrad NA 1999 Muscle-specific overexpression of FAT/CD36 enhances fatty acid oxidation by contracting muscle, reduces plasma triglycerides and fatty acids, and increases plasma glucose and insulin. *J Biol Chem* **274**:26761-26766
21. de Jong M, Visser TJ, Bernard BF, Docter R, Vos RA, Hennemann G & Krenning EP 1993 Transport and metabolism of iodothyronines in cultured human hepatocytes. *J Clin Endocrinol Metab* **77**:139-143
22. Kinugawa K, Minobe WA, Wood WM, Ridgway EC, Baxter JD, Ribeiro RC, Tawadrous MF, Lowes BA, Long CS & Bristow MR 2001 Signaling pathways responsible for fetal gene induction in the failing human heart: evidence for altered thyroid hormone receptor gene expression. *Circulation* **103**:1089-94
23. Klein I & Ojamaa K 2001 Thyroid hormone and the cardiovascular system. *N Engl J Med* **344**:501-509
24. Kragie L 1994 Membrane iodothyronine transporters. Part I: Review of physiology. *Endocr Res* **40**:319-341
25. Krenning EP, Docter R, Bernard B, Visser TJ & Hennemann G 1981 Characteristics of active transport of thyroid hormone into rat hepatocytes. *Biochem Biophys Acta* **676**:314-320
26. Lameloise N, Siegrist-Kaiser C, O'Connell M & Burger A 2001 Differences between the effects of thyroxine and tetraiodothyroacetic acid on TSH suppression and cardiac hypertrophy. *Eur J Endocrinol* **144**:145-154
27. Liang H, Juge-Aubry CE, O'Connell M & Burger AG 1997 Organ-specific effects of 3,5,3'-triiodothyroacetic acid in rats. *Eur J Endocrinol* **137**:537-544
28. Lim CF, Docter R, Krenning EP, van Toor H, Bernard B, de Jong M & Hennemann G 1994 Transport of thyroxine into cultured hepatocytes: effects of mild non-thyroidal illness and calorie restriction in obese subjects. *Clin Endocrinol* **40**:79-85
29. Mori Y, Nishikawa M, Toyoda N, Yonemoto T, Matsubara H & Inada M 1991 Iodothyronine 5'-deiodinase activity in cultured rat myocardial cells: characteristics and effects of triiodothyronine and angiotensine II. *Endocrinology* **128**:3105-3112
30. Moruzzi P, Doria E & Agostoni PG 1996 Medium-term effectiveness of L-thyroxine treatment in idiopathic dilated cardiomyopathy. *Am J Med* **101**:461-467
31. Mullis-Jansson SL, Argenziano M, Homma S, Weinberg AD, Williams M, Rose EA & Smith CR 1999 A randomized double-blind study of the effect of triiodothyronine on cardiac function and morbidity after coronary bypass surgery. *J Thorac Cardiovasc Surg* **117**:1128-1135
32. Muñoz A & Bernal J 1997 Biological activities of thyroid hormone receptors. *Eur J Endocrinol* **137**:433-445

33. Nauman A, Nauman J, Porta S & Grzesniuk W 1994 Iodothyronine-5'-deiodinase in rat heart, regulatory action of catecholamines. In: Braverman LE, Eber O & Langsteger W (eds) Heart and thyroid. Blackwell Scientific Publications Inc., Cambridge, MA, pp. 196-172
34. van Nieuwenhoven FA, Luiken JJFP, de Jong YF, Grimaldi PA, van der Vusse GJ & Glatz JFC 1998 Stable transfection of fatty acid translocase (CD36) in a rat muscle cell line (H9c2). *J Lipid Res* **39**:2039-2047
35. Peters A, Ehlers M, Blank B, Exler D, Falk C, Kohlmann T, Fruehwald-Schultes B, Wellhoener P, Kerner W & Fehm HL 2000 Excess triiodothyronine as a risk factor of coronary events. *Arch Intern Med* **160**:1993-1999
36. Rosic MA, Pantovic SB, Lucic AP, Ribarac-Stepic N & Andjelkovic I 2001 Kinetics of thyroxine (T_4) and triiodothyronine (T_3) transport in the isolated rat heart. *Exp Physiol* **86**:13-18
37. Sabatino L, Iervasi G, Ferrazzi P, Francesconi D & Chopra IJ 2000 A study of iodothyronine 5'-monodeiodinase activities in normal and pathological tissues in man and their comparison with activities in rat tissues. *Life Sci* **68**:191-202
38. Sherman SI & Ladenson PW 1992 Organ-specific effect of tiratricol: a thyroid hormone analog with hepatic, not pituitary, superagonist effects. *Clinical Endocrinology and Metabolism* **75**:901-905
39. Sherman SI, Ringel MD, Smith MJ, Kopelen HA, Zoghbi WA & Ladenson PW 1997 Augmented hepatic and skeletal thyromimetic effects of tiratricol in comparison with levothyroxine. *J Clin Endocrinol Metab* **82**:2153-2158
40. Spooner PH, Morkin E & Goldman S 1999 Thyroid hormone and thyroid hormone analogues in the treatment of heart failure. *Cor Art Disease* **10**:395-399
41. Stremmel W 1988 Fatty acid uptake by isolated rat heart myocytes represents a carrier-mediated transport process. *J Clin Invest* **81**:844-852
42. Sussman MA 2001 When the thyroid speaks, the heart listens. *Circ Res* **89**:557-559
43. Trost SU, Swanson E, Gloss B, Wang-Iverson DB, Zhang H, Volodarsky T, Grover GJ, Baxter JD, Chiellini G, Scanlan TS & Dillmann WH 2000 The thyroid hormone receptor-β-selective antagonist GC-1 differentially affects plasma lipids and cardiac activity. *Endocrinology* **141**:3057-3064
44. Vasilets LA, Darmer D, Isenberg G & Schwarz W 2001 RNA from heart of young and old rats leads to the expression of protein(s) in *Xenopus* oocytes that alter the transport activity of rat Na^+,K^+-ATPases differently. *Pflügers Arch* **443**:84-91
45. Yan Z & Hinkle PM 1993 Saturable, stereospecific transport of 3,5,3'-triiodo-L-thyronine and L-thyroxine into GH_4C_1 pituitary cells. *J Biol Chem* **268**:20179-20184
46. Yoshizumi T, Nozaki S, Fukuchi K, Yamasaki K, Fukuchi T, Maruyama T, Tomiyama

Y, Yamashita S, Nishimura T & Matsuzawa Y 2000 Pharmacokinetics and metabolism of ^{123}I-BMIPP fatty acid analog in healthy and CD36-deficient subjects. *J Nucl Med* **41**:1134-1138

Summary

Summary

Thyroid hormones, T_3 and T_4, are important for proper heart development and function. A small variation in serum levels of these hormones results in measurable changes in cardiac performance such as changes in resting heart rate and contractility. These changes are mediated by intracellular actions of T_3 and T_4 through association with nuclear thyroid hormone receptors and subsequent regulation of gene transcription. The first step in this sequence is the transport of T_3 and T_4 across the plasma membrane. It is generally accepted that this is mediated by specific carrier proteins. While the knowledge about these proteins in other organs is abundant, information about cardiac thyroid hormone uptake is scarce. Therefore, the aim of this thesis was to characterize the cell physiological properties of T_3 and T_4 uptake in heart (*Chapters 2 – 4*) and to obtain information on the molecular structure(s) (*Chapters 5 – 6*).

Previously, it has been shown that neonatal rat cardiomyocytes exhibit a specific mechanism for the uptake of T_3. This mechanism is further explored in the study described in *Chapter 2* and is energy- and temperature-dependent, but not Na^+-dependent. Furthermore, T_3 uptake is reduced in presence of tryptophan, a ligand for the amino acid system T, suggesting the presence of an accessory transport mechanism. In addition to the neonatal cardiomyocytes, T_3 uptake has been examined in the embryonic rat heart derived cell line H9c2(2-1) (*Chapter 4*) and is found to display similar characteristics as that of the neonatal cardiomyocytes. In *Chapter 2*, we also initiated studies on the characterization of T_4 uptake and showed that T_4 enters the cardiomyocyte by a temperature-dependent mechanism. Interestingly, unlabeled T_4 inhibited the uptake of T_3, though not significantly. These findings prompted us to continue the examination of T_4 uptake in H9c2(2-1) cells. T_4 uptake in the H9c2(2-1) cells is specific, energy-dependent, but not Na^+-dependent. Based on similar features of T_3 and T_4 uptake and their mutual inhibition, we suggested that heart cells share a common mechanism for the uptake of both T_3 and T_4.

A comparison based on the cell physiological properties of thyroid hormone uptake between pituitary, liver and heart suggests that thyroid hormone uptake is tissue-specific. Tissue-specificity of uptake may contribute to the development of new agents for the clinical treatment of patients with heart failure or thyroid disease. The metabolites Triac and Tetrac are used in the treatment of thyroid cancer. The

iodothyronine analogue DITPA is developed to improve heart function in patients with heart failure, but has only been tested in animal studies. Their effect on T_3 uptake was analyzed in neonatal rat cardiomyocytes (*Chapter 3*). This chapter shows that DITPA interfered with the plasma membrane transport of T_3, while Triac and Tetrac did not. Furthermore, examination of the uptake of Triac indicated that Triac is transported across the plasma membrane of the cardiomyocyte, but probably by a mechanism different from that of T_3.

In the embryonic heart T_3 and T_4 are essential for growth and development. In *Chapter 4* we hypothesized that thyroid hormone uptake mechanisms play an important role during differentiation. The H9c2(2-1) cell line propagates as undifferentiated myoblasts and forms differentiated myotubes upon reduction of the serum concentration. Two observations support our hypothesis: (1) T_3 and T_4 stimulated fusion at day 9 of culture. This was taken as an indicator of differentiation, based on morphological characteristics and the 31-fold rise in creatine kinase activity from day 2 to day 9. Furthermore, (2) the uptake of T_3 and T_4 was higher in myotubes compared with myoblasts.

Expression cloning in *Xenopus laevis* oocytes has been successfully used for the cloning and characterization of the molecular structure of many plasma membrane transporters. We adopted this method and explored T_3 uptake in *Xenopus laevis* oocytes injected with rat heart mRNA (*Chapter 5*). T_3 uptake was not significantly induced in oocytes injected with heart mRNA as compared with native oocytes. Also optimization of the signal to noise ratio either by using alternative ligands to diminish the background or by size-fractionation of the mRNA to enrich the signal was not successful. We cannot exclude that the abundance of mRNAs encoding the cardiac thyroid hormone transport protein was too low to stimulate the uptake of T_3.

Finally, in *Chapter 6* we tested a candidate iodothyronine transporter *i.e.* a protein with potential thyroid hormone transport activity. One of the important plasma membrane transport proteins in heart is the fatty acid translocase (FAT). Since FAT transports anionic fatty acids and iodothyronines are ligands for different organic anion transporters, we tested the hypothesis that FAT mediates the uptake of thyroid hormones. Uptake studies in oocytes injected with the cRNA encoding FAT demonstrated that FAT indeed induces thyroid hormone uptake. Furthermore, T_3 uptake by FAT did not depend on the Na^+ gradient which is in accordance with the cell

physiological studies described in this thesis.

The major findings of this thesis and suggestions for future research are described in *Chapter 7*. In summary, uptake of T_3 and T_4 in the heart is mediated by a common mechanism which is energy- and temperature dependent, but not Na^+-dependent. The molecular basis of this mechanism needs to be further elaborated as well as the physiological role of additional transporters like FAT. Furthermore, of special interest for future research is the tissue specificity of thyroid hormone uptake, since this may be beneficial for the development of new agents for clinical treatment of patients with heart failure.

Samenvatting

Samenvatting

De schildklierhormonen, T_3 en T_4, zijn belangrijk voor een goede ontwikkeling en het goed functioneren van het hart. Een kleine verandering in de serumconcentraties van deze hormonen resulteert al in meetbare veranderingen in hartfunctie zoals een verandering van de hartfrequentie en contractiekracht. Deze veranderingen komen tot stand door intracellulaire effecten van T_3 en T_4 via binding aan specifieke kernreceptoren en de daarop volgende regulatie van gen transcriptie. De eerste stap in deze opeenvolging van gebeurtenissen is de opname van T_3 en T_4 via het plasmamembraan. Een algemeen geaccepteerd idee is dat dit door middel van specifieke transporteiwitten gebeurt. Terwijl er steeds meer aanwijzingen zijn voor het bestaan van deze transporteiwitten in andere weefsels, is de informatie met betrekking tot het hart nog maar zeer beperkt. Het doel van deze studie was dan ook het karakteriseren van de celfysiologische aspecten van T_3 en T_4 transport in het hart (*Hoofdstuk 2 – 4*) en daarnaast het verkrijgen van informatie over de moleculaire structuur (*Hoofdstuk 5 – 6*).

Eerder is al aangetoond dat neonatale hartspiercellen van de rat een specifiek mechanisme bezitten voor de opname van T_3. Dit mechanisme is verder onderzocht in *Hoofdstuk 2* en bleek energie- en temperatuurafhankelijk, maar niet Na^+-afhankelijk. Daarnaast was de opname van T_3 verlaagd in aanwezigheid van tryptofaan. Tryptofaan wordt opgenomen door aminozuur transport systeem T, wat suggereert dat een deel van de T_3 opname via dit systeem verloopt. Naast de studies in neonatale hartspiercellen hebben we ook het transport van T_3 in de van oorsprong uit embryonaal hart afkomstige H9c2(2-1) cellijn bekeken (*Hoofdstuk 4*). De kenmerken van dit transport systeem kwamen overeen met dat van de neonatale hartspiercellen. Verder zijn we in *Hoofdstuk 2* gestart met het bestuderen van de opname van T_4. T_4 passeert het plasmamembraan van de hartspiercel via een temperatuurafhankelijk mechanisme. Een andere interessante bevinding in dit hoofdstuk is dat T_4 de opname van T_3 kan remmen. Deze resultaten stimuleerden ons verder te gaan met de karakterisering van het T_4 transport in H9c2(2-1) cellen. De T_4 opname in de H9c2(2-1) cellen was specifiek en energieafhankelijk, maar niet Na^+-afhankelijk. Gebaseerd op de overeenkomst in eigenschappen van het T_3 en T_4 transport kunnen we concluderen dat T_3 en T_4 middels een zelfde mechanisme worden opgenomen in de hartcellen.

Een vergelijking gebaseerd op de celfysiologische aspecten van schildklierhormoon opname tussen de hypofyse, lever en hart suggereert dat het mechanisme van schildklierhormoon opname weefselspecifiek is. Deze weefselspecificiteit zou kunnen bijdragen aan de ontwikkeling van nieuwe middelen voor de klinische behandeling van patiënten met hartfalen of een schildklier ziekte. De schildklierhormoon metabolieten Triac en Tetrac worden gebruikt voor de behandeling van schildklierkanker. Het analogon DITPA is ontwikkeld ter verbetering van de hartfunctie in patiënten met hartfalen, maar is voorlopig alleen nog toegepast in dieren. Het effect van deze stoffen op T_3 opname werd geanalyseerd in de neonatale hartspiercellen (*Hoofdstuk 3*). Dit hoofdstuk laat zien dat DITPA interfereert met het plasmamembraan transport van T_3, terwijl Triac en Tetrac geen effect hebben op de opname. Daarnaast hebben we de opname van Triac bekeken. Triac kan door de hartspiercellen opgenomen worden, waarschijnlijk via een ander mechanisme dan dat voor T_3.

In een embryonaal hart zijn T_3 en T_4 noodzakelijk voor groei en ontwikkeling. De hypothese dat de T_3 en T_4 opname mechanismen een belangrijke rol spelen tijdens de differentiatie werd onderzocht in een studie in H9c2(2-1) cellen beschreven in *Hoofdstuk 3*. De H9c2(2-1) cellen zijn in staat te differentiëren van myoblasten naar meerkernige, zogenaamde myotubes wanneer de serum concentratie verlaagd wordt. Twee bevindingen bevestigen onze hypothese: (1) T_3 en T_4 stimuleerden de fusie op dag 9 van de kweek. Dit kan beschouwd worden als een maat voor differentiatie op basis van morfologische kenmerken en de 31-voudige stijging in creatine kinase activiteit van dag 2 tot dag 9. (2) de opname van T_3 en T_4 was in de myotubes hoger vergeleken met de myoblasten.

De moleculaire structuur van een groot aantal plasmamembraan transporteiwitten is opgehelderd met behulp van expressie klonering in *Xenopus laevis* oocyten. Wij hebben deze techniek gebruikt en T_3 opname bestudeerd in oocyten geïnjecteerd met mRNA uit het hart van een rat (*Hoofdstuk 5*). De T_3 opname was niet significant hoger in de mRNA geïnjecteerde oocyten vergeleken met niet geïnjecteerde oocyten. Ook de optimalisatie van de signaal / ruis ratio door alternatieve T_3 analoga aan te bieden om het achtergrond signaal te verlagen of door het mRNA op grootte te fractioneren om het signaal te versterken, leidde niet tot een verbetering van de resultaten. We kunnen niet uitsluiten dat het aantal mRNAs coderend voor de hartspecifieke schildklierhormoon transporter te laag is om voldoende transporteiwitten

tot expressie te brengen en voldoende stimulatie van T_3 opname te kunnen meten.

Tot slot worden in *Hoofdstuk 6*, experimenten beschreven met een kandidaat schildklierhormoon transporter, dat wil zeggen een eiwit met potentiële schildklierhormoon transport activiteit. Een van de belangrijke plasmamembraan transporters in het hart is het vetzuur translocase (FAT). Omdat FAT vetzuren in de anionvorm kan transporteren en schildklierhormoon door anion transporters opgenomen kan worden, hebben we de hypothese getest dat FAT zo'n kandidaat is (Hoofdstuk 6). Opname studies in oocyten geïnjecteerd met cRNA coderend voor FAT demonstreren dat FAT inderdaad de opname van schildklierhormoon induceert. Daarnaast laten deze studies ook zien dat T_3 opname door FAT Na^+-onafhankelijk is, wat overeenkomt met de bevindingen in de celfysiologische studies beschreven in dit proefschrift.

De belangrijkste bevindingen en suggesties voor verder onderzoek zijn beschreven in *Hoofdstuk 7*. Samenvattend: T_3 en T_4 worden middels eenzelfde mechanisme opgenomen in het hart. Dit mechanisme is energie- en temperatuurafhankelijk, maar niet Na^+-afhankelijk. De moleculaire basis voor dit mechanisme zal verder onderzocht moeten worden evenals de fysiologische betekenis van een additioneel transport systeem zoals FAT. Van bijzonder belang voor verder onderzoek is de weefselspecificiteit van opname. Dit zou met name een grote rol kunnen spelen in de ontwikkeling van nieuwe medicijnen voor de klinische behandeling van patiënten met hartfalen.

List of Publications

Publications

van der Putten HHAGM, Joosten BJLJ, Klaren PHM, Everts ME 2001 Characterization of uptake and compartmentalization of 3,5,3'-triiodothyronine in cultured neonatal rat cardiomyocytes. *J Endocrinol* **171**:183-192

Bogerd J, Blomenröhr M, Andersson E, van der Putten HHAGM, Tensen CP, Vischer HF, Granneman JCM, Janssen-Dommerholt C, Goos HJT, Schultz RW 2001Discrepancy between molecular structure and ligand selectivity of a testicular follicle-stimulating hormone receptor of the African catfish (*Clarias Gariepinus*). *Biol Reprod* **64**, 1633-1643

Verhoeven FA, van der Putten HHAGM, Hennemann G, Lamers JMJ, Visser TJ, Everts ME 2002 Uptake of triiodothyronine and triiodothyroacetic acid in neonatal rat cardiomyocytes: effects of metabolites and analogs. *J Endocrinol* **173**:247-255

van der Putten HHAGM, Joosten BJLJ, Klaren PHM, Everts ME 2002 Uptake of T_3 and T_4 in myoblasts and myotubes of the embryonic heart cell line H9c2(2-1). *J Endocrinol , accepted*

Abstracts

van der Putten HHAGM, Joosten BJLJ, Friesema ECH, Klaren PHM, Everts ME 1999 Transport of T_3 in neonatal rat cardiomyocytes: a temperature and energy-dependent mechanism. Oral presentation at the 26th Annual Meeting of the European Thyroid Association in Milan, Italy. *J Endocrinol Invest* **22** (suppl. to no. 6):18.

van der Putten HHAGM, Joosten BJLJ, Klaren PHM, Everts ME 2000 Uptake of triiodothyronine (T_3), thyroxine (T_4) and reverse T_3 (rT_3) in neonatal rat cardiomyocytes. Poster presentation at the 12th International Thyroid Congress in Kyoto, Japan. *Endocrine J* **47**:250

van der Putten HHAGM, Joosten BJLJ, Klaren PHM, Everts ME 2001Comparison of $[^{125}I]T_3$ and $[^{125}I]T_4$ uptake in the embryonic heart cell line H9c2. Poster presentation

at the 27th Annual Meeting of the European Thyroid Association in Warsaw, Poland. *J Endocrinol Invest* **24**:105

van der Putten HHAGM, Slob S, Joosten BJLJ, Klaren PHM, Everts ME 2002 Effects of triiodothyronine and thyroxine on the differentiation of skeletal and heart muscle. Oral presentation at the 31th Meeting of the European Society for Muscle Research in Lunteren, the Netherlands. *J Muscle ResCell Motil* (in press)

Dankwoord

Curriculum vitae

Dankwoord

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en zeg,
mijn glorie was dat ik door vrienden werd bemind.
(William B. Yeats)

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Haidy

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Curriculum vitae

Haidy H.A.G.M. van der Putten werd geboren op 15 januari 1974 te Aarle-Rixtel. Na het behalen van het VWO-ß diploma in 1992 aan het Dr. Knippenbergcollege te Helmond, begon zij met de studie Biologie aan de Universiteit Utrecht. In november 1997 werd het doctoraal examen Biologie behaald. De bijbehorende afstudeerstages werden uitgevoerd te Utrecht op de afdeling Vergelijkende Endocrinologie, faculteit Biologie (begeleiding Prof.dr. H.J.Th. Goos, Dr. J. Bogerd) en op het Hubrecht Laboratorium (begeleiding Dr. G.J.C. Veenstra, Dr. M.H.W. Hooiveld). Aansluitend werd voor een periode van drie maanden een extra stage uitgevoerd op de afdeling Endocrinologie Moléculaire de la Reproduction, Université de Rennes I, Frankrijk (begeleiding Dr. O. Kah en Dr. C. Teitsma).

Vanaf maart 1998 tot en met september 2002 was zij aangesteld als Assistent in Opleiding (AIO) bij de hoofdafdeling Veterinaire Anatomie en Fysiologie, afdeling Fysiologie van de faculteit der Diergeneeskunde, Universiteit Utrecht. De moleculaire studies beschreven in dit proefschrift werden uitgevoerd op de afdeling Inwendige Geneeskunde van het Erasmus Universitair Medisch Centrum te Rotterdam in de periode januari 1999 tot oktober 2001. Het onderzoek werd verricht onder begeleiding van Prof.dr. M.E. Everts en Prof.dr.ir. T.J. Visser en werd gesubsidieerd door de Nederlandse Hartstichting.