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3,5,3′-Triiodothyronine deprivation affects phenotype and intracellular $[Ca^{2+}]_i$ of human cardiomyocytes in culture

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Abstract

Objective: A decrease in plasma T3 concentration is a frequent finding in patients with heart failure. However, the role of this 'low T3 syndrome' on disease evolution has never been clarified. As phenotypic and functional cardiomyocyte impairments are alterations that correlate with the failing myocardium, we studied the long-term effects of T3 deprivation on human cardiomyocyte structure and calcium handling. **Methods:** Atrial cardiomyocytes and myocardial tissue were cultured with or without 3 nM T3. Microscopical examination of structural features was followed by analysis of α-sarcomeric actinin and sarcoplasmic reticulum calcium ATP-ase (SERCA-2) content. Calcium handling was studied by $[Ca^{2+}]_i$ imaging. **Results:** When stimulated with cyclopiazonic acid, a SERCA-2 inhibitor, T3-deprived cardiomyocytes showed significantly faster ($P=0.03$) and more transient ($P=0.04$) increases in $[Ca^{2+}]_i$ than T3-supplemented cells. Moreover, in the T3-free cultures a significantly lower number of cells ($P=0.003$) responded to caffeine, a typical activator of sarcoplasmic reticulum Ca^{2+}-release channel. T3-deprived cardiomyocytes also presented altered morphology with larger dimensions than T3-supplemented cells ($P<0.0001$). Additionally, in T3-deprived samples α-sarcomeric actinin and SERCA-2 protein levels were reduced to 65.6±3% ($P<0.0001$) and 74.1±4% ($P=0.005$), respectively, when compared with the T3-supplemented group. **Conclusions:** Our data show that human cardiomyocyte calcium handling and phenotype are strongly influenced by T3 suggesting important implications of the 'low T3 syndrome' on the progression of heart failure.

Keywords: Cell culture/isolation; Myocytes; Hormones; Calcium (cellular); SR-function; Heart failure

1. Introduction

Several studies performed in animal models indicate that 3,5,3′-triiodothyronine (T3), the biologically active thyroid hormone, plays a crucial role in cardiac physiology [1,2]. Thyroid hormone responsive elements have been found in genes coding for myocardial contractile proteins and for regulators of intracellular calcium homeostasis [3,4]. Triiodothyronine has been proven to downregulate the fetal, slow-contracting β-myosin heavy chain (β-MHC) in favor of the adult fast-contracting α-myosin heavy chain (α-MHC) and to upregulate sarcoplasmic reticulum calcium ATP-ase (SERCA-2) [5–7]. Thyroid hormone is also involved in cell growth and development, as physiological levels of T3 are necessary for differentiation of many cell types including embryo cardiomyocytes [8–11]. In vivo, a switch from fetal to adult protein isoforms can be seen soon after birth and coincides with a significant surge in thyroid hormone concentrations [12].

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In humans, the effects of T3 on heart function are less clear, and have been mainly drawn from clinical studies [13,14]. In clinically euthyroid patients with advanced congestive heart failure a reduction of circulating T3 levels, secondary to impaired conversion of thyroxine (T4) into T3, is frequently associated with poor ventricular function and is a strong predictor of poor short-term outcome [15,16]. In view of the effects of T3, a depressed T3 condition could favor the cardiomyocyte reorganization and electro-mechanical dysfunction that is observed in progression of heart failure [17–23]. Except for a single case previously reported [24], the pathophysiological relationships between reduction of T3 and evolution of heart failure in humans have never been investigated.

In this study we have evaluated the effects of T3 deprivation on phenotype and calcium handling of human cardiomyocytes and myocardial tissue in culture. For this purpose we compared a state of T3 deprivation to a condition of T3 maintained at physiological concentrations. We found that T3-deprived preparations presented morpho-structural alterations and reduced calcium handling largely prevented by T3 supplementation.

2. Methods

2.1. Source of cells

Right atrial myocardial appendages were obtained from 20 children during pediatric corrective cardiac surgery (eight males, 12 females, age 25±14 months, mean±S.D., range 6–48 months). Procedures were approved by our Institutional Ethics Review Committee and the investigation conformed to the principles outlined in the Declaration of Helsinki. All the patients were clinically and chemically euthyroid (FT4 13.0±1.7 pg/ml, FT3 5.2±1.6 pg/ml and TSH 2.8±0.7 μUI/ml (mean±S.D.)). Patients had no clinical or hemodynamic signs of congestive heart failure but exhibited the following congenital heart diseases: tetralogy of Fallot (n=6), atrial septal defect (n=5), subaortic valvular stenosis (n=3), interventricular defect (n=4), ventricular septal defect (n=2).

2.2. Cell culture technique

Twenty to 50 mg of tissue (wet weight) were taken under sterile conditions and processed within 1 h. After washing in PBS and removing connective tissue, the myocardium was minced into fragments of about 2 mm^3 and plated in Iscove's Modified Dulbecco's Medium (IMDM, Sigma, St. Louis, MO) containing 10% fetal bovine serum (FBS, Sigma), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma). Tissue fragments were maintained at 37°C in 5% CO_2–95% air atmosphere. Fresh medium was carefully added after 3 days from plating, and renewed every 3 days thereafter. Cardiomyocytes were obtained for migration from the plated fragments.

2.3. T3 study protocol

To study the effects of T3, a randomly chosen portion of the minced myocardium, was plated and maintained in IMDM containing 10% FBS deprived of thyroid hormones (T3-free group); the remaining fragments were plated and maintained in the same thyroid hormones-deprived medium supplemented with 3 nM T3 (Sigma) (T3-supplemented group).

2.4. Depletion of T3 and T4 from FBS

FBS was absorbed overnight at 4°C on activated charcoal (100 μg/ml serum). Total and free T3 and T4 concentrations were measured by a completely automated AIA 600 system (Tosho Corporation, Tokyo, Japan) and IMx apparatus (Abbott Laboratories, Diagnostic Division, Abbott Park, IL, USA). Following charcoal absorption the thyroid hormones in FBS were undetectable.

2.5. Cardiac-specific proteins

After 10 days of culture, cardiomyocytes were lysed with hypotonic buffer (PBS:H_2O 1:6) and five cycles of freeze and thawing. Total protein content was determined by the method of Peterson [25]. The cardiac markers myoglobin and troponin I were assessed by a completely automated immunoenzymatic system (Sanofi Diagnostic Pasteur, Marnes-la-Coquette, France).

2.6. Histological and immunohistochemical protocols

Myocardial fragments were fixed with 10% formalin and embedded in paraffin; 5-μm serial sections were stained with hematoxylin and eosin for histological examination or processed for immunohistochemistry.

In the immunohistochemical study the sections were deparaffinized and rehydrated. To unmask the antigenic binding sites the slides were heated in a microwave oven in 10 mM citrate buffer (pH 6) at 700 W for a total of 15 min. Endogenous peroxidase activity was inhibited by a 5-min wash in 3% hydrogen peroxide. Unspecific binding was blocked with 10% normal goat serum (Sigma). The sections were then incubated overnight with a primary monoclonal antibody against α-sarcomeric-actinin (1:400, clone EA 53, Sigma). Successively, biotinylated secondary antibody was added at a concentration of 1:200. The slides were then incubated with peroxidase-conjugated avidin (1:300, Sigma). Cell labeling was revealed by using diaminobenzidine as a chromogen (fast DAB tablet kit; Sigma) according to the manufacturer's instructions. Images were acquired using a Leica microscope with a CCD camera (JVC, Victor Company of Japan, Japan). Pictures

were converted into grey scale, then optical density (OD) measurements were made with Scion image software (Scion Corporation, USA).

2.7. Immunocytochemistry

Myocardial biopsies were cultured in chamber-slides for 10 days, then myocardial tissue was discarded and the cardiomyocyte outgrowth was fixed in methanol at −20°C. After washing in PBS, the cells were permeabilized with 0.2% Triton-X100 in PBS for 4 min at 4°C. Endogenous peroxidases were inhibited with 0.3% hydrogen peroxide and 0.1% sodium azide. Unspecific binding was blocked with 5% BSA (Sigma). Cells were then incubated for 1 h with primary antibody against α-sarcomeric-actinin 1:400 or against human SERCA-2 (clone SA-185, Biomol, Plymouth, PA) 1:500. After washing, the cells were exposed to biotinylated anti-mouse secondary antibody (1:300, Vector, Burlingame, Canada) and incubated with avidin–peroxidase (1:500). Cell staining was revealed with diaminobenzidine. Images were acquired and analyzed as described above.

2.8. Western blot analysis

Cardiac muscle tissue and cardiomyocytes were solubilized in buffer containing 20 mM Tris–HCl (pH 8.0), 1 mM EDTA, 1 mM DTT and protease inhibitor cocktail (Sigma) using a Dounce homogenizer. The tissue homogenates were centrifuged at 500×*g* for 10 min in order to precipitate the tissue debris. Total protein content in homogenates was determined according to the method of Peterson [25].

Equal amounts of atrial proteins were resolved by SDS–PAGE on 8.0% gel and blotted electrophoretically onto a 0.2-μm nitrocellulose membrane. The membrane was blocked overnight at 4°C with 5% BSA in PBST (0.1% v/v Tween-20 in PBS) and then probed for 1 h with primary monoclonal antibodies against α-sarcomeric-actinin (1:3000), and SERCA-2 (1:2500). After washing in PBST, biotinylated secondary antibody was added at a dilution of 1:3000. The membrane was then washed and incubated with peroxidase conjugated avidin (1:500). Proteins were visualized with a chemiluminescence assay (ECL kit, Biorad, USA). Densitometric analyses were performed using Scion image software.

2.9. $[Ca^{2+}]_i$ imaging

Cardiomyocyte outgrowths were obtained on glass coverslips coated with Engelbreth Holm-Swarm mouse sarcoma extracellular matrix (ECM gel, Sigma, 1:40). After 10 days of culture, coverslips were transferred to a serum-free recording solution composed as follows (in mM): NaCl 130, KCl 3.1, K_2HPO_4 1.0, $NaHCO_3$ 4.0, dextrose 5.0, $MgCl_2$ 1.0, $CaCl_2$ 2.0, HEPES/NaOH 10, ascorbic acid 1.0, myo-inositol 0.5, pyruvic acid 2, (±)-sulfinpyrazone 0.02, pH 7.2–7.4. After 1-h stabilization, cells were loaded into a 4 μM solution of either fluo-3AM or fura-2AM (Molecular Probes, USA) for 40 min at room temperature. Coverslips were placed in a recording chamber and perfused at about 2 ml per min. Fluo-3 imaging was performed on a Leica TCS-NT confocal microscope. Images were transferred to a custom-made software in order to measure fluorescence changes within each cell profile. Fura-2 imaging was performed on a Zeiss Axioskop microscope, using an integrated CCD camera (PCO Sensi Cam, Germany). Images were acquired at excitation wavelengths of 340 and 380 nm and were stored every 2–4 s. Imaging Workbench software (Axon, USA) was used to calculate the ratio of fluorescence at the two excitation wavelengths for each pixel within a cell boundary. Ratio was then averaged in areas corresponding to the soma of cells in the field. The specific SERCA-2 inhibitor cyclopiazonic acid (50 μM) or the calcium mobilizer caffeine (10 mM) were dissolved in recording saline and were used to stimulate $[Ca^{2+}]_i$ increases through a fast application system.

2.10. Statistical analysis

Data are expressed as mean±S.E.M. Unless otherwise stated, differences between groups were compared by means of a two-tailed unpaired Student's *t*-test. Differences were considered significant at P less than 0.05.

3. Results

3.1. Validation of culture technique

Starting from 3 to 4 days of culture, cardiomyocytes began to migrate away from the myocardial fragments. At 7-10 days, the tissue fragments were surrounded by an outgrowth of cells with homogeneous morphology (Fig. 1, panel A).

Almost all the cells (more than 90%) were characterized as cardiomyocytes by immunocytochemical analysis with monoclonal antibodies against α-sarcomeric-actinin and sarcoplasmic reticulum calcium ATPase (SERCA-2) (Fig. 1, panels B and C).

Cell type identification was confirmed by the presence of the cardiomyocyte specific markers myoglobin (4.9±1.3 ng/mg protein, n=3) and troponin I (16.2±1.4 ng/mg protein, n=3) in cell homogenates; in order to exclude possible contamination from FBS, the same proteins were assayed in fresh medium and found to be, respectively, 0.7±0.2 and 3.1±0.3 ng/mg protein (n=3).

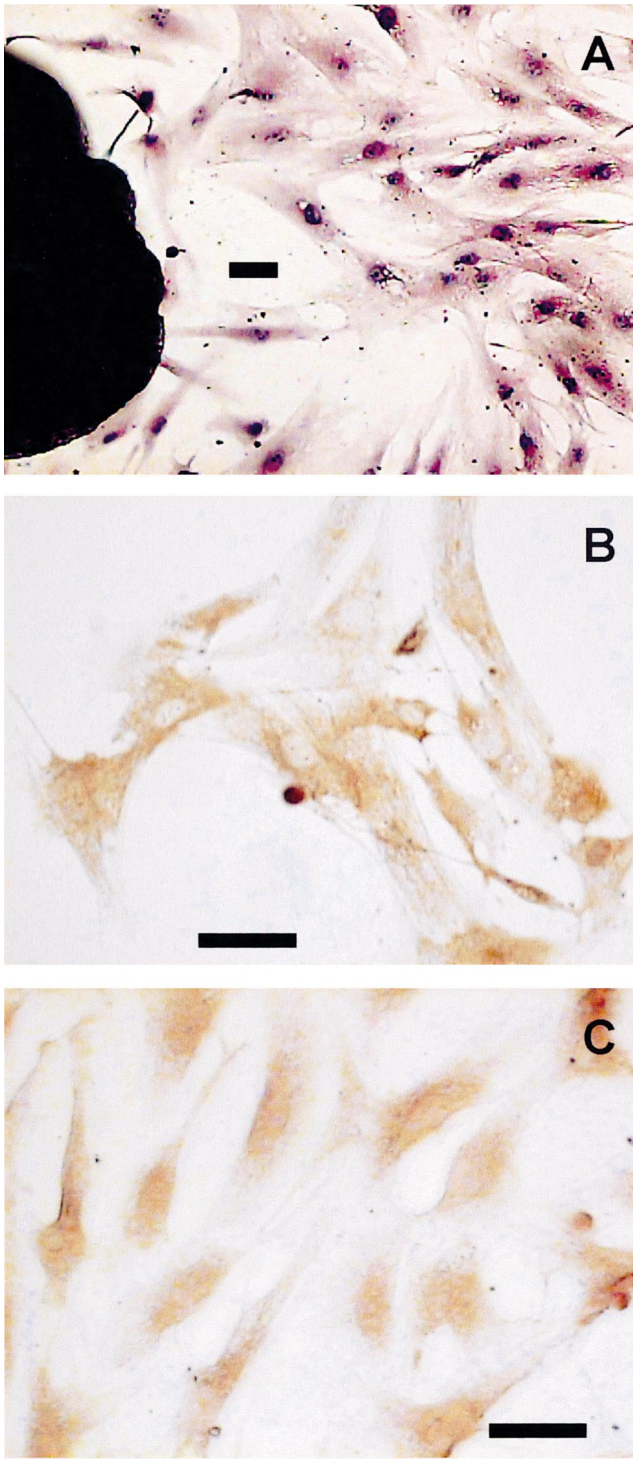

Fig. 1. Cell culture technique. Atrial myocardial fragments were plated by-passing the enzymatic isolation step. At 10 days after plating, cardiomyocyte outgrowth was observed around the tissue (A), staining was performed with hematoxylin-eosin. Outgrowing cardiomyocytes were immunocharacterized with monoclonal antibody against α-sarcomeric-actinin (B) and SERCA-2 (C); staining was performed with peroxidase using diaminobenzidine as a chromogen (scale bar=50 μm).

3.2. Effects of T3 supplementation

3.2.1. Analysis of cultured myocardium

Histological characterization allowed comparison of total protein staining and general tissue preservation of myocardial fragments cultured in the presence or absence of 3 nM T3. After only 4 days of culture untreated fragments presented a weaker staining when compared with those supplemented with T3 (Fig. 2, panels A and B) with an OD value of 31±2.7 vs. 69±2.9 (n=20, $P<$ 0.001). After 10 days of T3 deprivation OD values were further decreased and degenerative alterations, including cardiomyocyte atrophy, cell death and fibrosis had become prominent (Table 1 and Fig. 2, panels C and D). Therefore, the absence of T3 induces a progressive loss of proteins and decreases tissue viability.

The histological alterations were reflected in a reduction in myocardial-specific structural proteins. When immunostained for α-sarcomeric-actinin, T3-deprived myocardium exhibited a weaker immunoreactivity than T3-supplemented tissue (Table 2).

3.2.2. Analysis of outgrowing cardiomyocytes

The outgrowing cardiomyocytes were also profoundly affected by the culture conditions. Cells maintained for 10 days in T3-free medium, exhibited a more irregular shape with a larger surface area ($P<0.0001$) and transversal diameter ($P=0.005$) than the T3-supplemented cells (Fig. 2, panels E and F; Table 3). Moreover, untreated cardiomyocytes showed a weaker staining for α-sarcomeric-actinin (see Fig. 2, panels E and F and Table 2), indicating a smaller amount of cardiomyocyte-specific proteins.

3.2.3. Expression of cardiomyocyte-specific structural protein

The relative α-sarcomeric-actinin level was quantitated in 20-day-old tissue and cell preparations through Western blotting (Fig. 3, upper picture of panel A). The average α-sarcomeric-actinin signal in T3-free samples was 65.6±3% of the signal measured in the T3-supplemented samples (n=6, $P<0.0001$; Fig. 3, lower picture of panel A); mean values measured in cells and tissue homogenates are reported in Table 4.

3.2.4. Calcium handling

The effects of T3 on calcium handling were evaluated through Western blot analysis of SERCA-2 content and $[Ca^{2+}]_i$ imaging.

SERCA-2 detection was performed on tissue homogenates and cell samples after 20 days in culture (Fig. 3, upper picture of panel B). The average SERCA-2 specific signal in T3-free homogenates was 74.1±4% of the signal measured in T3-supplemented samples (n=6, P=0.005; Fig. 3, lower picture of panel B). Mean relative values detected in cell and tissue homogenates are reported in Table 4.

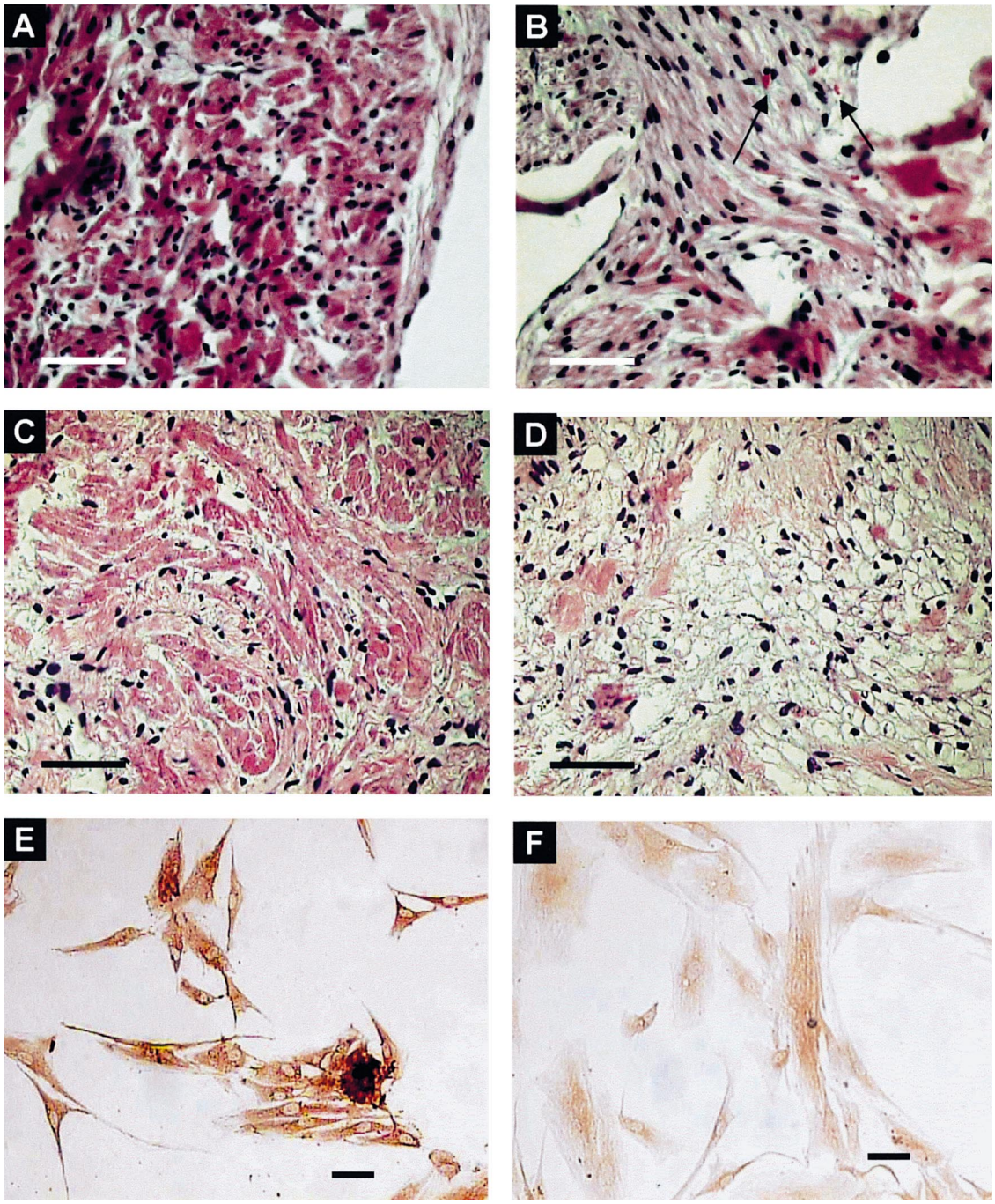

Fig. 2. Structural and phenotypic effects of T3 supplementation. On histological examination, T3-supplemented atrial myocardial fragments presented better preserved cardiomyocytes both at 4 (A) and 10 days of culture (C). Weaker staining and progressive atrophy was observed in the corresponding untreated samples (B and D, respectively); arrows indicate apoptotic bodies. Staining was performed with hematoxylin-eosin. After 10 days in culture, T3-supplemented cells (E) presented smaller dimension and stronger staining for α-sarcomeric-actinin with respect to the untreated cells (F). Immunocytochemical staining was performed with peroxidase using diaminobenzidine as a chromogen (scale bar=50 μm).

The different levels of expression of SERCA-2 in T3-free and T3-supplemented cells is likely to cause a difference in Ca^{2+} homeostasis. In particular, a high concentration of SERCA on the store membranes should result in a larger Ca^{2+} load inside the intracellular compartments. Since the kinetics of a transient $[Ca^{2+}]_i$ increase elicited by an exit of Ca^{2+} from the stores are determined by the equilibrium between Ca^{2+} leak and removal, a larger Ca^{2+} load would sustain the leak for longer time periods and result in a more long-lasting $[Ca^{2+}]_i$ duration. To investigate this hypothesis, cells were stimulated with a transient pulse of 50-μM cyclopiazonic acid, a specific SERCA-2 inhibitor. Under normal conditions the calcium leak from the intracellular stores is in

Table 1
Quantification of the histological results obtained from hematoxylin-eosin staining of human atrial myocardium

Days of culture	OD values		n^a	P
	T3+	T3−		
4	69±2.9	31±2.7	20	<0.001
10	67±3.4	28±1.5	20	<0.001

To compare total protein staining of T3+ and T3− samples, myocardial sections were colored with hematoxylin-eosin method. Images were acquired with a CCD camera and optical density (OD) was measured as described in the Methods section.
[a] Number of measures from three different experiments.

Table 2
Quantification of the immunohistochemical results obtained from α-sarcomeric-actinin staining of 10-day-old human atrial cardiomyocytes and myocardial tissue

Samples	n^a	OD values		P
		T3+	T3−	
Cell	20	50±12	20±5	<0.0001
Tissue	20	60±14	35±8	<0.0001

To compare the staining for a cardiomyocyte-specific protein in T3+ and T3− samples, cells and myocardial sections were immunostained for α-sarcomeric-actinin. Images were acquired with a CCD camera and optical density (OD) was measured as described in the Methods section.
[a] Number of measures from three different experiments.

equilibrium with the SERCA-2 mediated calcium inflow. Therefore the application of cyclopiazonic acid unmasks the leak and causes an increase in $[Ca^{2+}]_i$ in the cytosol.

In different experiments (n=4) we analyzed nine T3-supplemented and eight T3-free coverslips for a total of 81 T3-free cardiomyocytes and 94 T3-supplemented cells. In all the T3-free cells, the treatment with cyclopiazonic acid produced a faster and more transient increase in Ca^{2+} (Fig. 4 panel A and Fig. 5). The average time to peak was 112±16 s for T3-supplemented cells and only 64±5 s for T3-free cells (P=0.03) (Fig. 4, panel B). Moreover, in T3-deprived cardiomyocytes the response amplitude at 3 min after the response onset was 51±11% of the peak response, while in T3-supplemented cells the response amplitude after 3 min was 84±7% of the peak response (P=0.04) (Fig. 4, panel C). These data indicate that

Table 3
Surface area and transversal diameter in 10-day-old human atrial cardiomyocytes in culture

Parameters	T3−	T3+	P
Cell number	90[a]	90[a]	
Number of specimens	5	5	
Surface area (μm^2)	3300±137	1960±158	<0.0001
Transversal diameter (μm)	34±1.3	26±1.4	0.0005

[a] Cells from eight different fields were analyzed with a mean of 11 cells/field.

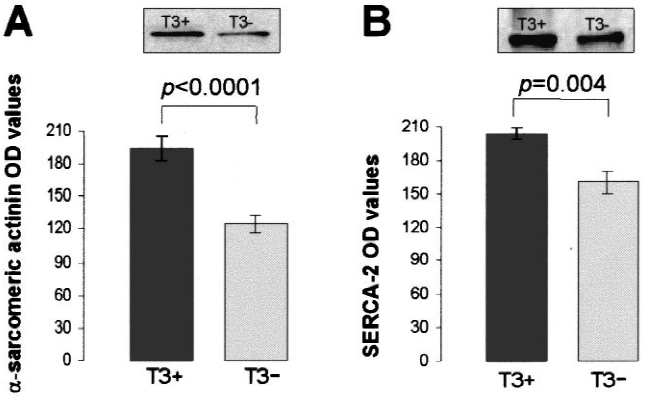

Fig. 3. Western blot analysis of α-sarcomeric-actinin and SERCA-2 in homogenates of cardiomyocytes and myocardial tissue. After 20 days of culture in T3-supplemented (T3+) medium, cell and tissue samples showed a stronger signal for both α-sarcomeric-actinin and SERCA-2 when compared to untreated samples (T3−) (upper picture of A and B). As shown in the lower picture of A and B, the average densitometric signals were significantly lower in the untreated samples than in the treated group (n=6). P was determined on absolute OD values by two-tailed unpaired Student's t-test; data are expressed as mean±S.E.M.

cyclopiazonic acid induced a more sustained $[Ca^{2+}]_i$ increase in T3-supplemented cells.

The altered Ca^{2+} homeostasis of T3-free cardiomyocytes was also observed when ryanodine sensitive stores were stimulated with caffeine (10 mM). Indeed, T3-free cultures showed a significantly lower number of cells responsive to caffeine with respect to T3-supplemented cultures (median in T3-free 2.3%, 0–6.7% 25th–75th percentile, 279 cells, nine coverslips; median in T3-supplemented 10.8%, 4.1–20.5% 25th–75th percentile, 182 cells, eight coverslips. Chi-square test with Yates correction, 1 degree of freedom, P=0.003).

4. Discussion

The main finding of this study is evidence for a crucial role of T3 in maintaining morpho-structure and calcium

Table 4
Analysis of α-sarcomeric actinin and SERCA-2 content in homogenates from 20-day-old human atrial cardiomyocytes and myocardial tissue

Samples	α-sarcomeric-actinin			SERCA-2		
	n^a	T3+[b]	T3−[c]	n[a]	T3+[b]	T3−[c]
Cell	3	100	69.4±6	3	100	64.3±5
Tissue	3	100	61.8±3	3	100	80.7±1

OD values (%) of α-sarcomeric actinin and SERCA-2 protein bands measured by scanning densitometry.
[a] Number of sample homogenates.
[b] The optical signal measured in T3-supplemented samples was considered 100%.
[c] In the T3-free group OD values are expressed as a percentage of the corresponding T3-supplemented values.

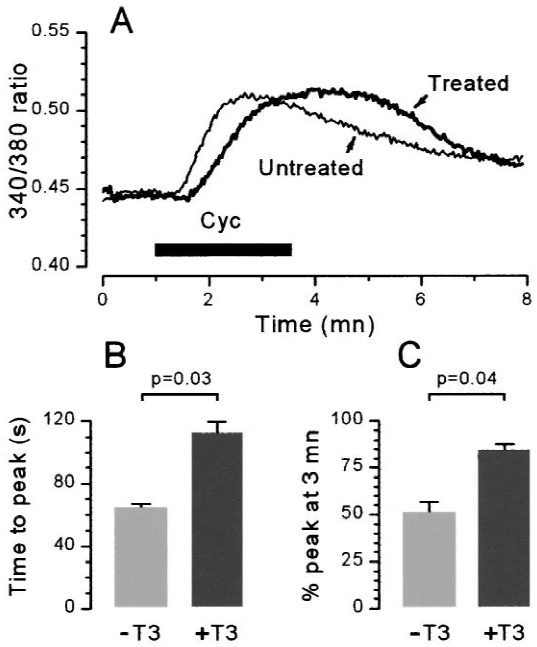

Fig. 4. (A) Quantification of a typical $[Ca^{2+}]_i$ imaging experiment. The black bar indicates the time and duration of treatment with cyclopiazonic acid. Sister cultures were loaded with fura2 and imaged under identical conditions. (B and C) Quantification of all the experiments. When compared to T3-supplemented cardiomyocytes, T3-free cardiomyocytes presented faster time to peak (B) and lower amplitude of signal measured at 3 min after the response onset expressed as a percentage of the response at peak (C). *P* was determined using the two-tailed unpaired Student's *t*-test; data are expressed as mean±S.E.M.

handling of human atrial cardiomyocytes and myocardial tissue in vitro.

The culture system we adopted allowed us to gain insight in the pathogenesis of human heart failure. With all the problems in transferring in vitro data to intact in vivo myocardium, atrial culture of human myocytes has been found to represent an attractive model with which to study pathophysiological properties of the human cardiomyocyte [26].

In our model, the absence of T3 leads to disorganization of cultured myocardium and phenotypical remodeling of isolated cardiomyocytes, which resembles gross and cellular structural impairment observed during heart failure progression [17,18]. At molecular level, T3-untreated samples show a significant reduction in the α-sarcomeric-actinin content indicating that the T3-modulatory effect on cell shape and structure is mediated, at least in part, through the regulation of this molecule. Alpha-sarcomeric-actinin is essential in order to maintain sarcomeric organization and, according to recent reports, a reduction of its levels is responsible, among other things, for myocardial remodeling in human heart failure [27,28]. Thus one can speculate that the 'low T3 syndrome' observed in heart failure evolution might influence the overall myocardial architecture by affecting the level of α-sarcomeric-actinin.

In chronic heart failure morpho-structural alterations are strongly related to reduced myocardial performance [27]. A number of papers have suggested the relevance of altered calcium handling in the pathophysiology of myocardial failure [19–23]. SERCA-2 has been documented to have an important role in calcium cycling as a major determinant of Ca^{2+} uptake into the sarcoplasmic reticulum. A reduction in expression or activity of this pump contributes to abnormal intracellular Ca^{2+} handling in both animal and human models of heart failure [21–23]. Therefore we investigated the long-term effects of T3 deprivation on SERCA-2 content and Ca^{2+} homeostasis. Remarkably, we found a significant decrease in SERCA-2 protein in all our T3-free samples, in agreement with a recent animal study in which a low-T3 syndrome was demonstrated to reduce SERCA-2 expression in cardiac tissue [29]. The low concentration of SERCA on the store membranes of the T3-untreated cardiomyocytes should result in a reduced Ca^{2+} load inside the intracellular compartments. Consistent with this hypothesis, in T3-free cardiomyocytes the pharmacological block of SERCA-2 elicited a more transient intracellular $[Ca^{2+}]_i$ increase as compared to T3-supplemented cells. These data indicate a reduction in Ca^{2+} storage capability in T3-free cardiomyocytes suggesting an important role for T3 in the regulation of cardiomyocyte Ca^{2+} homeostasis. The altered calcium handling of the T3-free preparation was also confirmed by the reduced Ca^{2+} responsiveness of untreated cells to caffeine, a receptor-dependent agonist that triggers Ca^{2+} release from stores.

From a pathophysiological point of view, impaired Ca^{2+} storage and release capabilities determine a poor calcium cycling, which in turn has been found to be associated with reduced frequency potentiation of contractile force, an important marker of human heart failure [30].

To our knowledge this is the first report on the effects of T3 on calcium handling in human cardiomyocytes in vitro. Thyroid hormone action has previously been investigated in animal models [31–35]; however, it is important to underline that the electrophysiological properties of animal myocardium are considerably different from human myocardium [36], thus, results from animals cannot be directly extrapolated to humans. In addition, in most animal studies pharmacological concentrations of T3 were used [6,31,34] and, in some instances, T3 was added a few days after the cardiomyocytes were plated [6,33]. Owing to the rapid onset of dedifferentiation in vitro, the time when T3 supplementation occurs represents a critical step in the experimental design. For this reason, in the present study, T3 was added at the beginning of culture, before the dedifferentiation took place.

Finally important therapeutic implications derive from our findings. The overall data described above confirm the hypothesis that the 'low T3 syndrome' observed in heart failure can be considered a true hypothyroid-like cardiac condition that worsens per se cardiomyocyte remodeling and dysfunction. On the basis of our results, the treatment

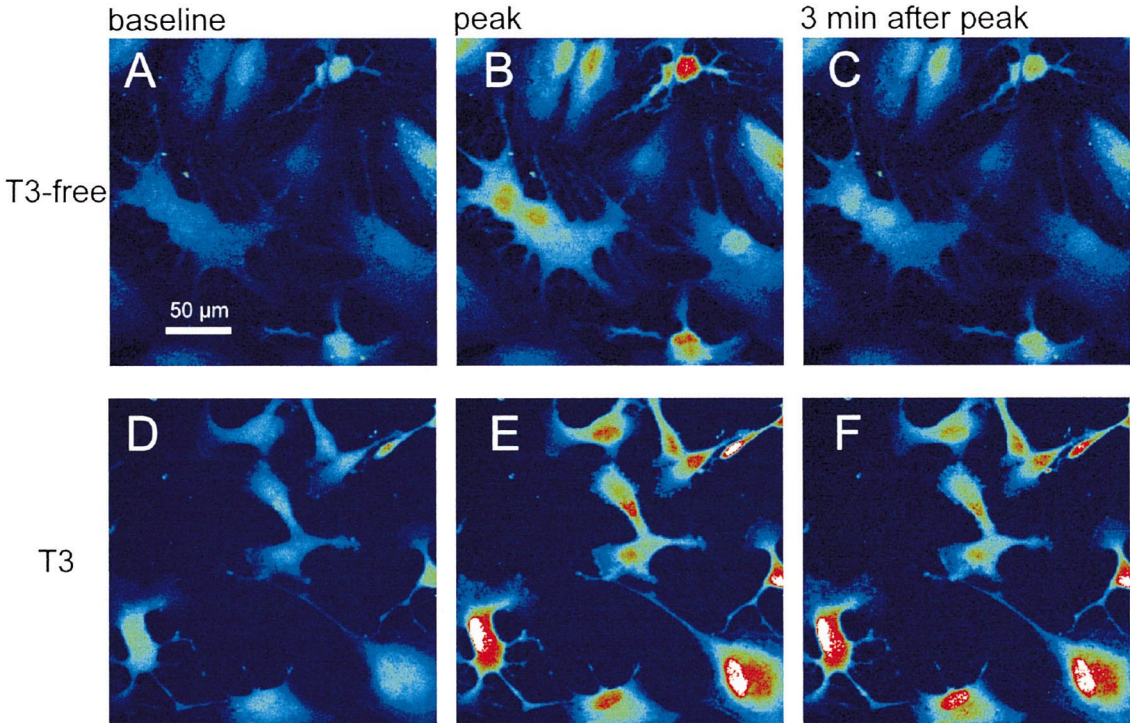

Fig. 5. Sister cultures (T3-free: upper panels; T3-supplemented: lower panels) loaded with fluo-3 and imaged on the confocal microscope. Coverslips were loaded together and imaged sequentially under identical conditions. Baseline fluorescence prior to the application of the SERCA-2 inhibitor cyclopiazonic acid were similar in T3-free and T3-supplemented cultures (A and D, respectively). At the peak of the response (B and E) and 3 min after the response onset (C and F) fluorescence of T3-free cells was lower when compared to the corresponding treated cells. Refer to Fig. 4 for quantification of the effects of T3 supplement on $[Ca^{2+}]_i$ response time course (scale bar=50 μm).

with substitutive doses of T3 might represent a promising therapeutic strategy to improve myocardial functions during the evolution of heart failure. Preliminary reports [16] on the benefits of T3 treatment in patients with cardiac dysfunction encourage this approach.

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