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Depressed contractile reserve and impaired calcium handling of cardiac myocytes from chronically unloaded hearts are ameliorated with the administration of physiological treatment dose of T3 in rats

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Abstract

Aim: Chronic cardiac unloading causes a time-dependent upregulation of phospholamban (PLB) and depression of myocyte contractility in normal rat hearts. As thyroid hormone is known to decrease PLB expression, we examined whether thyroid hormone restores the depressed contractile performance of myocytes from chronically unloaded hearts.

Methods: Cardiac unloading was induced by heterotopic heart transplantation in isogenic rats for 5 weeks. Animals were treated with either vehicle or physiological treatment dose of 3,5,3′-triiodo-L-thyronine (T3) that does not cause hyperthyroidism for the last 3 weeks ($n = 20$ each).

Results: In vehicle-treated animals, myocyte relaxation and $[Ca^{2+}]_i$ decay were slower in unloaded hearts than in recipient hearts. Myocyte shortening in response to high $[Ca^{2+}]_o$ was also depressed with impaired augmentation of peak-systolic $[Ca^{2+}]_i$ in unloaded hearts compared with recipient hearts. In vehicle-treated rats, protein levels of PLB were increased by 136% and the phosphorylation level of PLB at Ser16 were decreased by 32% in unloaded hearts compared with recipient hearts. By contrast, in the T3-treated animals, the slower relaxation, delayed $[Ca^{2+}]_i$ decay, and depressed contractile reserve in myocytes from unloaded hearts were all returned to normal levels. Furthermore, in the T3-treated animals, there was no difference either in the PLB protein level or in its Ser16-phosphorylation level between unloaded and recipient hearts.

Conclusion: These results suggest that the treatment with physiological treatment dose of thyroid hormone rescues the impaired myocyte relaxation and depressed contractile reserve at least partially through the restoration of PLB protein levels and its phosphorylation state in chronically unloaded hearts.

Keywords calcium handling, cardiac myocytes, phospholamban, thyroid hormone, transplantation.

While the effects of long-term pressure overload on normal hearts have been extensively studied in animals and humans (Hasenfuss 1998, Hunter & Chien 1999, Ito *et al.* 2000, Faber *et al.* 2006), little is known about the response of the normal hearts to chronic mechanical unloading (Welsh *et al.* 2001, Ito *et al.* 2003). We have recently reported that chronic left ventricular (LV) unloading of the normal heart causes a time-dependent

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depression of contractile reserve and calcium handling in rats (Ito *et al.* 2003). Also, studies of human astronauts immediately after long-term spaceflight suggest that chronic unloading induces cardiac atrophy and depression of cardiac performance (Bungo *et al.* 1987). However, no effective treatment has yet been developed to prevent the myocardial dysfunction in chronically unloaded hearts. The administration of thyroid hormone is known to improve cardiac function and calcium handling by regulating the expression of calcium-cycling proteins in both normal and failing hearts (Chang *et al.* 1997, Klein & Ojamaa 2001, Pantos *et al.* 2004). Namely, thyroid hormone up-regulates protein levels of sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2a) and down-regulates those of phospholamban (PLB) by modifying both gene transcription and translation (Dillmann 1990, Kiss *et al.* 1994, Klein & Ojamaa 2001). While mild hypothyroidism has been demonstrated after space flight (Strollo 1999, Grimm *et al.* 2002) and administration of thyroid hormone has been shown to upregulate the gene expressions of α-myosin heavy chain (MHC) and SERCA2a in unloaded rat hearts (Ojamaa *et al.* 1992), the effect of thyroid hormone on myocyte contractility and calcium handling in unloaded hearts remains to be examined. In the present study, we thus tested our hypothesis that thyroid hormone ameliorates depressed contractility in unloaded normal hearts, using the rat model of heterotopic heart transplantation (Ono & Lindsey 1969). From a clinical point of view, we specifically aimed to examine the beneficial effect of physiological treatment dose of thyroid hormone that has no effect on body weight, LV weight, haemodynamics, or the serum level of thyroid-stimulating hormone (TSH) (Ojamaa *et al.* 2000a).

Materials and methods

Animal model of LV unloading

A total of 80 male Lewis rats (7 weeks old, 230–250 g; Japan SLC, Hamamatsu, Japan) were used in the present study. LV unloading was induced by heterotopic heart transplantation to the abdominal aorta of isogenic recipient rat as previously reported (Ono & Lindsey 1969). Briefly, in this rat model, the ascending aorta of the donor is anastomosed end-to-side on the abdominal aorta of the recipient, and the pulmonary artery of the donor is anastomosed end-to-side on the inferior vena cava of the recipient. The donor heart is perfused through the coronary arteries and beats, but the LV is kept unloaded and flaccid. Therefore, both recipient hearts and transplanted unloaded (donor) hearts are perfused with the same blood and subjected to similar circulatory neurohormonal environments until killing. This model has been widely used to study the effect of mechanical unloading on LV mass, structure, and gene expression of the non-failing heart (Klein & Hong 1986, Korecky *et al.* 1986, Ojamaa *et al.* 1992, Rakusan *et al.* 1997, Depre *et al.* 1998, Ito *et al.* 2003). These previous studies showed that the size of the transplanted heart in this model is determined by cardiac workload, namely the decrease in the size of unloaded hearts is mainly due to decreased mechanical stress (Klein *et al.* 1991, Korecky & Masika 1991). Animals were studied at 5 weeks after the transplantation in the present study (Ito *et al.* 2003).

Administration of thyroid hormone

At 2 weeks after the transplantation, animals were randomly assigned to two groups, and either vehicle or 3,5,3′-triiodo-L-thyronine (T3; 1.2 μg day^{-1}; Sigma, St Louis, MO, USA), a physiologically active form of thyroid hormone, was given subcutaneously using an osmotic pump during the last 3 weeks ($n = 20$ each). Danzi *et al.* (2005) reported that this dose of T3 is sufficient to normalize serum T3 level in hypothyroid rats when administered constantly by osmotic pump. In a preliminary study, we confirmed that this concentration of T3 does not affect body weight, LV weight, haemodynamics, or the serum levels of TSH. We initiated the treatment with T3 at 2 weeks after the transplantation because we intended to administer T3 in a stable haemodynamic condition after the transplantation, and our previous study have demonstrated that neither contractility nor calcium handling in myocytes from unloaded hearts have yet been impaired at 2 weeks after the transplantation while both of them are deteriorated at 5 weeks (Ito *et al.* 2003). Blood pressure and heart rate were measured before killing. Blood samples were obtained at 5 weeks after the transplantation to measure serum levels of TSH, T3, and L-thyroxine (T4) by chemiluminescent immunoassay (Chemilumi-ACS-TSH-III, Chemilumi-ACS-FT3-II and Chemilumi-ACS-FT4, respectively; Bayer Medical Ltd, Tokyo, Japan). All procedures were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of Tohoku University.

Simultaneous measurement of cell motion and $[Ca^{2+}]_i$

Left ventricular myocytes were dissociated from both recipient hearts and transplanted unloaded hearts from animals treated with either vehicle or T3 ($n = 7$–8 per group). Cell motion and $[Ca^{2+}]_i$-sensitive fluorescence with fluo-3 were measured simultaneously. The procedures of cell dissociation, instrumentation and calibration of $[Ca^{2+}]_i$ are described elsewhere (Ito *et al.* 1999, Tajima *et al.* 1999). At baseline, myocytes were paced

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with field stimulation at 0.5 Hz with 1.2 mM $[Ca^{2+}]_o$ at 36 °C. To study contractile reserve at high work states, the $[Ca^{2+}]_o$ was increased to 2.5 and 4.0 mM at a constant pacing frequency of 0.5 Hz. Measurements were made after 3 min at each level of elevated $[Ca^{2+}]_o$.

Protein levels in LV tissue

Left ventricular tissues of both recipient hearts and transplanted unloaded hearts from the same recipient animals were frozen and stored at −80 °C until use. Western blot analysis was performed to assess protein levels of SERCA2a, PLB, and phosphorylated PLB (n = 5 each) by a modification of the methods of Nakayama *et al.* (2003). To assess the protein expression level of phosphorylated PLB, anti-phosphorylated PLB (Ser 16) and anti-phosphorylated PLB (Thr 17) antibodies were used by a modification of the methods of Sabbah *et al.* (2003). Sarcoplasmic reticulum-enriched membrane samples (1 μg per lane, $n = 5$ for each group) were separated by sodium dodecyl sulphate polyacrylamide gels (SDS-PAGE; 15% for PLB and phosphorylated PLB, and 10% for SERCA2a), and electroblotted onto polyvinylidine difluoride membranes. Primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) except for anti-PLB antibody (Affinity Bio Reagents, Golden, CO, USA). Electrophoretic analysis of MHC proteins was performed to separate the cardiac MHC isoforms; α- and β-MHC using 3 μg of homogenized LV tissue per lane ($n = 5$ for each group) (Laemmli 1970, Talmadge & Roy 1993, Blough *et al.* 1996, Reiser & Kline 1998).

Histopathological analysis

Left ventricular samples were prepared with Masson–Goldner trichrome stain ($n = 5$ per group). At ×10 magnification (ECLIPSE E600; Nikon, Tokyo, Japan), the images were analysed with a personal computer using an ACT-1 software (Nikon, Tokyo, Japan) and Adobe Photoshop 7.0 software (Adobe Systems, Mountain View, CA, USA) for microscopic image processing, and Image J ver.1.34n software (National Institutes of Health, Bethesda, MD, USA) for measuring the area of interstitial fibrosis (Kitamura *et al.* 1997).

Statistical analysis

Results are expressed as mean ± SEM. Comparisons among the groups were analysed using either the Student *t*-test or ANOVA followed by a *post hoc* test using the Bonferroni correction. Two-way ANOVA with repeated measures was used to assess the response (intervention term) to the sequential stepped increases in $[Ca^{2+}]_o$ and to compare any differences between the transplant and control groups (group term). Statistical significance was accepted at the level of $P < 0.05$.

Results

Effects of physiological treatment dose of thyroid hormone

The physiological treatment dose of T3 used in the present study (1.2 μg day^{-1}) did not affect body weight, blood pressure, or heart rate at 5 weeks after the transplantation and 3 weeks after the initiation of the treatment (Table 1). Beating rate of unloaded hearts was comparably decreased when compared with that of corresponding recipient hearts in both control and T3-treated rats. Serum levels of T4 were depressed in T3-treated group compared with control group while serum levels of TSH were comparable in both groups (Table 1). The LV-to-body weight ratio was lower in transplanted unloaded hearts than in recipient hearts in both groups (Table 1).

Myocyte function

The baseline characteristics of contraction, and $[Ca^{2+}]_i$ transients in myocytes from both recipient and unloaded hearts at 3 weeks after the initiation of the treatment with T3 or vehicle are shown in Table 2. Myocyte size decreased significantly but to a compar-

Table 1 Characteristics of the animals at 3 weeks after the initiation of T3

	Control ($n = 20$)	T3-treated ($n = 20$)	*P*-values
BW, g	320 ± 4	319 ± 2	N.S.
Recipient heart			
LV weight, mg	668 ± 20	660 ± 18	N.S.
LV/BW, mg g^{-1}	2.07 ± 0.06	2.07 ± 0.05	N.S.
HR, bpm	487 ± 5	481 ± 6	N.S.
Unloaded heart			
LV weight, mg	307 ± 14*	346 ± 24*	N.S.
LV/BW, mg g^{-1}	0.95 ± 0.04*	1.08 ± 0.07*	N.S.
HR, bpm	451 ± 9*	445 ± 12*	N.S.
SBP, mmHg	117 ± 3	116 ± 3	N.S.
Thyroid function ($n = 9$ each)			
TSH, μIU mL^{-1}	0.94 ± 0.09	0.86 ± 0.09	N.S.
free-T3, pg dL^{-1}	3.26 ± 0.18	3.64 ± 0.14	N.S.
free-T4, ng dL^{-1}	2.74 ± 0.09	2.40 ± 0.08	<0.05

Values are mean ± SEM.
Recipient, recipient hearts; Unloaded, unloaded hearts; LV, left ventricle; LV/BW, ratio of left ventricular weight to body weight; BW, Body weight; SBP, Systolic blood pressure; HR, Heart rates; TSH, Thyroid stimulating hormone; T3, Triiodo-L-thyronine; T4, L-thyroxine; N.S., not statistically significant.
*$P < 0.05$ vs. recipient hearts of the same animals.

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Table 2 Baseline parameters of myocyte function at 3 weeks after the initiation of T3

	Control		T3-treated	
	Recipient	Unloaded	Recipient	Unloaded
Myocyte length (end-diastole), μm	112 ± 3	97 ± 3*	116 ± 2	105 ± 2†
Fractional cell shortening, %	6.9 ± 0.5	5.7 ± 0.5	5.5 ± 0.4	6.0 ± 0.5
Time to peak shortening, ms	89 ± 3	94 ± 3	86 ± 2	89 ± 3
Time to 50% relengthening, ms	35 ± 2	45 ± 3**	36 ± 2	35 ± 2
Peak systolic $[Ca^{2+}]_i$, nmol L^{-1}	500 ± 20	517 ± 19	484 ± 18	497 ± 22
End diastolic $[Ca^{2+}]_i$, nmol L^{-1}	84 ± 3	79 ± 4	79 ± 3	70 ± 2
Time to peak $[Ca^{2+}]_i$, ms	32 ± 1	31 ± 2	25 ± 1	27 ± 1
Time to 50% decline in $[Ca^{2+}]_i$, ms	62 ± 1	77 ± 5**	58 ± 2	57 ± 5

Results were obtained from eight control (29 myocytes) and seven T3-treated (32 myocytes) rats, and are expressed as mean ± SEM.
Recipient, LV myocytes from the recipient heart after receipt of the heterotopic transplanted heart. Unloaded, LV myocytes from the transplanted unloaded heart. *$P < 0.001$ vs. control recipient hearts and T3-treated recipient hearts; †$P < 0.01$ vs. T3-treated recipient hearts; **$P < 0.01$ vs. all groups.

able extent in both vehicle-treated and T3-treated unloaded hearts when compared with recipient hearts. In the control group, fractional cell shortening and levels of peak-systolic and end-diastolic $[Ca^{2+}]_i$ under baseline conditions were similar in myocytes from unloaded and recipient hearts. However, time to 50% relengthening and time to 50% decline in $[Ca^{2+}]_i$ were prolonged in myocytes from unloaded hearts compared with those from recipient hearts. By contrast, both slower relengthening and delayed $[Ca^{2+}]_i$ decay in myoyctes from unloaded hearts were normalized by the T3 treatment. Contractile function at high work states was studied by increasing $[Ca^{2+}]_o$ to 2.5 and 4.0 mM in myocytes. Representative tracings are shown in Figure 1. In the control group, fractional cell shortening of unloaded myocytes was severely depressed (9.1 ± 1.1% vs. 12.1 ± 0.9% at 4.0 mM $[Ca^{2+}]_o$, $P < 0.01$) associated with impaired augmentation of peak-systolic $[Ca^{2+}]_i$ (598 ± 35 vs. 780 ± 44 nM at 4.0 mM $[Ca^{2+}]_o$, $P < 0.01$) (Fig. 2a and c). By contrast, in the T3-treated group, there was no difference in calcium-dependent contractile reserve between myocytes from unloaded hearts and those from recipient hearts (Fig. 2b and d). However, there was no difference in the myofilament responsiveness to calcium between recipient and unloaded hearts in both control and T3-treated groups (Fig. 2e and f).

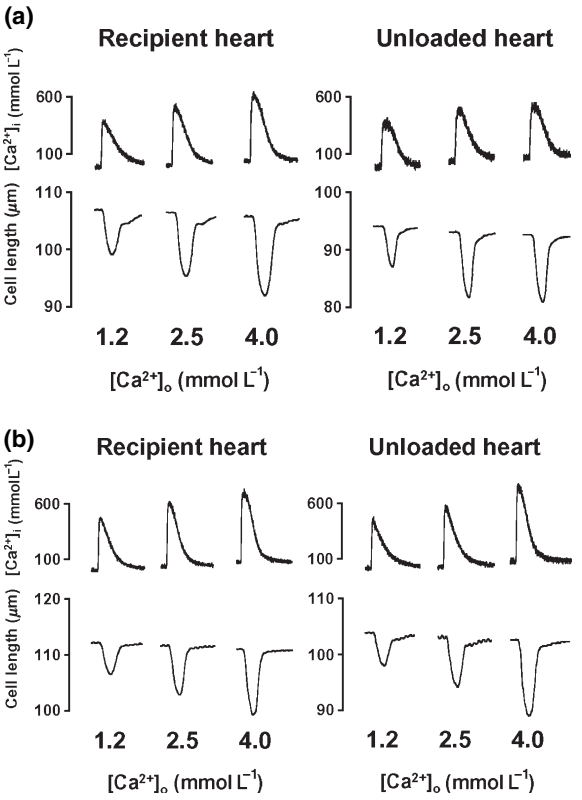

Figure 1 Representative tracings of myocytes from (a) vehicle-treated control animals and (b) T3-treated rats in response to elevation of perfusate Ca^{2+} concentration ($[Ca^{2+}]_o$).

Expression levels of calcium-cycling proteins

Protein expression levels of SERCA2a were not affected by cardiac unloading in the control group. The treatment with the physiological treatment dose of T3 tended to increase the expression of SERCA2 in unloaded hearts when compared with recipient hearts, but the extent of increase did not reach statistical significance (Fig. 3). By contrast, protein levels of PLB were significantly increased in unloaded hearts compared with recipient hearts in the control group. Although protein expression levels of PLB in unloaded hearts tended to be increased when compared with recipient hearts in the T3-treatment group, the extent of

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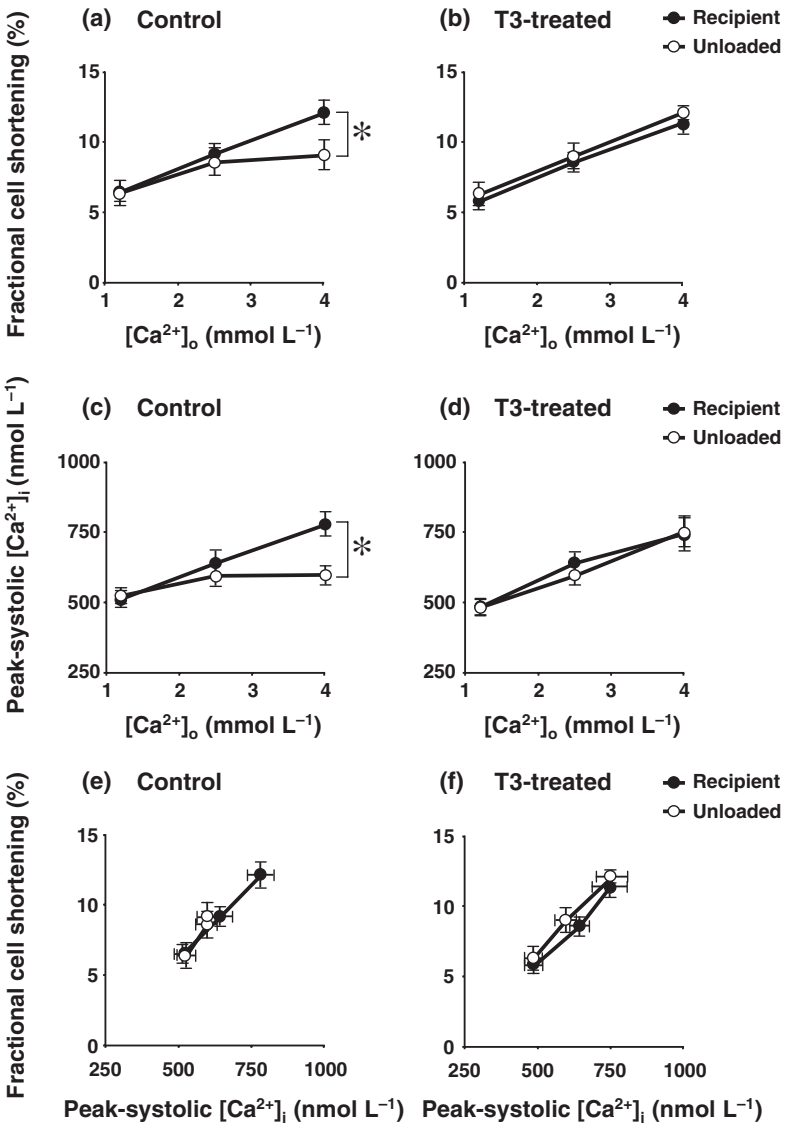

Figure 2 Ca^{2+}-dependent contractile reserve. Relationship between $[Ca^{2+}]_o$ and fractional cell shortening in isolated left ventricular (LV) myocytes from control group ($n = 11$–14) (a) and T3-treated group ($n = 15$) (b). Relationship between $[Ca^{2+}]_o$ and peak-systolic $[Ca^{2+}]_i$ in isolated LV myocytes from control group ($n = 11$–14) (c) and T3-treated group ($n = 15$) (d). Relationship between peak-systolic $[Ca^{2+}]_i$ and fractional cell shortening in isolated LV myocytes from control group ($n = 11$–14) (e) and T3-treated group ($n = 15$) (f). Ca^{2+}-dependent contractile reserve is depressed in myocytes from unloaded hearts compared with recipient hearts in control group (a and c). However, there was no difference in the myofilament responsiveness to calcium between recipient and unloaded hearts in both control and T3-treated groups (e and f). Recipient and unloaded indicate myocytes from recipient and unloaded hearts, respectively. Results are expressed as mean ± SEM. *$P < 0.01$ vs. recipient hearts.

increase was not statistically significant (Fig. 3). The SERCA2a/PLB ratio, which is a major determinant of cardiac contractile performance (Brittsan & Kranias 2000), was severely depressed in unloaded hearts compared with recipient hearts in the control group, whereas the decrease in the SERCA2a/PLB ratio was normalized in T3-treated animals (Fig. 3). Although there was no difference in the protein levels of SERCA2a and PLB between control unloaded and T3-treated unloaded hearts, the SERCA2a/PLB ratio tended to be higher in T3-treated unloaded hearts than in control unloaded hearts ($P = 0.058$) (Fig. 3). The phosphorylation state of PLB is also physiologically important in the regulation of SERCA2a function because PLB inhibits SERCA2a in its unphosphorylated state. In control animals, the expression level of Ser 16-phosphorylated PLB was significantly lower in unloaded hearts than in recipient hearts (Fig. 4). By contrast, in T3 treated animals, there was no difference in the levels of Ser 16-phosphorylated PLB between recipient and unloaded hearts (Fig. 4). The levels of Thr 17-phosphorylated PLB did not differ between recipient and unloaded hearts in both groups (Fig. 4). We also assessed the extent of Ser-16 and Thr-17 phosphorylations of PLB by calculating Ser-16 phosphorylated PLB/total PLB and Thr-17 phosphorylated PLB/total PLB ratios, respectively (Fig. 4c). T3 treatment attenuated the reduction in the Ser-16 phosphorylated PLB/total PLB ratio, but not that of the Thr-17 phosphorylated PLB/total PLB ratio in unloaded hearts. These data combined with those in Figure 3 suggest that the level of unphosphorylated PLB that suppresses SERCA2a was increased in unloaded hearts compared with recipient hearts in the control group, and that the

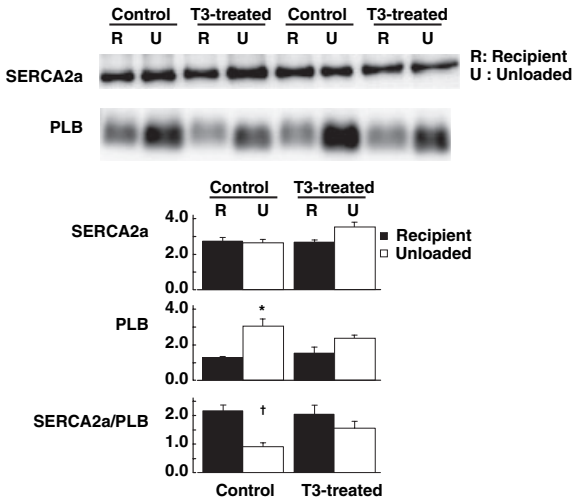

Figure 3 Upper panel, representative Western blots of SERCA2a and phospholamban (PLB) in unloaded and recipient hearts from control or T3-treated groups. Lower panel, left ventricular (LV) protein levels of SERCA2a and PLB, and the SERCA2a-to-PLB protein ratio (SERCA2a/PLB). Phospholamban was up-regulated and SERCA2a/PLB was severely depressed in control unloaded hearts compared with recipient hearts. In contrast, there was no difference in the PLB level or the SERCA2a/PLB ratio between unloaded and recipient hearts in T3-treated group. There was no difference in the protein levels of SERCA2a and PLB between control unloaded hearts and T3-treated unloaded hearts. However, SERCA2a/PLB tended to be higher in T3-treated unloaded hearts than in control unloaded hearts ($P = 0.058$). Recipient and unloaded indicate myocytes from recipient and unloaded hearts, respectively. The vertical axis represents the densitometric values in arbitrary units. Results are expressed as mean SEM. $^*P < 0.01$, $\dagger P < 0.005$ vs. recipient hearts.

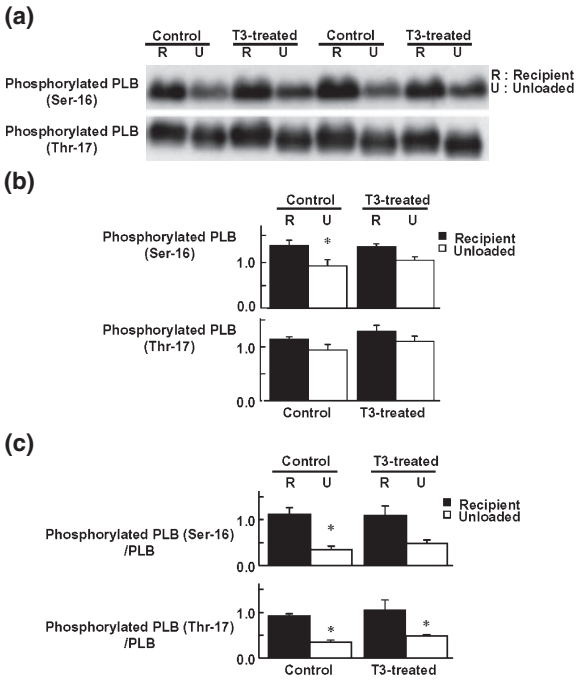

Figure 4 (a) Representative Western blots of phosphorylated phospholamban (PLB) (Ser 16) and phosphorylated PLB (Thr 17) in unloaded and recipient hearts from control or T3-treated groups. (b) LV tissue protein levels of phosphorylated PLB (Ser 16) and phosphorylated PLB (Thr 17). In control animals, the expression of Ser 16-phosphorylated PLB was lower in unloaded hearts than in recipient hearts. By contrast, in T3 treated animals, there was no difference in the levels of Ser 16-phosphorylated PLB between recipient and unloaded hearts. T3 treatment did not affect the level of Thr 17-phosphorylated PLB. (c) Ser-16 phosphorylated PLB/total PLB and Thr-17 phosphorylated PLB/total PLB ratios in both recipient and unloaded hearts. T3 treatment attenuated the reduction of Ser-16 phosphorylated PLB/total PLB ratio but not that of Thr-17 phosphorylated PLB/total PB ratio. Recipient and unloaded indicate myocytes from recipient and unloaded hearts, respectively. The vertical axis represents the densitometric values in arbitrary units or their ratios. Results are expressed as mean ± SEM. $^*P < 0.01$ vs. recipient hearts.

increase in unphosphorylated PLB in unloaded hearts was attenuated in the T3 treated group.

Isoform shift of MHC

Cardiac unloading induced isoform shift from α-MHC to β-MHC both in the control and T3-treated groups (Fig. 5). However, there was no difference in the levels of β-MHC protein or the ratio of β-MHC/total MHC protein of unloaded hearts between the control and T3-treated groups. These results suggest that the physiological treatment dose of T3 employed in the present study did not affect the isoform profile of MHC protein and that the isoform shift of MHC cannot explain the differences in myocyte shortening and relaxation between the control and T3-treated groups.

Histopathological observation

The magnitude of fibrosis tended to be higher in unloaded hearts than in recipient hearts with or without the T3 treatment (Fig. 6). However, there was no difference in the degree of fibrosis in unloaded hearts between the control and T3-treated groups ($P = 0.85$). These results suggest that the treatment with this dose of T3 did not affect the degree of fibrosis in unloaded hearts.

Discussion

The novel finding of the present study was that the treatment with the physiological treatment dose of thyroid hormone normalized the impaired relaxation and depressed contractile reserve in chronically unloaded hearts through restoration of calcium handling,

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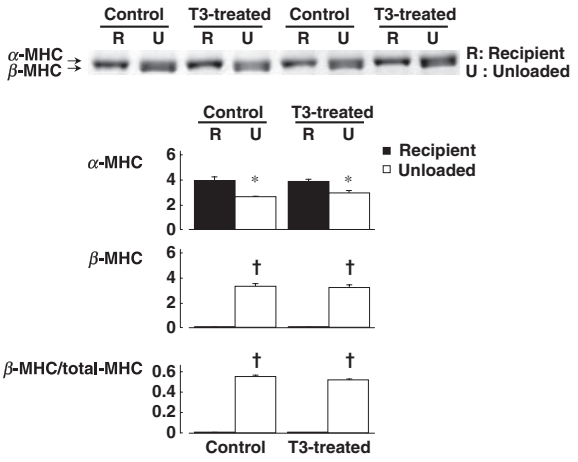

Figure 5 Representative electrophoretic analysis of myosin heavy chain (MHC) isoforms (upper panel) and left ventricular tissue protein levels of α-MHC, β-MHC, and β-MHC/total MHC protein (lower panel). Cardiac unloading equally induced isoform shift from α-MHC to β-MHC both in the control and T3-treated groups. Recipient and unloaded indicate myocytes from recipient and unloaded hearts, respectively. The vertical axis represents the densitometric values in arbitrary units. Results are expressed as mean ± SEM. *$P < 0.01$, †$P < 0.001$ vs. recipient hearts.

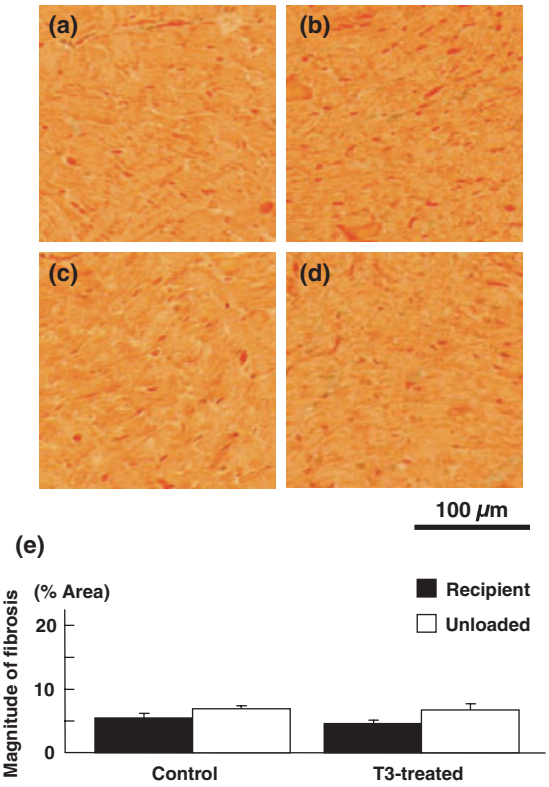

Figure 6 Masson's trichrome-Goldner stained sections of control recipient left ventricle (LV) (a), control unloaded LV (b), T3-treated recipient LV (c), and T3-treated unloaded LV (d). Original magnification ×20; scale bar = 100 μm. Magnitude of interstitial fibrosis (e) ($n = 5$ for each group). The magnitude of fibrosis tended to be higher in unloaded hearts than in recipient hearts with or without the T3 treatment. However, there was no significant difference in the degree of fibrosis between recipient and unloaded hearts in both groups.

which may be explained at least partially by amelioration of SERCA2a/PLB protein ratio and phosphorylation state of PLB protein. To the best of our knowledge, this is the first study that demonstrates the usefulness of the physiological treatment dose of thyroid hormone to restore myocardial function under chronically unloaded conditions.

Cardiac unloading and myocyte function

In the present study, we showed the depression of contractile reserve and calcium handling in myocytes from 5-week LV unloaded hearts. On the other hand, Welsh *et al.* (2001) reported that contractile function is preserved in myocytes from 2-week LV unloaded hearts using the same animal model as ours. We previously reported that myocyte function was impaired at 5 weeks after the initiation of LV unloading, but not at 2 weeks after the initiation (Ito *et al.* 2003). Therefore, the discrepant results between the report by Welsh *et al.* (2001) and ours (Ito *et al.* 2003) would be due to the difference in the duration of cardiac unloading.

Thyroid hormone and contractile function

The SERCA2a function is inhibited by PLB in its unphosphorylated form. We have recently reported that chronic cardiac unloading causes a time-dependent depression of myocyte contractile function mainly due to a decrease in the SERCA2a/PLB protein ratio (Ito *et al.* 2003), and confirmed our previous observations in the present study. We aimed further to prevent the myocardial dysfunction caused by chronic unloading with pharmacological intervention. For this purpose, we employed a simultaneous treatment with thyroid hormone as the clinical and experimental studies reported that the hormone could improve cardiac performance in both normal and failing hearts (Chang *et al.* 1997, Ojamaa *et al.* 2000a,b, , Klein & Ojamaa 2001, Pantos *et al.* 2004). For instance, Ojamaa *et al.* (2000a) reported that treatment with a physiological treatment dose of T3 (1.2 μg day^{-1}) for 2 weeks significantly improved the ejection fraction of a rat myocardial infarction model compared with no treatment. Indeed, we were able to demonstrate that the treatment with this dose of thyroid hormone restored slower myocyte relaxation and delayed

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$[Ca^{2+}]_i$ decline at baseline (Table 2), depressed myocyte contractile reserve (Figs 2–4), and dysregulated protein expression of PLB and its phosphorylation state (Figs 5 and 6) in unloaded hearts without affecting systemic haemodymanics or inducing LV hypertrophy.

Thyroid function is sensitively regulated by a feedback system through the alteration of TSH release. Although free T4 levels were decreased by 12% in T3-treated group when compared with control group in the present study, TSH levels were similar in both control and T3-treated groups in the present study, suggesting that the physiological treatment dose of T3 used here was low enough not to affect the feedback system that regulates thyroid function.

Mechanisms for the beneficial effects of physiological treatment dose of thyroid hormone

Cardiac unloading induced isoform shift from α-MHC to β-MHC (Fig. 5). As thyroid hormone is reported to increase the expression of α-MHC and to decrease that of β-MHC (Ojamaa *et al.* 1992), an increase in the proportion of α-MHC might contribute to the T3-induced normalization of contractile properties in myocytes from unloaded hearts. However, the treatment with this dose of T3 did not affect the isoform profile of MHC *protein* in the present study. These data suggest that the dose of T3 employed in the present study (1.2 μg day^{-1}) was too low to elicit an increase in the proportion of α-MHC as compared with the established dose (>2.5 μg day^{-1}) demonstrated to alter the MHC isoform profile (Danzi *et al.* 2005). Thyroid hormone is also reported to enhance cardiac performance by inducing LV hypertrophy (Degens *et al.* 2003). However, the treatment with the physiological treatment dose of T3 did not alter the LV/BW ratio in either recipient or unloaded hearts in the present study. Therefore, neither MHC isoform shift nor induction of LV hypertrophy explains the beneficial effects of this dose of thyroid hormone. By contrast, delayed $[Ca^{2+}]_i$ decay in myocytes from unloaded hearts was normalized by the T3 treatment. In addition, depressed augmentation of peak-systolic $[Ca^{2+}]_i$ levels at high $[Ca^{2+}]_o$ in myocytes isolated from unloaded hearts was also restored by the T3 treatment. SERCA2a/PLB ratio, which is a major determinant of cardiac contractile performance (Brittsan & Kranias 2000), was severely depressed in unloaded hearts compared with recipient hearts in the control group. As shown in Figure 3, the remarkably depressed SERCA2a/PLB ratio in unloaded hearts of the control group was predominantly due to the increase in PLB expression levels. By contrast, in T3-treated rats, both SERCA2a and PLB expressions in unloaded hearts tended to be increased when compared with recipient hearts, resulting in the amelioration of the SERCA2a/PLB ratio and possibly in the restoration of contractile reserve. Moreover, the increased expression of PLB protein and decreased expression of Ser 16-phosphorylated PLB in unloaded hearts, which elicit the suppression of SERCA2a function, were ameliorated by the treatment with this dose of T3. Decreased expression of Ser 16-phosphorylated PLB and no change in that of Thr 17-phosphorylated PLB have been also reported by Brixius *et al.* (2003) in myocardium from patients with dilated cardiomyopathy when compared with non-failing hearts. Furthermore, Zinman *et al.* (2006) reported that thyroid hormone increased Ser 16-phosphorylation of PLB, which was predominantly induced by cyclic AMP dependent protein kinase A (PKA) in cardiomyocytes. Therefore, it is possible that the physiological treatment dose of T3 employed in the present experiment increased PKA activity, and maintained Ser 16-phosphorylated state of PLB. The results of our present study suggest that the beneficial effects of thyroid hormone on relaxation and contractile reserve in myocytes isolated from unloaded hearts are mainly due to restoration of calcium handling which may be explained at least partially by normalizing the expression of PLB protein and its phosphorylation state. This notion is consistent with the past reports, which demonstrated that the overexpression of PLB impairs myocyte contractility while both the ablation of PLB and increased phosphorylation of PLB accelerate contractile performance (Brittsan *et al.* 2000, Brittsan & Kranias 2000, MacLennan & Kranias 2003, Antoons *et al.* 2006).

Recently, it is reported that expression levels of thyroid hormone receptors are altered in cardiac hypertrophy as well as in myocardial infarction (Kinugawa *et al.* 2001, Pantos *et al.* 2005) and that the pretreatment with thyroid hormone protects rat hearts against ischaemic stress at least in part by enhancing the expression of heart shock protein 27 (Pantos *et al.* 2002, 2003, 2006). Although we did not measure the expression levels of thyroid hormone receptors in the present study, it is possible that the altered thyroid hormone signalling in unloaded hearts might also explain the beneficial effects of T3 treatment. Further studies are needed to elucidate this issue.

Clinical implications

The present findings may have clinical implications in humans such as astronauts subjected to cardiac unloading during a prolonged spaceflight as echocardiographic evaluation of astronauts immediately after the long-term spaceflight revealed that chronic unloading elicits cardiac atrophy and depression of cardiac performance (Bungo *et al.* 1987) and mild hypothyroidism has been

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demonstrated after space flight (Strollo 1999, Grimm *et al.* 2002). Patients treated with left ventricular assist device (LVAD) are also subjected to cardiac unloading (Deng *et al.* 2005, Mancini & Burkhoff 2005, Burkhoff *et al.* 2006). Although LVADs are primarily used as a bridge to cardiac transplantation, weaning and removal of LVAD have recently been performed (Kumpati *et al.* 2001, Birks *et al.* 2004, Wohlschlaeger *et al.* 2005). However, after successful weaning and removal of LVAD, recurrent myocardial dysfunction is frequently noted in patients with severe heart failure, resulting in a re-implantation of LVAD or heart transplantation (Helman *et al.* 2000, Hetzer *et al.* 2001, Farrar *et al.* 2002, Margulies 2002, Razeghi *et al.* 2002). Yacoub *et al.* reported that a combined therapy of LVAD support and pharmacotherapy using beta-2 adrenergic agonist clenbuterol successfully improved the rate of LVAD removal without the need for a re-implantation of LVAD or heart transplantation (Yacoub 2001, Barton *et al.* 2005). Although precise mechanisms of the beneficial effects of clenbuterol are not fully understood, Tsuneyoshi *et al.* (2005) demonstrated that clenbuterol improved the papillary muscle function of unloaded normal hearts using the same animal model as ours. In this regard, a combined therapy of LVAD support and the physiological treatment dose of T3 might also provide a novel strategy to treat patients with severe heart failure.

Limitations of the study

Several limitations should be mentioned for the present study. First, although previous studies have shown that the present rat model of cardiac unloading is useful to examine unloaded conditions of hearts (Klein & Hong 1986, Korecky *et al.* 1986, Klein *et al.* 1991, Korecky & Masika 1991, Ojamaa *et al.* 1992, Rakusan *et al.* 1997, Depre *et al.* 1998, Ito *et al.* 2003), it does not completely mimic the clinical conditions in the spacecraft or in heart failure patients with LVAD. Secondly, more than 90% of the Ca^{2+} extrusion from the cytosol of cardiac myocytes during diastole depends on the SR calcium uptake in rodents, whereas in large animals, Na^{+}/Ca^{2+} exchangers as well as SR calcium uptake contribute to the process (Bers 2002). Thus, the present findings should be re-evaluated in a future study with large animals (e.g. pigs). Thirdly, we employed different perfusate Ca^{2+} concentrations, but not various pacing rates, to assess contractile reserve of isolated myocytes. Although we believe that the contractile reserve of isolated myocytes can be tested either by increasing pacing frequency or perfusate Ca^{2+} concentration (Ito *et al.* 2000), this issue should be elucidated in a future study. Finally, although there was a difference in the isoform expression of MHC between recipient and unloaded hearts, the time to peak shortening of cardiac myocytes was not significantly different between the two groups. Some unknown compensatory mechanisms, e.g. a decrease in microtubules (Philpott *et al.* 1990) or activation of phosphatidylinositol 3-kinase signalling (Cao *et al.* 2005, Furuya *et al.* 2006), might play a role in cardiac myocytes from unloaded hearts. This issue also remains to be elucidated.

In conclusion, we were able to demonstrate that the treatment with the physiological treatment dose of thyroid hormone restores depressed contractile reserve in myocytes from chronically unloaded hearts. Our finding may have a clinical implication, as we will face more frequently the condition of chronic cardiac unloading in the treatment of heart failure with LVAD as well as in the space industry in the future.

Conflict of interest

There are no conflicts of interest.

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