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A physiological role for glucuronidated thyroid hormones: Preferential uptake by H9c2(2-1) myotubes

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Abstract

Conjugation reactions are important pathways in the peripheral metabolism of thyroid hormones. Rat cardiac fibroblasts produce and secrete glucuronidated thyroxine (T4G) and 3,3′,5-triiodothyronine (T3G). We here show that, compared to fibroblasts from other anatomical locations, the capacity of cardiofibroblasts to secrete T4G and T3G is highest. H9c2(2-1) myotubes, a model system for cardiomyocytes, take up T4G and T3G at a rate that is 10–15 times higher than that for the unconjugated thyroid hormones. T3 and T4, and their glucuronides, stimulate H9c2(2-1) myoblast-to-myotube differentiation. A substantial β-glucuronidase activity was measured in H9c2(2-1) myotubes, and this confers a deconjugating capacity to these cells, via which native thyroid hormones can be regenerated from glucuronidated precursors. This indicates that the stimulatory effects on myoblast differentiation are exerted by the native hormones. We suggest that glucuronidation represents a mechanism to uncouple local thyroid hormone action in the heart from that in other peripheral tissues and in the systemic circulation. This could represent a mechanism for the local fine-tuning of cardiac thyroid hormone action.

Keywords: Thyroxine; Tri-iodothyronine; Glucuronidation; Myoblast; H9c2(2-1) cells; Cellular uptake; Differentiation

1. Introduction

The thyroid gland mainly secretes thyroxine (T4), which is enzymatically deiodinated in the periphery to yield the potently biologically active hormone 3,5,3′-triiodothyronine (T3). Besides deiodination, conjugation reactions are important pathways in the peripheral metabolism of thyroid hormones. Enzymatically catalyzed sulfation and glucuronidation reactions produce iodothyronine sulfates and glucuronides, respectively (Visser, 1996; Wu et al., 2005). Glucuronidation reactions, catalyzed by uridine 5′-diphosphate-glucuronyltransferases (UGTs), transfer a glucuronosyl group to the phenolic hydroxyl group of the iodothyronine molecule. UGTs constitute a superfamily of many enzyme isoforms. Based on amino acid similarities two subfamilies, UGT1 and UGT2, can be identified (Mackenzie et al., 1997). When classified by substrate specificity, three UGT isoforms have been implicated to be involved in the glucuronidation of thyroid hormones, *viz.* phenol-UGT (UGT1A9) and bilirubin-UGT (UGT1A1) that both conjugate T4 and reverse T3 (rT3, 3,3′,5′-triiodothyronine), and androsterone-UGT (UGT2B7) that conjugates T3 (Beetstra et al., 1991; Visser et al., 1993a,b). The substrate specificity of UGT isoforms is, however, not fully characterized.

Glucuronidated iodothyronines are considered to be biologically inactive, and the increased water solubility facilitates biliary excretion. The liver is the predominant site of expression of many UGT isoforms (Shelby et al., 2003), but extrahepatic sources of UGT activity have been found in other organs such as the kidney, skin, lung and small intestine (Grams et al., 2000; Turgeon et al., 2001) and, in smaller amounts, spleen, thymus, brain and heart (Burchell and Coughtrie, 1989). We have demonstrated that glucuronidated iodothyronines are synthesized and secreted by cardiac fibroblasts, not cardiomyocytes, obtained from neonatal rat heart (van der Heide et al., 2002). These observations strongly hint at a role of glucuronidating mechanisms other than merely the facili-

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tation of detoxification and plasma clearance through biliary excretion.

The extensive peripheral metabolism, by deiodination and conjugation, of thyroid hormones can be regarded as analogous to the posttranslational modifications that determine the unique biological actions of many mature peptide hormones. Glucuronidation profoundly affects the physico-chemical properties of substrates. Compared to native iodothyronines, conjugates possess highly different biological activities and reactivities towards enzymes, transporters, biological membranes, receptors and binding proteins (Hays and Hsu, 1988; Visser, 1990). For example, T3 glucuronide (T3G) is resistant to inner and outer ring deiodination, whereas T4G is not (Eelkman Rooda et al., 1989; Hays and Cavalieri, 1992). Glucuronidated thyroid hormones are still susceptible to deconjugation by β-glucuronidase activity to yield the native hormones (Hays and Hsu, 1987; van der Heide et al., 2002), and it has been suggested that this constitutes an alternative pathway, *i.e.* deiodination of T4G to T3G followed by deconjugation, for the generation of T3 in rat brain and liver (Hays and Cavalieri, 1992). Although conclusive evidence is lacking, concerted thyroid hormone conjugations and deconjugations could well represent a mechanism for the local or systemic regulation of thyroid hormone bioavailability.

Cardiofibroblasts are the most abundant cell type in the heart (Agocha and Eghbali-Webb, 1997; Nair et al., 1968), and have the capacity to metabolize thyroid hormones and secrete their metabolites (van der Heide et al., 2002, 2004). This would confer to cardiofibroblasts a role, by endocrine or paracrine interactions, in cardiac physiology. We here investigated the effect of thyroid hormone glucuronidation on cellular uptake and differentiation of the myogenic cell line H9c2(2-1) from embryonic rat heart ventricle.

2. Materials and methods

Three-day-old (hereafter referred to as neonatal) and 6-week-old (hereafter referred to as adult) Wistar rats were obtained from laboratory stock (Utrecht University, The Netherlands). Rats were killed by decapitation, organs were quickly dissected and processed as described below. All animal procedures were approved by the local ethical review committee.

2.1. Materials

All reagents used for cell isolation and cell culture were obtained from Life Technologies BV (Breda, The Netherlands), with the exception of trypsin (Boehringer, Mannheim, Germany). Sephadex LH-20 was purchased from Amersham Pharmacia Biotech Benelux (Roosendaal, The Netherlands). Polystyrene 24-wells culture dishes were from Corning Costar Europe (Badhoeve-dorp, The Netherlands). 3,5,3′-Triiodothyronine, 3,3′,5′-triiodothyronine (reverse T3, rT3) and thyroxine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Radiolabeled [^{125}I]T3 (specific radioactivity: 81.4 TBq/mmol), [^{125}I]rT3 (29.9 TBq/mmol) and [^{125}I]T4 (4.3 TBq/mmol) were obtained from NEN Life Science Products Inc. (Boston, MA, USA). To remove free iodide traces, radiolabeled iodothyronines were purified on Sephadex LH-20 columns with a 2.5 ml bed volume shortly before use (van der Heide et al., 2002). The purified hormone was collected in 1 M NH_3/96% ethanol, lyophilized at room temperature under a stream of N_2 and redissolved in the appropriate medium for cellular uptake studies and free hormone fraction determination. All other chemicals were analytical grade and obtained from commercial suppliers.

2.2. Cell cultures

2.2.1. Primary fibroblast cultures

Primary cultures of neonatal rat cardiac fibroblasts were prepared as described previously (Everts et al., 1996). Hearts were digested using trypsin to obtain a cell suspension. Cardiac fibroblasts were obtained by preplating the cell suspension for 2 h in culture flasks, and cultured at 37 °C in a 5% CO_2 atmosphere in DMEM supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin. Two days after isolation the culture medium was replaced with fresh medium. Fibroblasts thus obtained were grown for 6 days, after which period they were harvested with 0.25% trypsin in Ca^{2+}-free buffer and seeded into 24-wells culture plates at a density of 1.0×10^6 cells per well.

Cardiac fibroblasts of adult rats were isolated using a modification of the Langendorff perfusion. Isolation was performed at 25 °C under calcium-free conditions, buffers were saturated with carbogen. The heart was perfused for 20–25 min with Krebs-Heinseleit saline containing 9 mg/100 ml liberase blendzyme type IV (Roche Diagnostics Corporation, Mannheim, Germany) and 25 mM $CaCl_2$. Then the tissue was diced in 1-mm^3 pieces and incubated for 10 min in Krebs-Heinseleit saline containing liberase blendzyme type IV, 0.2% fatty acid-free BSA and 25 μg/ml Dnase. The incubate was filtered through cheese cloth, and the filtrate was centrifuged ($25 \times g$, 5 min). As judged by light microscopy inspection, the pellet thus obtained contained cardio-myocytes, whereas the supernatant contained fibroblasts. The supernatant was centrifuged at $325 \times g$ (5 min), and the pellet was seeded in a culture flask. Cardiac fibroblasts were cultured in DMEM supplemented with 10% FCS until cellular confluence was reached.

Selected organs of adult rats were quickly dissected and collected in ice-cold phosphate-buffered saline. Explants with a size of approximately 1 mm^3 were transferred to gelatine-coated Petri dishes and cultured in DMEM supplemented with 5% FCS, 5% human serum and 1% penicillin/streptomycin. The culture medium was replaced with fresh medium every 48 h. Fibroblast monolayers were harvested with 0.25% trypsin in Ca^{2+}-free buffer and then cultured in a 75-cm^2 culture flask until confluence. Fibroblasts were successfully obtained from lung and skin explants, cultures consisted for >90% of fibroblasts, as judged from immunocytochemistry using an anti-vimentine antibody (DakoCytomation, Glostrup, Denmark) (results not shown). We were unsuccessful to grow fibroblasts from m. gastrocnemius, liver, kidney and pancreas explants.

2.2.2. Cell lines

We used three different commercially available cell lines (European Collection of Cell Cultures, Salisbury, UK). H9c2(2-1) is a myogenic cell line from embryonic rat heart ventricle (Kimes and Brandt, 1976). H9c2(2-1) myoblasts were seeded in 24-well culture dishes at a density of 0.5×10^5 cells/well and cultured in low glucose DMEM supplemented with 34.5 mM $NaHCO_3$, 10% FCS and 1% penicillin/streptomycin (van der Putten et al., 2002). Two days after seeding, serum content was reduced from 10% to 1% in the incubation medium, which successfully induced myotube formation (results not shown). C2C12 mouse myoblast cells were cultured as described for the H9c2(2-1) myoblasts. The 27FR rat embryonic skin fibroblast cell line was cultured in "high glucose" (25 mM) DMEM supplemented with 2 mM glutamine, 10% FCS and 1% penicillin/streptomycin.

2.2.3. Cellular uptake studies fibroblasts

Cellular uptake was measured in 24-wells culture plates. After removal of the culture medium, fibroblasts, at a density of 1.0×10^6 cells/well, were preincubated in Krebs-Ringer buffer supplemented with 0.5% BSA (30 min, 37 °C). The incubation medium was identical to the preincubation medium, but now 100 nM T4 or T3, and, respectively, [^{125}I]T4 or [^{125}I]T3 to a radioactive concentration of 10^6 cpm/ml were added. Incubation was performed at 37 °C, and was terminated by rapid aspiration of the incubation medium. Cells were washed with ice-cold isotonic saline to remove free tracer, dissolved in 1 ml 0.1 M NaOH and counted for ^{125}I-activity in a gamma-counter (Packard Cobra II, Packard Instruments Co., Meriden, CT, USA).

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2.2.4. H9c2(2-1) myotubes

After removal of the culture medium, differentiated H9c2(2-1) myoblasts were preincubated in Krebs-Ringer buffer (30 min, 37 °C) supplemented with 0.1% BSA for the measurement of the uptakes of T4 and T4G, and with 0.5% BSA for the measurement of the uptakes of T3 and T3G. The incubation medium was identical to the pre-incubation medium to which, substrates were added at a total concentration of 10 nM. When the uptake of T4 and T3 was measured, [^{125}I]T4 and [^{125}I]T3 were added at a radioactive concentration of 2×10^6 cpm/ml. Radiolabeled glucuronidated iodothyronines were synthesized as described below, and were added to the incubation medium at a total concentration of 10 nM [^{125}I]T4G or [^{125}I]T3G. The incubation was quenched, and the incubate analysed as described above. The effects of native iodothyronines on the uptake of their glucuronidated metabolites, at concentrations of 10 μM and 10 nM, respectively, were assessed in 10-min endpoint assays.

2.3. Synthesis of thyroid hormone glucuronides

Thyroid hormone glucuronides were produced in a culture of adult rat cardiofibroblasts, using a procedure that we have described for neonatal rat cardiac fibroblasts (van der Heide et al., 2002). Fibroblasts, grown in a 75-cm^2 culture flask to 80% confluence, were washed with 5 ml phosphate-buffered saline, and then incubated in 5 ml DMEM culture medium to which 100 nM T3 or 100 nM T4 was added, with a radioactive concentration of 2×10^6 cpm/ml [^{125}I]T3 or [^{125}I]T4 tracer, respectively. Substrate concentrations were chosen so as to prevent substrate depletion during the 2-day incubation period. Cells were incubated at 37 °C in a 5% CO_2 atmosphere, and after 48 h the medium was collected. Iodothyronine metabolites were resolved by Sephadex LH-20 column chromatography as described below. Water-soluble metabolites, that we previously identified as glucuronides (van der Heide et al., 2002), were pooled and evaporated to dryness under a stream of N_2. The amount of glucuronides in the dried fractions was calculated from the ^{125}I-radioactivity measured and the specific activity of the tracer added. We typically obtained 3.6 ± 0.5 nmol T4 glucuronide (T4G), and 6.6 ± 0.3 nmol T3 glucuronide per culture.

2.4. Free hormone fraction analysis

Free hormone fractions were measured by equilibrium dialysis as described by Sterling and Brenner (1966). Radiotracers were purified as described above. The free fractions of T4 and T4G were measured at a total concentration of 10 nM in Krebs-Ringer buffer supplemented with 0.1% BSA. The free fractions of T3 and T3G were measured at the same total concentration in Krebs-Ringer buffer supplemented with 0.5% BSA.

2.5. Determination of fusion index

At days 1 and 7 after initiating differentiation, cultures were fixated with, subsequently, 70% and 96% ethanol, and stained with haematoxylin. Nuclei were counted using an inverted microscope (Nikon TMS-F, Bunnik, The Netherlands). Images were acquired using a Nikon Coolpix 990 digital camera. Myotubes were defined as a plasmalemma containing three or more nuclei. The fusion index was calculated from five microscopic fields, and was calculated by dividing the number of nuclei in the myotubes by the total number of nuclei counted.

2.6. Sephadex LH-20 column chromatography and post-analysis

Columns consisted of Sephadex LH-20 (10%, w/v) with a 2.5 ml bed volume in a glass pipette, and were equilibrated with 3× 1 ml 0.1 M HCl before use. Samples were acidified with one sample volume 1.0 M HCl, and chromatography was performed according to Mol and Visser (1985). Iodide, water-soluble conjugates and native hormones were collected in 3× 1 ml 0.1 M HCl, 8× 1 ml H_2O and 3× 1 ml 35 mM NH_3 in 96% ethanol, respectively. Collected fractions were analyzed for ^{125}I-activity in a gamma-counter. Water fractions from Sephadex LH-20 minicolumns were collected and evaporated to dryness as described above. The residue was dissolved in 2 ml 50 mM phosphate-buffered saline (pH 6.8), and divided into two equal aliquots. To one aliquot, 50 μg β-glucuronidase was added, the other received vehicle only, and both were incubated at 37 °C for 24 and 48 h. The effects of β-glucuronidase digestion were assessed by Sephadex LH-20 column chromatography as described above.

2.7. Effects of thyroid hormone glucuronides on H9c2(2-1) myoblast differentiation

H9c2(2-1) myoblasts were seeded at a density of 50×10^3 cells per well in 24-wells culture plates and cultured in DMEM supplemented with 10% FCS and 1% penicillin/streptomycin for 2 days until 80% cellular confluence was reached. Then, the culture medium was aspirated and replaced by fresh medium with supplements as indicated in the legends to the figures. To avoid possible confounding effects from thyroid hormones present in FCS, we prepared a stripped FCS from which the iodothyronines were removed according to the protocol of Samuels et al. (1979). The effects of thyroid hormone metabolites on the differentiation of H9c2(2-1) cells were assessed in the presence of a reduced serum concentration of 1%.

2.8. β-Glucuronidase

β-Glucuronidase activity was assayed according to Fishman (1974). In short, 100 μl of scraped H9c2(2-1) myotubes, with a protein concentration of 1 mg/ml, was incubated with 1 mM phenolphthalein-β-glucuronide in a total volume of 250 μl incubation medium (70 mM acetic acid at pH 5.0) for 60 min at 37 °C. Incubations without protein were used as a negative control, a homogenate from rat liver was used as a positive control. Phenolphthalein served as a standard. The reaction was quenched by adding 250 μl ice-cold 5% TCA. The pH of the incubate was adjusted to 10.2–10.5 by adding 500 μl of a glycine/NaOH solution (174 mM glycine, 228 mM NaCl, 318 mM NaOH, pH 12.9). Absorbance was measured at λ = 540 nm. The specific β-glucuronidase-UGT activity was defined as nmol phenolphthalein/h/mg protein.

2.9. Immunocytochemistry

Primary polyclonal rabbit anti-human UGT1A1 and anti-human UGT2B7 were obtained from BD Biosciences/Gentest (Woburn, MA); monoclonal mouse anti-bovine vimentin (a fibroblast-specific marker) was from DakoCytomation (Glostrup, Denmark). Primary cultures of adult rat cardiofibroblasts were grown on sterile glass coverslips in 24-wells plates until confluence, washed in phosphate-buffered saline and fixated in 4% paraformaldehyde. Aspecific binding sites were blocked with 15% goat serum (20 min at room temperature), and cells were incubated with anti-vimentin (1:200 dilution), and either anti-UGT1Al (1:500 dilution) or anti-UGT2B7 (1:500 dilution) (2 h, room temperature). Secondary antibodies were from Molecular Probes Europe BV (Leiden, The Netherlands). Highly cross-adsorbed Alexa Fluor 488-conjugated goat anti-rabbit IgG, and Alexa Fluor 568-conjugated goat anti-mouse IgG were used to detect UGT and vimentin primary antibody binding, respectively, according to manufacturer's instructions, and were incubated for 90 min at room temperature. Finally, coverslips were inbedded in FluorSave™ Reagent (Calbiochem, San Diego, CA), and investigated using a Leica TCS SP confocal laser scanning microscope.

2.10. Analysis and statistics

Cellular uptake data were analyzed using a weighted non-linear regression data analysis program (Leatherbarrow, 1987) in which Marquardt's algorithm for least squares estimation of parameters (Marquardt, 1963) is employed. Uptake progress curves were fitted to a single first-order rate equation: $f(t) = \text{Limit} \cdot (1 - e^{-kt})$, where the Limit represents the uptake at time (t) approaching ∞, and k is a first-order rate constant. Data points were not transformed prior to analysis, and the S.E.M. was used as an explicit weighting value for each data point. Statistical significance was evaluated with Student's t-test, or by one-way ANOVA followed by Tukey's multiple comparison test, where appropriate. Significance was accepted at $P < 0.05$ (two-tailed), probabilities are indicated by asterisks ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

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3. Results

Fig. 1 shows the progress curves of the uptake of T4 and T3 in cultured neonatal cardiac fibroblasts. The uptake of both iodothyronines is well described by a single first-order exponential equation, with calculated rate constants of 0.03 and 0.04 min^{-1}, respectively. Data points and calculated parameters converge well on a linearization of the single exponential, indicative of one active component in the net cellular uptake of T4 and T3. Measured at a total iodothyronine concentration of 100 nM, the calculated limit for the uptake of T3 is 1.7-fold higher than that for T4. From the slope of the tangents to the progress curves at time = 0 min, the maximum uptake rates can be calculated at 0.3 and 0.7 fmol/min per μg protein for T4 and T3, respectively, which is a 2.3-fold difference.

Fig. 2 shows that cardiac fibroblasts from adult rat secrete water-soluble metabolites upon 48 h exposure to 100 nM T4 and T3. Following β-glucuronidase treatment, 79 ± 2% of the water-soluble T4 metabolites was digested after 24 h ($n = 3$), and >90% after 48 h. Similarly, 54 ± 2% of the water-soluble T3 metabolites was digested after 24 h ($n = 3$), and >80% after 48 h. Based on their susceptibility to β-glucuronidase digestion we identified these metabolites as glucuronides. This is corroborated by our observation of cytosolic immunoreactivities for both UGT1A1 and –2B7 in vimentin-positive cells in our cultures (results not shown). No increases in ^{125}I-radioactivity in the HCl eluates, containing free iodide, were detected, indicating that no appreciable outer ring deiodination of thyroid hormones by cardiofibroblasts occurred. These results show that the conjugating and deiodinating activities of cardiofibroblasts from adult rat heart are similar to those reported for neonatal rat cardiofibroblasts (van der Heide et al., 2002).

Neonatal and adult cardiac fibroblasts secrete the highest amount of T4 glucuronide and T3 glucuronide compared to fibroblasts from other sources, and compared to two myoblast cell lines, as shown in Figs. 3A and 4A. Glucuronidation rates are the net result of a cell's capacity for substrate uptake, glucuronidation, and extrusion of the glucuronidated metabolite. We therefore have defined the normalized glucuronidation rate as the ratio of the amount of secreted glucuronides per total amount of hormone (native and glucuronidated) in the cell. When corrected for uptake, neonatal and adult cardiac fibroblasts still have the highest glucuronidation rates compared to the other cell types investigated (Figs. 3B and 4B).

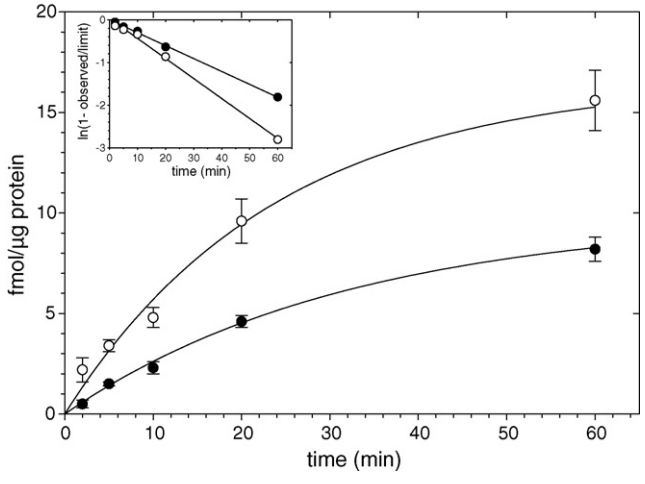

Fig. 1. Progress curve of the uptake of T4 (●) and T3 (○), both at 100 nM in neonatal cardiac fibroblasts cultured in medium containing 0.5% BSA ($n = 3$–4, average values ± S.E.M. are shown). Data points were fitted to a single first-order rate equation (see text); calculated kinetic parameters for T4 uptake are: Limit = 10 fmol/μg protein, $k = 0.03\ min^{-1}$, and for the uptake of T3: Limit = 17 fmol/μg protein, $k = 0.04\ min^{-1}$. The inset shows linearizations of the single exponential equations.

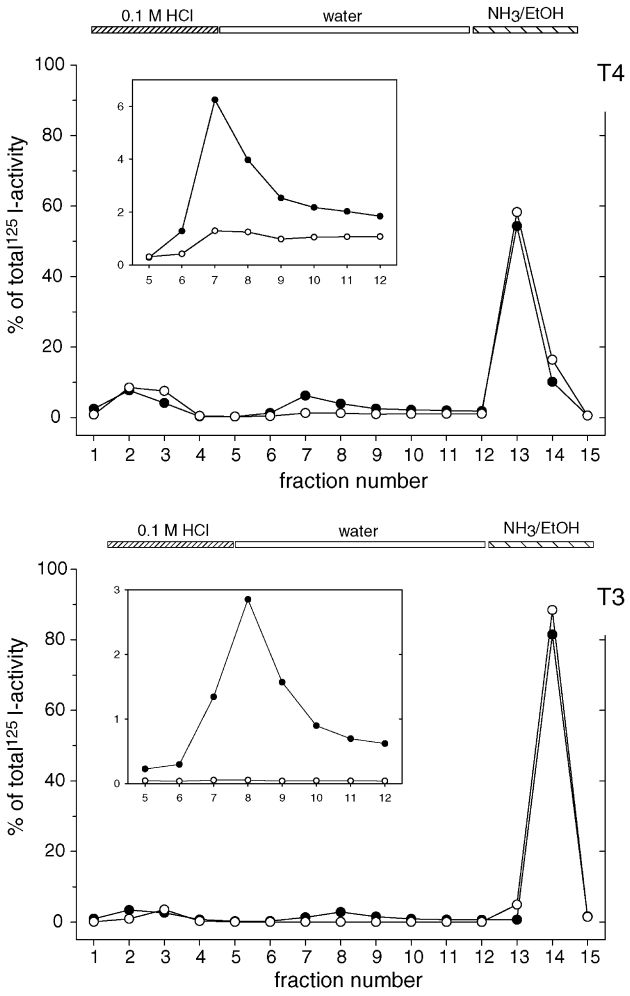

Fig. 2. Sephadex LH-20 chromatograms of T4 glucuronides (top panel) and T3 glucuronides (bottom panel) produced by adult cardiac fibroblasts exposed for 48 h to 100 nM T4 and T3, respectively. Typical results of six different experiments are shown. Control incubations (○) were performed without cells. The insets show fractions 5–12, containing water-soluble glucuronides, in more detail.

Fig. 5A and B shows the progress curves of the uptake of T4G and T3G and native iodothyronines in differentiated H9c2(2-1) myotubes. Glucuronidated T4 and T3 are preferentially taken up by myotubes, as evidenced by the equilibrium levels for T4G and T3G that are 12- and 4-fold higher than for native T4 and T3, respectively. In contrast with the uptake of iodothyronines in neonatal rat cardiac fibroblasts (Fig. 1), progress curves are not adequately described by single exponential equations, indicating the presence of multiple transport pathways for glucuronidated

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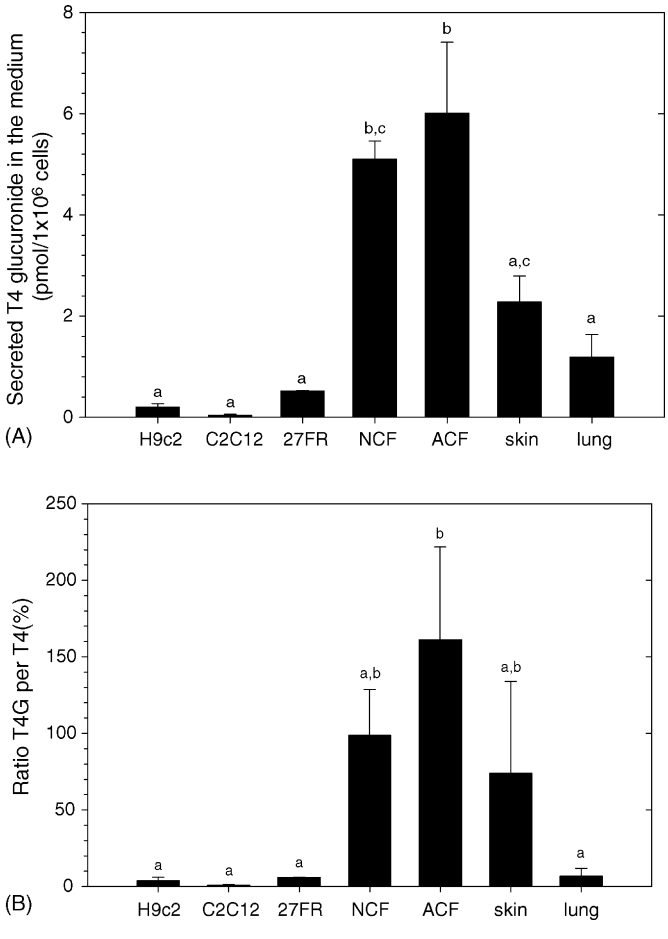

Fig. 3. (A) Amount of T4 glucuronide secreted by cultured myoblasts and fibroblasts of different origin after 48 h of incubation with 100 nM T4 ($n = 3$–12, mean ± S.E.M.). (B) Normalized glucuronidation rate, expressed as the ratio of the amount of T4 glucuronides secreted per the amount of T4 in the cell (native and glucuronides) ($n = 3$–12, mean ± S.E.M.). Bars with the same letter do not differ significantly. NCF: neonatal rat cardiac fibroblasts; ACF: adult rat cardiac fibroblasts.

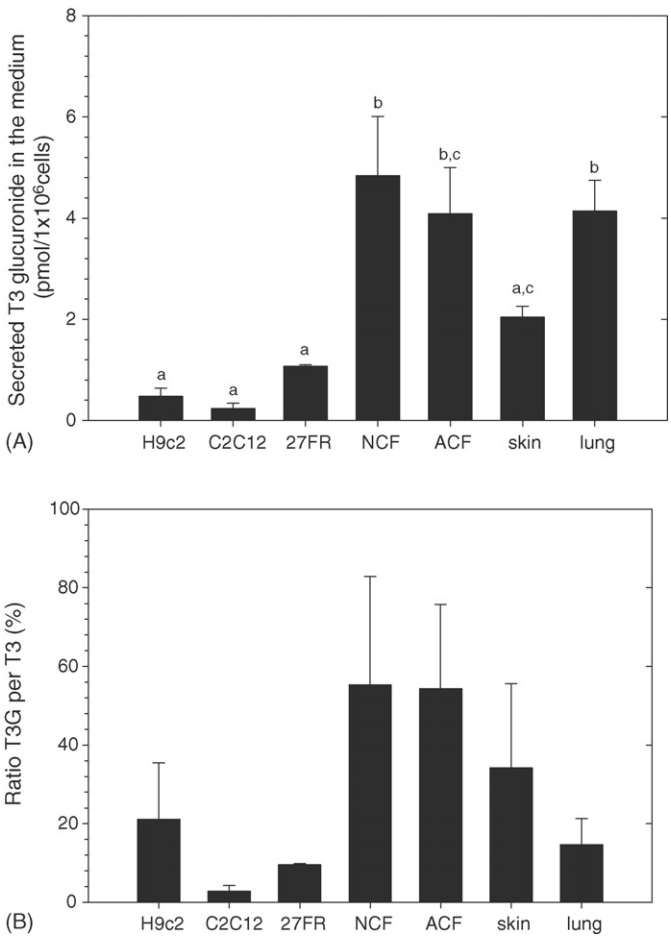

Fig. 4. (A) Amount of T3 glucuronide secreted by cultured myoblasts and fibroblasts of different origin after 48 h of incubation with 100 nM T3 ($n = 3$–12, mean ± S.E.M.). (B) Normalized glucuronidation rate, expressed as the ratio of the amount of T3 glucuronides secreted per the amount of T3 in the cell (native and glucuronides) ($n = 3$–12, mean ± S.E.M.). Bars with the same letter do not differ significantly.

and native thyroid hormones in H9c2(2-1) myotubes. The free, dialyzable fractions of T4 and T3 and their glucuronides in the incubation media for cellular uptake do not differ significantly (Table 1).

The uptake of T4G and T3G is virtually unaffected by a 1000-fold surplus concentration of T3. In contrast, a 1000-fold surplus of T4 inhibits the uptake of T3G by 90% ($P = 0.029$) (Fig. 6). The uptake of T4G is inhibited by 31% by T4, but this result does not reach statistical significance ($P = 0.27$).

Table 1
Free fractions of 10 nM T4 and T3, and their glucuronides, in media for the measurement of cellular uptake (Krebs-Ringer buffer supplemented with BSA)

Iodothyronine species	BSA supplementation (%)	Free fraction (%)
T4	0.1	4.6 ± 0.4
T4 glucuronide	0.1	4.6 ± 0.6
T3	0.5	4.0 ± 0.3
T3 glucuronide	0.5	3.6 ± 1.5

Mean ± S.D. are shown ($n = 3$).

The effects of T3, T4 and their respective glucuronides on H9c2(2-1) myoblast-to-myotube differentiation are most prominent after 7 days of incubation (Fig. 7). The myoblast fusion index rises two- to three-fold compared to the control incubation, with no significant differences between the native hormones and their glucuronides. H9c2(2-1) myotubes contain a specific β-glucuronidase activity of 376 ± 57 nmol/h per mg protein (mean ± S.D., $n = 3$).

4. Discussion

We here show that, compared to fibroblasts from other sources, cultured cardiac fibroblasts from neonatal and adult rat heart have the highest capacity to glucuronidate T4 and T3. This property is primarily attributable to the glucuronidating capacity of these cells, and not to the capacity for the uptake of native T4 and T3. Although the spectrum of anatomical locations from where we could obtain our primary cultures of fibroblasts is rather limited, the glucuronidating capacity of cardiac fibroblasts reflects the topographic differentiation of the transcriptome of fibroblasts from different anatomical sites or donor sources

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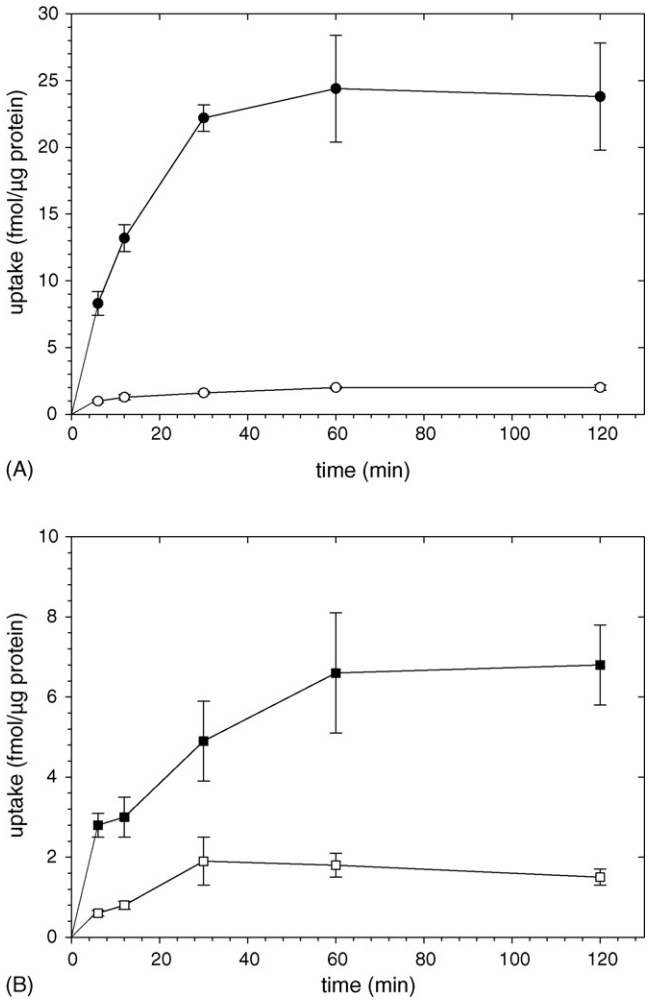

Fig. 5. (A) Time course of the uptake of 10 nM T4 (◯) and 10 nM T4 glucuronide (T4G) (●) in differentiated H9c2(2-1) myoblasts in 0.1% BSA. (B) Time course of the uptake of 10 nM T3 (□) and 10 nM T3 glucuronide (T3G) (■) in differentiated H9c2(2-1) myoblasts in 0.5% BSA. Mean ± S.E.M. ($n = 6$) are shown. Note the difference in y-axis scales between panels (A) and (B).

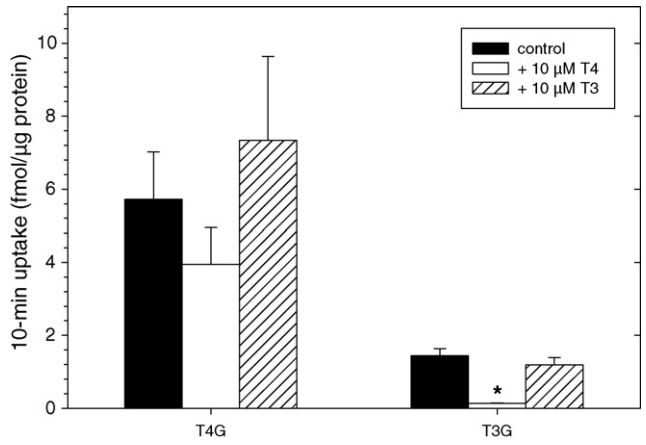

Fig. 6. Effects of 10 µM T4 or T3 on the 10-min uptake of 10 nM T4 glucuronide (T4G) or T3 glucuronide (T3G) in differentiated H9c2(2-1) myoblasts (mean ± S.E.M. of 2–8 triplicate observations).

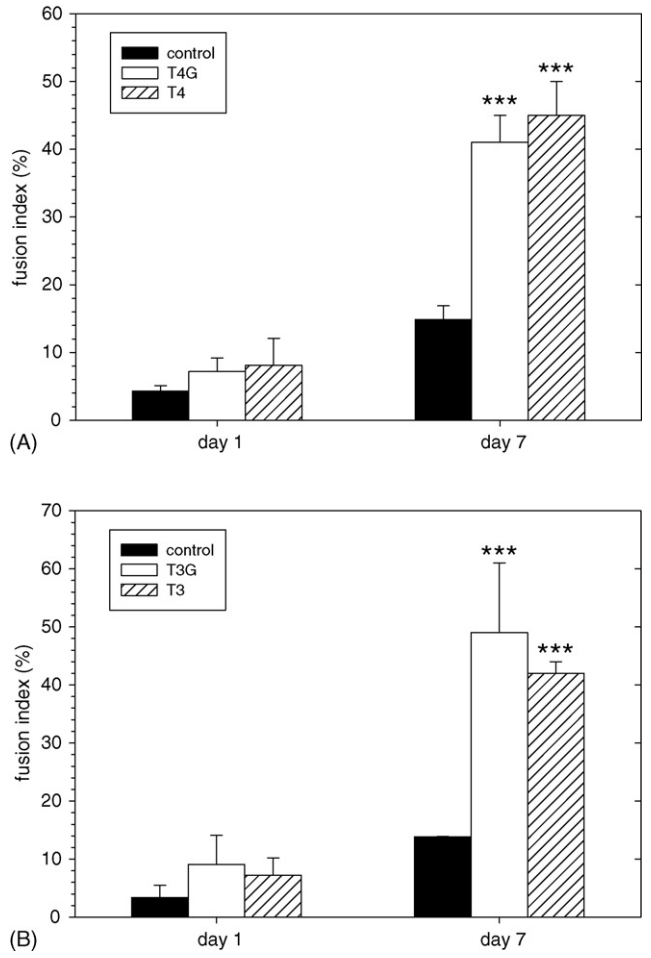

Fig. 7. Effect of 10 nM T4 and T4 glucuronide (T4G) (top panel), and 10 nM T3 and T3 glucuronide (T3G) (bottom panel) on the differentiation of H9c2(2-1) myoblasts to multinucleated myotubes (mean ± S.D., triplicate observations of two experiments). Control incubations were without thyroid hormones and contained vehicle only. Asterisks indicate statistically significant differences with respect to control.

(Chang et al., 2002). The cellular uptake of thyroid hormones by neonatal rat cardiofibroblasts occurs via a single-site pathway, as indicated by our kinetic analyses. The maximum uptake rate and the limit for the net uptake of T3 are approximately twice that of T4, and these kinetic differences of the cardiofibroblast's transport system can be regarded as adaptive to the physiological total T3 concentrations that are considerably lower than those of T4. The capacities for the transmembrane transport of, respectively, T4 and T3 in neonatal cardiofibroblasts, as judged from the equilibrium values of the time course progress curves, are five- and nine-fold higher than those measured in differentiated H9c2(2-1) myotubes (this study). The differences between the limits for the net uptake of T4 and T3, and between iodothyronine uptake between cell types reflect the activities of iodothyronine- or cell specific transporters involved in uptake and extrusion pathways. Also, cellular binding properties could be different between iodothyronines and cell types, resulting in different cellular loading capacities and uptake limits. From the first-order rate constants that describe the uptake of T4 and T3 in neonatal cardiofibroblasts, half-time values ($t_{1/2}$) of, respectively, 23

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and 17 min can be calculated ($t_{1/2} = (\ln 2)/k$). We estimate half-time values that are roughly similar, *i.e.* 15–55 min, from the time courses of the cellular uptake of T3 by neonatal cardiomyocytes that were incubated in the presence of approximately 100 pM to 10 μM T3 (van der Putten et al., 2001; Verhoeven et al., 2002). The capacities for the uptake of thyroid hormones in rat cardiofibroblasts and cardiomyocytes are thus well comparable. Docter et al. (1987), reporting on the results of a comparative study between cultured human fibroblasts and rat hepatocytes, reached a similar conclusion. Taken together, these results indicate that cardiofibroblasts are well equipped for the uptake and metabolism of thyroid hormones.

The glucuronidation of iodothyronines in the rat heart is clearly a feature of cardiofibroblasts and not of cardiomyocytes, as we demonstrated earlier that the latter cell type does not have the property to produce thyroid hormone glucuronides *in vitro* (van der Heide et al., 2002). This is corroborated by our observations that the myoblast cell lines H9c2(2-1) and C2C12 secreted only minimal amounts of thyroid hormone glucuronides upon exposure to 100 nM T4 or T3. The capacity for thyroid hormone glucuronidation is already present in the neonatal cardiofibroblast, and remains at a high level during postnatal development.

Differentiated H9c2(2-1) cells displayed a clear preference for the uptake of T4G and T3G over the respective native, unconjugated iodothyronines. This effect cannot be explained by altered substrate availability in the incubation media, as equilibrium dialysis showed that the concentrations of free hormone and free thyroid hormone glucuronide were similar. The results from our equilibrium dialyses differ from those reported by Hays and Hsu (1988) who measured dialyzable percentages of T4G that were increased 3.5- to 5-fold compared to native T4 in cat and human plasma, respectively. No significant differences were observed between the dialyzable percentages of T3G and T3 in cat and human plasma (Hays and Hsu, 1988). Most probably, the discrepancies between the free iodothyronine concentrations in our BSA-supplemented incubation media and human and cat plasma are explained by the presence of the specific thyroid hormone binding proteins transthyretin and thyroxine-binding globulin in the plasma preparations. Still, the glucuronidation of T4 is likely to result in an increased free plasma concentration of T4G *in vivo*, and thus an increased fraction that is putatively available for transporter proteins and metabolizing enzymes.

The cellular uptake of T4G and T3G by H9c2(2-1) myotubes was inhibited by T4, whereas T3 was without effect. This indicates that the preferential uptake of thyroid hormone glucuronides is mediated partly via a common pathway shared with T4, and partly by transporters that are specific for glucuronidated iodothyronines. There is a wide array of transporters that can play a role in transmembrane transport of thyroid hormone (see Friesema et al., 2005 for a review). Organic anion transporters from the Na^+/taurocholate cotransporting polypeptide (NTCP) and organic anion transporting polypeptide (OATP) families (Abe et al., 1998; Friesema et al., 1999; Fujiwara et al., 2001; Hagenbuch and Meier, 2004), and L-type and T-type amino acid transporters (LAT, TAT) (Blondeau et al., 1993; Friesema et al., 2001; Lakshmanan et al., 1990; Zhou et al., 1990, 1992) have all been implicated in transmembrane thyroid hormone transport. Specifically in cardiac tissue, a fatty acid translocase (FAT) is expressed (Pelsers et al., 1999), and expression of rat FAT cRNA in *Xenopus laevis* oocytes increased the uptake of T3 (van der Putten et al., 2003). The monocarboxylate transporter-8 (MCT8), a member of the T-type amino acid transporter family, was identified as a specific thyroid hormone transporter, and was found to be highly expressed in rat heart (Friesema et al., 2003). The presence of a conjugated group on the iodothyronine molecule greatly affects the characteristics of transmembrane thyroid hormone transport. Indeed, sulfation of the phenolic hydroxyl group of T3 results in a T3 sulfate (T3S) which has a greatly reduced affinity for transport by FAT and MCT8 expressed in *Xenopus* oocytes (Friesema et al., 2003; van der Putten et al., 2003). The characteristics of iodothyronine glucuronide transport by FAT and MCT8 transporters, and by other transporters from the organic anion transporters and LAT/TAT families have not yet been investigated, but it can be anticipated that transport affinities and capacities will be affected by the presence of a glucuronosyl moiety in the iodothyronine's molecular structure.

Members from the multidrug resistance protein (MRP) family play a prominent role in the cellular export of glucuronides (Keppler et al., 1999, 2000; König et al., 1999). Based on transport inhibition studies, at least three different transporters with distinct glucuronide substrate affinities were identified in rat liver microsomes (Csala et al., 2004), and the differential expression of MRPs could thus confer a specific glucuronide transport modality to a cell. Rat cardiac tissue differentially expresses *mdr2* but not *mdr1b* mRNA (Brown et al., 1993), and also in mouse and human heart the expression of specific MRPs has been demonstrated (Cox et al., 2002; Flens et al., 1996; Meissner et al., 2004; Tsuruoka et al., 2002). We suggest that cardiofibroblasts express a specific MRP that confers the specific export capacity for thyroid hormone glucuronides.

It has been shown earlier that the uptake of radiolabeled T4 and T3 in primary cultures of rat cardiomyocytes (Everts et al., 1996) and in H9c2(2-1) myoblasts and myotubes (van der Putten et al., 2002) is not completely inhibited by the addition of a surplus of unlabeled hormone, suggesting that the transport of T4 and T3 is not mediated by a single transport protein. We here show that native, unconjugated T4 partly inhibits the uptake of T4G in H9c2(2-1) cells, probably by competition for the same transporter site. The cellular uptake of T3G was not affected by native T3. This indicates that target cells possess multiple transport pathways, and that the uptake of iodothyronine glucuronides occurs via a different transporter than that of native thyroid hormones. The organic anion transporter family is involved in the uptake of, *e.g.*, bilirubin glucuronide in human embryonic kidney cells (Cui et al., 2001), and estradiol 17-β-D-glucuronide in human hepatocytes (Shitara et al., 2003). The expression of specific organic anion transporters could confer a selective capacity for the uptake of glucuronidated thyroid hormones to H9c2(2-1) cells and cardiomyocytes.

Our results show that thyroid hormones and their glucuronides have equally potent effects on H9c2(2-1) myoblast-to-myotube differentiation. Extrapolating from the observations

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that H9c2(2-1) cells preferentially take up thyroid hormone glucuronides, and that these stimulate the myoblast differentiation, it can be hypothesized that cardiac muscle cells are targets for a paracrine action of cardiofibroblasts mediated by glucuronidated metabolites of thyroid hormones. We measured considerable β-glucuronidase activities in adult-rat whole-heart homogenates (van der Heide et al., 2004) and H9c2(2-1) myotubes (this study), and this indicates that cardiomyocytes can regenerate thyroid hormones by deconjugation of the glucuronidated conjugates. Moreover, adult rat cardiomyocytes and H9c2(2-1) cells contain an iodothyronine deiodinase type 1 activity (van der Heide et al., 2004; van der Putten et al., 2002; Yonemoto et al., 1999), and cardiomyocytes from rat and pig a deiodinase type 2 activity as well (van der Heide et al., 2004; Wassen et al., 2004). Most probably, the stimulatory effect on myoblast differentiation upon exposure to thyroid hormone glucuronides are ultimately caused by T3.

In conclusion, cardiofibroblasts can modulate cardiac thyroid hormone bioavailability by the production and secretion of thyroid hormone metabolites that are preferentially taken up by cardiomyocytes. This would partly uncouple thyroid hormone bioavailability in cardiac tissue from the thyroid hormone concentrations in the systemic circulation, and could well represent a mechanism for fine-tuning local, cardiac thyroid hormone action.

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