Uptake of tri-iodothyronine and thyroxine in myoblasts and myotubes of the embryonic heart cell line H9c2(2-1)

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Abstract

Uptake of tri-iodothyronine (T_3) was compared with that of thyroxine (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. When expressed per well, T_3 and T_4 uptake were, compared with myoblasts, 1.9- to 2-fold and 3.1- to 4-fold higher in myotubes respectively. On the other hand, the characteristics of T_3 and T_4 uptake were similar in myoblasts and myotubes. 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 type I activity, while deiodinase type II 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.

Journal of Endocrinology (2002) **175**, 587–596

Introduction

Thyroid hormones, thyroxine (T_4) and tri-iodothyronine (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 with 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). In both humans and 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 non-genomic cellular actions via the plasma membrane (Huang *et al.* 1999, Davis *et al.* 2002). The action of T_4 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,b), 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 DII 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 with 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 ischemia 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 T₃ responsive.

In the present study, T₃ and T₄ uptake was characterized and compared in the H9c2(2-1) cells. To understand the role of thyroid hormone uptake mechanisms during cardiac differentiation, T₃ and T₄ uptake was compared in undifferentiated myoblasts and differentiated myotubes. In addition, we examined the effects of T₃ and T₄ on the fusion index as an indicator of the degree of differentiation.

Materials and Methods

Materials

Cell culture reagents were purchased from Life Technologies (Breda, The Netherlands). Bovine serum albumin (BSA; fraction V) was obtained from Roche (Mannheim, Germany). L-tri-iodothyronine (T₃), L-thyroxine (T₄), L-tryptophan (Trp) and oligomycin were purchased from Sigma (St Louis, MO, USA), L-isomers of [¹²⁵I]T₃ (24.4 TBq/mmol), [¹²⁵I]T₄ (4.3 TBq/mmol) and [¹²⁵I]reverse T₃ (rT₃; 28.0 TBq/mmol) were from NEN (Boston, MA, USA). Prior to use [¹²⁵I]T₄ and [¹²⁵I]rT₃ 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, Wilts, UK). 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% CO₂ atmosphere in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS) and 100 U/ml penicillin–streptomycin. At day 2 after seeding, half of the culture was used for uptake experiments, while the other half was kept for 7 days in DMEM containing

1% (v/v) FCS and 100 U/ml penicillin–streptomycin to obtain fused myotubes. At day 2, the cultures were 80% confluent, presumably corresponding to around 2×10^5 cells/well. Medium was changed every 48 h. For the deiodinase assays, cells were seeded in 75 cm² culture flasks at a density of 2×10^6 cells/flask and further processed under the same conditions as described above for cells seeded in 24-well culture dishes.

Cellular uptake studies

Uptake experiments were performed at days 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 CaCl₂, 1.4 mM KH₂PO₄, 1.4 mM MgSO₄, 5.0 mM glucose; prepared in 25 mM Tris at pH 7.4) supplemented with 0.1% BSA for determination of T₄ uptake or 0.5% BSA for T₃ 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 c.p.m./ml [¹²⁵I]T₄, 10 nM T₄ and 0.1% BSA, or 4×10^5 c.p.m./ml [¹²⁵I]T₃, 10 nM T₃ 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 ¹²⁵I activity in a γ-counter (Packard Cobra II; Packard Instruments Co., Meriden, CT, USA). The time-course of T₃ and T₄ 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 T₃ and T₄ 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 Trp).

The amount of [¹²⁵I]T₃ or [¹²⁵I]T₄ associated with the cells, representing total cellular uptake, was expressed as fM/pM free hormone (see Free hormone fraction section). Results were corrected for the amount of ¹²⁵I activity associated with the walls of the culture wells. In experiments where uptake was measured in the presence of 10 μM unlabeled hormone, the saturable part of [¹²⁵I]T₃ uptake varied between 56 and 73% in myotubes and myoblasts respectively. For [¹²⁵I]T₄ uptake, these values varied between 33 and 39% in myoblasts and myotubes respectively.

Light microscopy and determination of fusion index

At days 2, 7 and 9 after seeding, cultures were fixed subsequently with 70% (v/v) and 96% (v/v) ethanol and

stained with 100% hematoxylin. 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 μ l 0.5% (v/v) Triton X-100 in phosphate-buffered saline and mildly agitated for 10 min 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 an 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 c.p.m./ml [125 I] T_3 , 10 nM T_3 and 0.5% BSA, the free T_3 fraction was $3.9 \pm 0.3\%$ ($n=5$). Addition of 10 μ M T_3 , 10 μ M T_4 , 10 μ M oligomycin or a combination of oligomycin and T_3 (both at 10 μ M) changed the free T_3 fraction to $4.6 \pm 0.1\%$ ($n=3$), $4.7 \pm 0.0\%$ ($n=4$), $3.8 \pm 0.1\%$ ($n=4$) and $4.9 \pm 0.1\%$ ($n=4$) respectively. The free T_4 fraction in Krebs–Ringer medium with 4×10^5 c.p.m./ml [125 I] T_4 , 10 nM T_4 and 0.1% BSA was $3.3 \pm 0.5\%$ ($n=3$) and changed in the presence of 10 μ M T_4 , 10 μ M T_3 , 10 μ M oligomycin or a combination of oligomycin and T_4 (both at 10 μ M) to $5.1 \pm 0.4\%$ ($n=3$), $4.1 \pm 0.3\%$ ($n=4$), $3.5 \pm 0.1\%$ ($n=4$) and $5.4 \pm 0.5\%$ ($n=4$) 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 $4.4 \pm 0.4\%$ ($n=3$) and $3.0 \pm 0.1\%$ ($n=3$) respectively.

Deiodinase assays

DI and DII activities were determined in myoblasts and myotubes and compared with 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 and 1 mM dithiothreitol (DTT). Subsequently, the cells were homogenized using a Potter–Elvehjem device (Heidolph RZR 2020, Schwabach, Germany) equipped with a Teflon pestle. For measurement of DI activity, 100 μ l aliquots of the homogenates (10 μ g protein in a total assay volume of 200 μ l) were incubated in triplicate for 15 min at 37 °C in 100 μ l incubation medium (100 mM phosphate buffer, 2 mM EDTA, 10 mM DTT) containing 1×10^6 c.p.m./ml [125 I] rT_3 and 1 μ M rT_3 in the presence or absence of 100 μ M propylthiouracil (PTU). The reaction was quenched by adding 100 μ l 5% (w/v) ice-cold BSA followed by 500 μ l 10% (v/v) trichloroacetic acid for deproteinization. Samples were centrifuged for 15 min at 4 °C and 1500 g (Heraeus labofuge 400R, Hanau, Germany) and 500 μ l of the supernatant was acidified with 500 μ l 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 γ -counter (Packard Cobra II). Specific DI activity was defined as the PTU-inhibitable part of iodide production. For measurement of DII activity, 100 μ l aliquots of homogenates (10 μ g protein in a total assay volume of 200 μ l) were incubated in triplicate under the same conditions as for the DI assay, using 1×10^6 c.p.m./ml [125 I] T_4 and 0.5 μ M T_4 with 20 mM DTT. PTU was omitted from the incubation medium.

Statistics

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

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 (Fig. 1A and C). At day 9, DNA content of the cells had reached a constant level, characteristic of 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 days 2, 7 and 9 is shown in Fig. 1B. CK activity increased 9-fold from day 2 to day 7

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 2 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. (A) DNA ($n=3$) and (C) protein ($n=3$) content of the cells was determined throughout the culture period. (B) The CK activity per well ($n=10$) and (D) the degree of fusion in percent (means $\pm$ S.D., $n=2$) were determined at days 2, 7 and 9 of culture.

($P<0.001$) and 3.5-fold from day 7 to day 9 (not significant). The total rise from day 2 to day 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 $18 \pm 4\%$ at day 7 and further increased to $28 \pm 3\%$ at day 9, the only statistical significant increase being from day 2 to day 7 ($P<0.01$).

In Fig. 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 3A shows the time-course of [125 I] T_3 uptake in myoblasts and myotubes. In myoblasts, uptake showed a

steep increase up to 1.5 h, and then slowly leveled off. In myotubes, uptake also leveled off after 1.5 h of incubation. When expressed per well, [125 I] T_3 uptake in myotubes was around 1.9- to 2.5-fold higher than in myoblasts. However, when the data were expressed relative to the protein content, [125 I] T_3 uptake in myotubes was around 1- to 1.3-fold higher compared with myoblasts. [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 (Fig. 3A).

T_4 uptake in myoblasts and myotubes as a function of incubation time leveled off after 1 h of incubation (Fig. 3B). [125 I] T_4 uptake per well was 3.1- to 4-fold higher in 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 1.6- to 2.2-fold higher than in myoblasts ($P<0.05$). This is somewhat higher than 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 (Fig. 3B).

Figure 2 Phase-contrast pictures ($10\times$) of H9c2(2-1) (A) myoblasts and (B) myotubes. Myoblasts were seeded at a density of 5×10^5 cells/well and cultured for 2 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.

Comparison of [125 I] T_3 and [125 I] T_4 uptake

Figure 4 shows 30-min uptake of [125 I] T_3 (left side) and [125 I] T_4 (right side) in myoblasts. The control values for uptake of [125 I] T_3 and [125 I] T_4 were 0.040 ± 0.001 fM/pM free T_3 and 0.072 ± 0.003 fM/pM free T_4 respectively, a 1.8-fold difference ($P<0.001$). [125 I] T_3 uptake was significantly inhibited by 10 μ M unlabeled T_3 (73%; $P<0.001$) and 10 μ M unlabeled T_4 (48%; $P<0.001$) (Fig. 4, left side). Oligomycin, added at a concentration of 10 μ M, significantly inhibited [125 I] T_3 uptake by 20% ($P<0.01$). Co-incubation with 10 μ M unlabeled T_3 and 10 μ M oligomycin resulted in a 65% ($P<0.001$) reduction of T_3 uptake, comparable with the effect of unlabeled T_3

alone. Similar experiments were performed with [125 I] T_4 uptake in myoblasts (Fig. 4, right side). [125 I] T_4 uptake was significantly reduced by 10 μ M unlabeled T_4 (33%; $P<0.001$) and 10 μ M unlabeled T_3 (60%; $P<0.001$). [125 I] T_4 uptake in myoblasts was not inhibited when 10 μ M oligomycin was added to the incubation medium. Co-incubation of 10 μ M unlabeled T_4 and 10 μ M oligomycin resulted in an inhibition comparable with 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 (Fig. 5) and showed the following control values: 0.088 ± 0.005 fM/pM free T_3 and 0.180 ± 0.008 fM/pM free T_4 respectively, a 2-fold difference ($P<0.05$). Unlabeled T_3 (10 μ M) 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% ($P<0.001$) and 39% ($P<0.001$) respectively. When myotubes were incubated with 10 μ M oligomycin, [125 I] T_3 uptake was reduced by 31% ($P<0.001$) (Fig. 5, left side) and [125 I] T_4 uptake was reduced by 26% ($P<0.01$) (Fig. 5, right side). The effect of co-incubation of oligomycin and unlabeled T_3 on [125 I] T_3 uptake in myotubes was comparable with the effect of unlabeled hormone alone (56%; $P<0.001$). This was also found for [125 I] T_4 uptake in the 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 fM/pM free T_3 to 0.032 ± 0.001 fM/pM free T_3 in the presence of 2 mM Trp (49%, $P<0.001$).

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 days 2, 3, 7 and 9 (Fig. 6). In general, the fusion index increased from day 2 to day 9, reflecting the morphological differentiation of the cells (see also Fig. 1D). Only at day 9 did exposure to T_3 and T_4 significantly stimulate fusion: T_3 treatment by $35 \pm 2\%$ ($P<0.01$ vs control at day 9), and T_4 by $40 \pm 2\%$ ($P<0.001$ vs control at day 9), as compared with $26 \pm 1\%$ in the control cultures. In addition, the effect of T_3 was not statistically different from that of T_4 .

Figure 3 Time-course of (A) $[^{125}\text{I}]\text{T}_3$ uptake and (B) $[^{125}\text{I}]\text{T}_4$ 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 $[^{125}\text{I}]\text{T}_3$ was measured in Krebs-Ringer medium with Na^+ (solid symbols) or in Krebs-Ringer in which Na^+ was replaced by choline (open symbols), both supplemented with 0.5% BSA. For uptake of $[^{125}\text{I}]\text{T}_4$, the media were supplemented with 0.1% BSA. Data show the means $\pm$ S.E.M. of 7–13 observations of five experiments for $[^{125}\text{I}]\text{T}_3$ uptake and of 6 observations from two experiments for $[^{125}\text{I}]\text{T}_4$ uptake.

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 Fig. 1.

Deiodinase activity

DI activity was 0.32 ± 0.05 fmol/ μg protein per min in myoblasts ($n=3$) and 0.22 ± 0.07 fmol/ μg protein per 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 per min and less than 1 fmol/ μg protein per min respectively.

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 the 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 with 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), fibroblasts (Docter *et al.* 1987, Benvenga & Robbins 1990) and skeletal muscle (van Hardeveld & Kassenaar 1978). T_4 uptake in these cell types is mediated by a saturable and energy-dependent mechanism. T_4 uptake showed a steeper time-course compared with T_3 . On the other hand, the characteristics of T_3 and T_4 uptakes were comparable in myotubes and myoblasts. First, T_3 and T_4 uptakes were inhibited roughly to the same extent by oligomycin (most clearly in myotubes) and, thus, T_3 and T_4 uptakes depend on the energy status of the cell. Secondly, T_3 and T_4 uptakes did not depend on the Na^+ gradient. Thirdly, 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 compared with that of T_4 . Together these findings are consistent with the idea that T_3 and T_4 share the same transporter in cardiac cells, but our results do not

Figure 4 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 μ M) were only present during incubation, whereas OM (10 μ M) 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. The difference between the solid and the hatched bars (absence (control; Ctrl) and presence of 10 μ M unlabeled hormone) represents the saturable part of $[^{125}I]T_3$ and $[^{125}I]T_4$ uptake. Data are presented as means $\pm$ S.E.M. of 6–18 observations from three experiments. * $P < 0.001$ vs control; # $P < 0.01$ vs control.

allow any conclusion about the affinity of the transporter for T_3 and T_4 to be drawn. 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 are presumably 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 Harveldt & Kassenaar 1978, Pontecorvi & Robbins 1986) and in a skeletal muscle cell line (Pontecorvi *et al.*

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 myotubes. Experiments were performed exactly as described for myoblasts (Fig. 4). The difference between the solid and the hatched bars (absence (control; Ctrl) and presence of 10 μ M unlabeled hormone) represents the saturable part of $[^{125}I]T_3$ and $[^{125}I]T_4$ uptake. Data are presented as means $\pm$ S.E.M. of 6–18 observations from three experiments. * $P < 0.001$ vs control; # $P < 0.01$ vs control.

Table 1 Effects of Trp on 30-min uptake of $[^{125}I]T_3$ and $[^{125}I]T_4$ in myoblasts. H9c2(2-1) myoblasts were preincubated (30 min) and incubated in the absence or presence of Trp. Data show the means $\pm$ S.E.M. from two independent experiments

	$[^{125}I]T_3$ uptake (fM/pM free T_3)	Effect (%)	$[^{125}I]T_4$ uptake (fM/pM free T_4)	Effect (%)
No additions	0.035 $\pm$ 0.001		0.112 $\pm$ 0.003	
Trp (0.5 mM)	0.014 $\pm$ 0.001*	–60	0.077 $\pm$ 0.003*	–31
Trp (2 mM)	0.011 $\pm$ 0.001*	–69	0.032 $\pm$ 0.007*	–71

* $P < 0.001$, Trp vs no additions.

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

Figure 6 Effect of T₃ and T₄ on fusion index. Cells were cultured from day 2 to day 9 in DMEM supplemented with 1% FCS and 5 nM T₃ (open bars) or 50 nM T₄ (hatched bars). Fusion index was determined at days 2, 3, 7 and 9 as described in Materials and Methods. Data are presented as means $\pm$ S.E.M. of two independent experiments. # $P < 0.01$ and * $P < 0.001$, T₃ or T₄ vs control at day 9.

uptake was comparable with the effect of unlabeled T₃: 69% and 73% in myoblasts, 49% and 60% in myotubes respectively. In general, incubations with excess unlabeled T₃ distinguish membrane-associated T₃ 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 T₃ uptake in H9c2(2-1) cells. Trp also inhibited the uptake of T₄ to the same extent as that of T₃ (71% and 69% respectively). When compared with the inhibition by unlabeled T₄ (33%), the effect of Trp was higher (71%), but comparable with the inhibitory effect of unlabeled T₃ on T₄ uptake (60%). These findings further support the idea that T₄ and T₃ 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 T₃ or T₄ (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.

T₃ and T₄ significantly increased the fusion index at day 9, indicating that the T₃ and T₄ uptake mechanisms are

important for the availability of thyroid hormones during differentiation. This is further supported by the observation that thyroid hormone uptake, most evident that of T₄, is higher in myotubes compared with myoblasts. Gonçalves *et al.* (1989) also reported a higher uptake in differentiated cells compared with undifferentiated cells. Although actions of T₄ on differentiation have been described (Brik & Shainberg 1990, Nakashima *et al.* 1998a, 1998b), it is generally thought that T₃ and not T₄ is the main regulator of such processes. The affinity of T₄ for the nuclear T₃ receptor is one-tenth that of T₃ (Muñoz & Bernal 1997). The effects on differentiation of the H9c2(2-1) cells were examined under conditions in which the free T₄ concentration was 7-fold higher than that of T₃, i.e. 1.5 and 0.2 nM respectively. Thus, theoretically, T₄ may have stimulated differentiation of the H9c2(2-1) cells by association with the nuclear T₃ receptor. If T₄ by itself does not induce differentiation, it should be deiodinated to T₃ to allow binding to the nuclear T₃ 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 by 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 T₄ into T₃, it is thought to play a minor role in the local production of T₃ (Visser 1996). Local T₃ 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 the 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. Finally, non-genomic effects of T₄ have been reported (Davis *et al.* 2002). T₄ appears to activate signal transducing kinases (Davis *et al.* 2002), amongst them those involved in differentiation such as the mitogen-activating protein kinase pathway (Chun *et al.* 2000).

In summary, the H9c2(2-1) cell line provides a useful model system for examination of thyroid hormone uptake in cardiac cells. Uptake of T₃ and T₄ is regulated by a specific and energy-dependent mechanism, and it appears that T₃ and T₄ share a common transport mechanism in H9c2(2-1) cells. Furthermore, the ability of both T₃ and T₄ 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 a therapeutic approach to target the heart with T₃, T₄ 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).

Acknowledgements

The authors thank T J Visser and E C H Friesema (Department of Internal Medicine, Erasmus University Medical School, Rotterdam, The Netherlands) for constructive discussions. This work was supported by The Netherlands Heart Foundation (grant no. 96.175) and The Netherlands Organization for Scientific Research (grant no. 903.40.194).

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Received in final form 20 August 2002

Accepted 21 August 2002