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RESEARCH PAPER

Biological actions of green tea catechins on cardiac troponin C

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BACKGROUND AND PURPOSE

Catechins, biologically active polyphenols in green tea, are known to have a protective effect against cardiovascular diseases. In this study, we investigated direct actions of green tea catechins on cardiac muscle function to explore their uses as potential drugs for cardiac muscle disease.

EXPERIMENTAL APPROACH

The effects of catechins were systematically investigated on the force-pCa relationship in skinned cardiac muscle fibres to determine their direct effects on cardiac myofilament contractility. The mechanisms of action of effective catechins were investigated using troponin exchange techniques, quartz crystal microbalance, nuclear magnetic resonance and a transgenic mouse model.

KEY RESULTS

(-)-Epicatechin-3-gallate (ECg) and (-)-epigallocatechin-3-gallate (EGCg), but not their stereoismers (-)-catechin-3-gallate and (-)-gallocatechin-3-gallate, decreased cardiac myofilament Ca^{2+} sensitivity probably through its interaction with cardiac troponin C. EGCg restored cardiac output in isolated working hearts by improving diastolic dysfunction caused by increased myofilament Ca^{2+} sensitivity in a mouse model of hypertrophic cardiomyopathy.

CONCLUSIONS AND IMPLICATIONS

The green tea catechins, ECg and EGCg, are Ca^{2+} desensitizers acting through binding to cardiac troponin C. These compounds might be useful compounds for the development of therapeutic agents to treat the hypertrophic cardiomyopathy caused by increased Ca^{2+} sensitivity of cardiac myofilaments.

Abbreviations

cTn, cardiac muscle troponin; EC, (-)-Epicatechin; ECg, (-)-Epicatechin-3-gallate; EGC, (-)-Epigallocatechin; EGCg, (-)-Epigallocatechin-3-gallate; fsTn, fast skeletal muscle troponin; HCM, hypertrophic cardiomyopathy; QCM, quartz crystal microbalance; Tn, troponin

Introduction

A number of studies suggest that consumption of green tea decreased the risk of several pathological conditions, including cardiovascular disease (Wang *et al.*, 1995; Arts *et al.*, 2001; Mukamal *et al.*, 2002). Green tea contains catechins as biologically active polyphenols. Major catechins in green tea are (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin-3-gallate (ECg), and

(-)-epigallocatechin-3-gallate (EGCg). ECg and EGCg have been shown to be effective against cardiovascular and other diseases (Chyu *et al.*, 2004; Tachibana *et al.*, 2004; Sasazuki *et al.*, 2008). Mechanisms involved in the prevention of cardiovascular diseases by EGCg have so far been suggested to be associated with its anti-oxidative effect (Chyu *et al.*, 2004), anti-inflammatory effect (Ludwig *et al.*, 2004) and vasorelaxant effect (Lorenz *et al.*, 2004) on the cardiovascular system.

In the present study, we systematically investigated the effects of catechins on the force-pCa relationship in membrane-permeabilized (skinned) cardiac muscle fibres and found that ECg and EGCg have Ca^{2+}-desensitizing effects on the muscle contraction (i.e. effects of decreasing the myofilament Ca^{2+} sensitivity). Hypertrophic cardiomyopathy (HCM) is a cardiac muscle disease characterized by a reduced diastolic function leading to heart failure. Recent genetic investigations revealed that a majority of HCM is caused by mutations in genes for sarcomeric proteins, and increased myofilament Ca^{2+} sensitivity was demonstrated to be a primary functional defect triggering the pathogenesis of HCM (Harada and Morimoto, 2004; Ahmad *et al.*, 2005; Morimoto, 2008; Morimoto, 2009). Although cardioprotective agents such as β-blockers or Ca^{2+} antagonists have been used in the treatment of HCM, there is no reliable evidence that these drugs protect patients with HCM from sudden death and no effective pharmacotherapy is established at present. In this study, we examined the effect of EGCg on the cardiac haemodynamics in a mouse model of HCM, caused by a troponin (Tn) mutation. The results suggest that the green tea catechins might be useful for the development of therapeutic agents to treat the HCM associated with an increased cardiac myofilament Ca^{2+} sensitivity. Part of this work has been reported previously in abstract form (Tadano *et al.*, 2005a,b).

Methods

Animals

All animal care in this investigation conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication no. 85–23, revised 1996). The experimental protocol was reviewed by the Committee of Ethics on Animal Experiments at the Faculty of Medicine, Kyushu University, and carried out according to the *Guidelines for Animal Experiments*, Faculty of Medicine, Kyushu University, and The Law (No. 105) and Notification (No. 6) of the Japanese Government.

Skinned muscle preparation and whole Tn exchange in skinned muscle fibres

Skinned muscle fibres were prepared from the left ventricular trabeculae and the back muscle of young male albino rabbits (2–2.5 kg) as described previously (Morimoto *et al.*, 1998). Endogenous Tn in cardiac and fast skeletal muscle skinned muscle fibres were exchanged with whole rabbit fast skeletal muscle Tn (fsTn) and cardiac muscle Tn (cTn), respectively, as described previously (Mirza *et al.*, 2005). After force measurements, fibre samples were analysed by a 5–20% gradient SDS-PAGE, stained with silver, and the extent of Tn exchange was determined by an optical densitometric scan of the gel (Morimoto *et al.*, 2002).

Preparation of cTn subunits

Human cTn subunits, cTnT, cTnI and cTnC, were expressed in *Escherichia coli* strain BL21(DE3)-CodonPlus-RP (Stratagene, La Jolla, CA, USA) using the expression vector pET-3a (EMD Biosciences, Madison, WI, USA). cTnC was purified with DEAE-Toyopearl 650M (Tosoh, Tokyo, Japan) and an FPLC gel filtration column, Superdex75 26/60 (GE Healthcare Bio-Sciences Corp, Piscataway, NJ, USA). cTnT was purified with DEAE-Toyopearl 650 M (Tosoh) and CM-Toyopearl 650M (Tosoh) in the presence of 6 M urea. cTnIs were purified with CM-Toyopearl 650M (Tosoh) and an FPLC ion-exchange column, MonoS (GE Healthcare) in the presence of urea. After removing urea by dialysis, cTnT and cTnI were used for the experiments.

EGCg binding to cTn subunits

Binding of EGCg to cTnT, cTnI and cTnC were measured using a 27 MHz quartz crystal microbalance (QCM, Initium, Inc., Tokyo, Japan), which is a very sensitive mass measuring apparatus (Okahata *et al.*, 1998a,b; Lu *et al.*, 2003), Biotinylated cTnT, cTnI or cTnC was immobilized on the avidin-coated QCM Au electrode and the sensor tips were immersed in a solution consisting of (in mM) 50 MOPS/KOH (pH 7.0), 300 KCl, 1 $MgCl_2$ and 4 EGTA: high ionic strength conditions were adopted to prevent cTnT and cTnI from being in abnormal conformations. The bindings of EGCg to cTnT, cTnI and cTnC were detected from the frequency changes (ΔF) due to changes in mass on the electrode at sub-nanogram levels upon cumulative injection of EGCg into the bathing solution.

Binding of cTnI N-terminal peptide to cTnC

A synthetic peptide for the N-terminal helix region of cTnI (AKKKSKISASRKLQLKTLLLQIAKQELE) was purchased from Greiner Bio-One (Tokyo, Japan).

Biotinylated cTnC was immobilized on the avidin-coated QCM Au electrode and the sensor tips were immersed in a solution consisting of (in mM) 43 MOPS/KOH (pH 7.0), 500 KCl, 0.9 $MgCl_2$ and 3.5 EGTA. Binding of the cTnI N-terminal peptide to cTnC were detected from ΔF upon cumulative injection of cTnI N-terminal peptide into the bathing solution. High ionic strength conditions were adopted to reduce non-specific binding of the N-terminal peptide to electrodes.

Generation of a transgenic mouse model of HCM

Cloning and mutagenesis of human cTnT cDNA were carried out as described previously (Morimoto *et al.*, 1998; 2002). About 1.3 kb of the upstream promoter region of the mouse cTnT gene obtained from genomic mouse DNA by PCR was replaced into an MHC class I promoter fragment of the cDNA expression vector pLG1 (Morimoto *et al.*, 2002), and designated as pTG. The recombinant cDNA encoding the WT or ΔE160 mutant cTnT was introduced into the cDNA cloning site of the plasmid pTG. The SpeI-XhoI fragment isolated as a transgene was then microinjected into the pronucleus of fertilized eggs of C57BL/6 mice. Identification of the transgene in founder mice and their progeny was performed by Southern blot analysis and PCR using genomic DNA isolated from tail. Homozygous transgenic mice were produced by mating between heterozygous ΔE160 cTnT transgenic mice and were used in this study. Homozygosity was determined by genotyping using PCR. Human cTnT transgene mRNA expression was detected by RT-PCR using a set of primers (5′-ACC ACC TTC TGA TAG GCA G and 5′-TCT GAC ATA GAA GAG GTG GTG), which amplify a 902 bp DNA fragment. Expression level of the cTnT transgene protein was determined by immunoblot analyses of the skinned cardiac muscle fibres using the monoclonal anti-human cTnT antibody 2D10 (Research Diagnostic, Concord, MA, USA), which does not react with mouse cTnT and the monoclonal anti-TnT antibody JLT-12 (Oncogene Science, Cambridge, MA, USA), which cross-reacts with human and mouse cTnTs equally. The transgenic mice were fed with standard rodent chow and water provided *ad libitum*.

Analysis of working isolated hearts

Hearts were excised from mice after anaesthesia with pentobarbital sodium (50 $mg \cdot kg^{-1}$, i.p.) and heparinization (15 U i.v.). The working hearts were prepared and analysis was carried out as described previously (Mizukami *et al.*, 2008).

Measurements of Ca^{2+} transient in isolated cardiomyocytes

Cardiomyocytes isolated from mouse left ventricle were loaded with Fura-2 acetoxymethyl ester, and $[Ca^{2+}]_i$ was monitored using a fluorescence recording system (IonOptix LLC, Milton, MA, USA) as described previously (Du *et al.*, 2007).

Data analysis

Data are presented as mean ± SEM. Mean values for more than three groups were compared by one-way analysis of variance, followed by a *post hoc* Dunnett's or Tukey's multiple comparison test. The difference between two group means was analysed with an unpaired Student's *t*-test.

Materials

Catechins were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Results

Figure 1 shows the structures of catechins examined in this study. Figure 2A shows the force-pCa relationships in skinned cardiac muscle fibres determined in the presence of epicatechin derivatives EC, EGC, ECg and EGCg. ECg and EGCg were found to decrease Ca^{2+} sensitivity, as shown by rightward shifts of the force-pCa relationships with significant reduction in pCa_{50}. EGCg had a greater Ca^{2+}-desensitizing effect than ECg. EC and EGC had no significant effects on cardiac myofilament Ca^{2+}-sensitivity, indicating that the galloyl group in ECg and EGCg has a critical role in the Ca^{2+}-desensitizing effects. On the other hand, closely related catechin compounds, including (-)-catechin-3-gallate and (-)-gallocatechin-3-gallate, which are diastereomers of ECg and EGCg, respectively, had no significant effects on the Ca^{2+} sensitivity of skinned cardiac muscle fibres (Figure 2B), strongly suggesting that Ca^{2+}-desensitizing effects of ECg and EGCg derived from a specific stereoselective molecular interaction with a target molecule in cardiac muscle. These epicatechin and catechin derivatives had no significant effects on the maximum force in skinned cardiac muscle fibres (data not shown). EGCg decreased the Ca^{2+} sensitivity of skinned cardiac muscle fibres in a concentration-dependent manner (Figure 2C).

EGCg was found to have a much weaker effect on the Ca^{2+} sensitivity of fast skeletal muscle compared with cardiac muscle (Figure 3A). Because cardiac and fast skeletal muscle contraction is regulated by specific isoforms of Tn, cTn and fsTn, respectively, we tested the possibility that the cardiac isoform of Tn

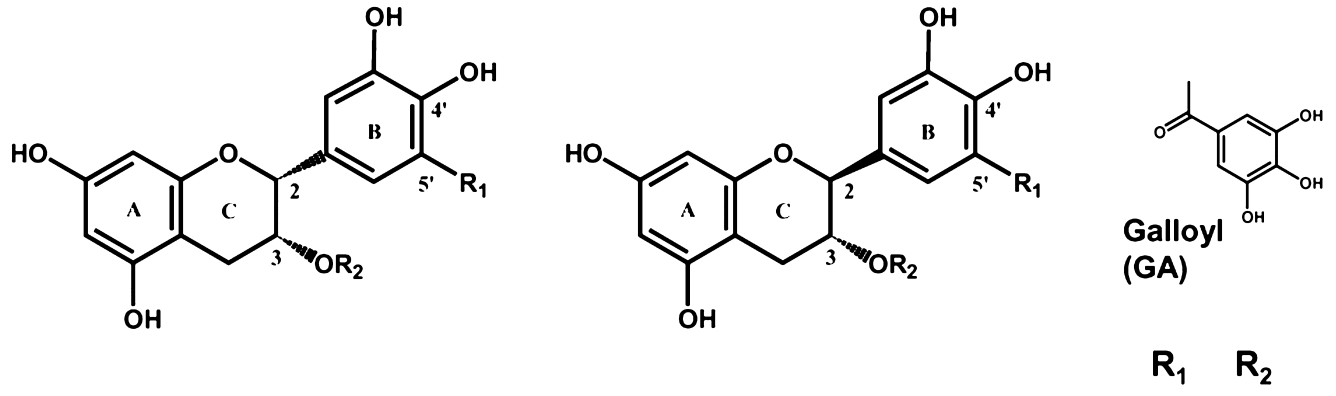

Figure 1

The structures of epicatechin and catechin derivatives used in this study.

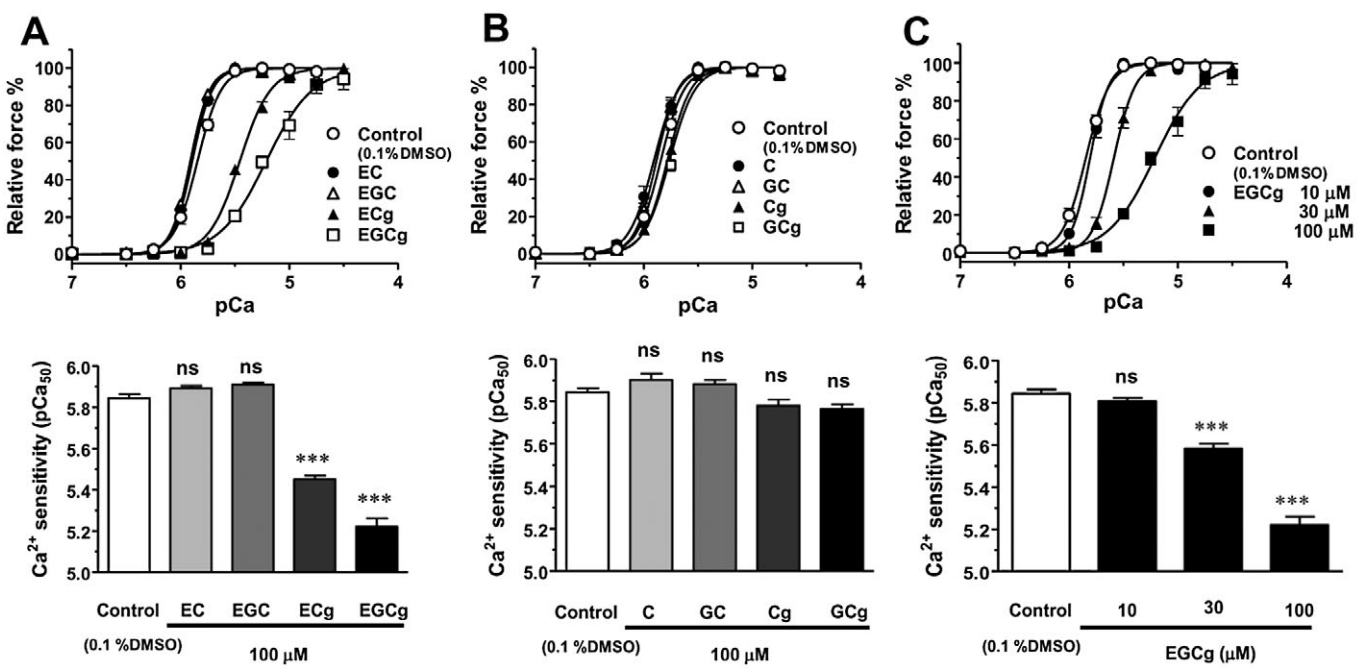

Figure 2

Effects of catechins on force generation in skinned cardiac muscle fibres. (A) Upper panel: Force-pCa relationships determined in the presence of 100 μM epicatechin derivatives. Lower panel: Effects of epicatechin derivatives on the Ca^{2+} sensitivity (pCa_{50}) of force generation in skinned cardiac muscle fibres. (B) Upper panel: Force-pCa relationships determined in the presence of 100 μM catechin derivatives. Lower panel: Effects of catechin derivatives on the Ca^{2+} sensitivity (pCa_{50}) of force generation in skinned cardiac muscle fibres. (C) Upper panel: Force-pCa relationships in skinned cardiac muscle fibres determined in the presence of 10, 30 and 100 μM EGCg. Lower panel: Concentration-dependent effects of EGCg on the Ca^{2+} sensitivity (pCa_{50}) of force generation in skinned cardiac muscle fibres. The data represent the means ± SE of measurements on 10 and 5 fibres for control and samples, respectively. ***$P < 0.001$ versus control (Dunnett's multiple comparison test). C, catechin; Cg, (-)-catechin-3-gallate; EC, (-)-Epicatechin; ECg, (-)-Epicatechin-3-gallate; EGC, (-)-Epigallocatechin; EGCg, (-)-Epigallocatechin-3-gallate; GC, gallocatechin; GCg, (-)-gallocatechin-3-gallate.

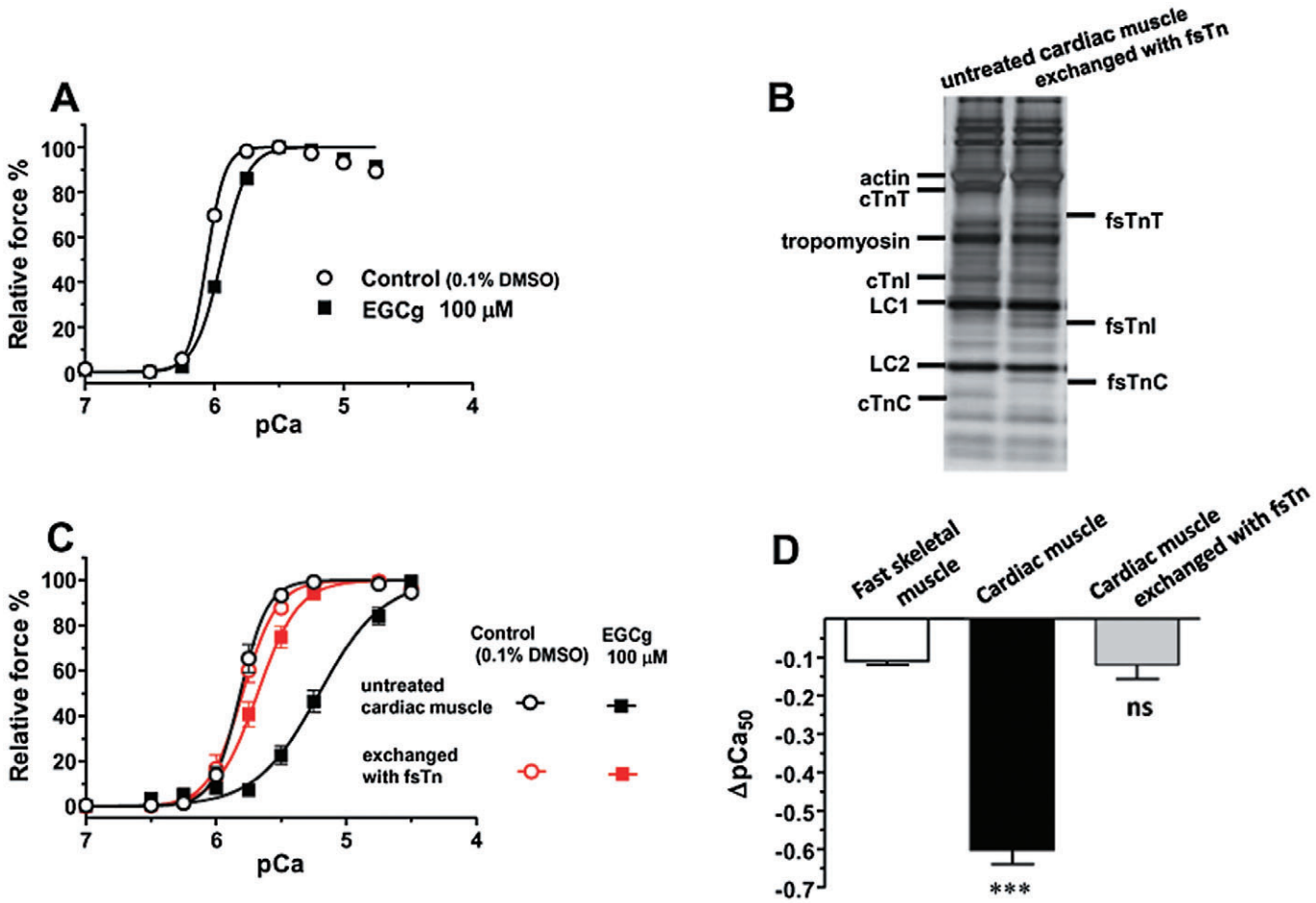

Figure 3

Role of Tn in determining the differential sensitivity to EGCg in cardiac and fast skeletal muscle. (A) Effect of EGCg on force-pCa relationships in skinned fast skeletal muscle fibres. The data represent the means ± SE of measurements on 5 fibres. (B) SDS-PAGE analysis of skinned cardiac muscle fibres in which endogenous cTn was exchanged with fsTn. Note that the decrease in cTnI was not apparent due to the presence of other protein(s) with similar mobility. Densitometric analyses of TnC isoforms in skinned fibres indicated that 83.5 ± 3.0% ($n = 7$ fibres) of endogenous Tn was exchanged with fsTn. LC1 and LC2, ventricular myosin light chains 1 and 2, respectively. (C) Effect of EGCg on force-pCa relationships in skinned cardiac muscle fibres exchanged with fsTn. The data represent the means ± SE of measurements on 7 fibres. (D) EGCg-induced decrease in the Ca^{2+}-sensitivity (ΔpCa_{50}) in skinned cardiac muscle fibres exchanged with fsTn. The data represent the means ± SE of measurements on 7 fibres. ***$P < 0.001$ versus fast skeletal muscle (Dunnett's multiple comparison test). EGCg, (-)-Epigallocatechin-3-gallate; fsTn, fast skeletal muscle troponin; Tn, troponin.

might be responsible for the greater effect of EGCg on cardiac muscle, by exchanging whole Tn complex in skinned fibres (Figure 3B). Exchanging endogenous cTn in skinned cardiac muscle fibres with fsTn reduced the Ca^{2+}-desensitizing effect of EGCg to almost the same level as that observed in the fast skeletal muscle fibres (Figures 3C,D). These results provide strong evidence that cTn is a specific target of EGCg in cardiac muscle that determines the greater effect of EGCg on the Ca^{2+} sensitivity of cardiac muscle, compared with its effects on fast skeletal muscle.

We next measured the binding of EGCg to the cTn subunits, using a quartz crystal microbalance (QCM), a very sensitive mass measuring device. QCM analyses indicated that EGCg bound to cTnC, but not to cTnI or cTnT (Figure 4A). Using nuclear magnetic resonance (NMR) spectroscopy, we have previously demonstrated that EGCg bound to the C-lobe of cTnC (Tadano *et al.*, 2005a,b). As the C-lobe of cTnC is known to interact with an N-terminal α-helical region of cTnI, we measured the binding of the N-terminal peptide of cTnI ($cTnI_{35–62}$) to cTnC, in the absence and presence of EGCg (Figure 4B). The results indicated that EGCg enhanced the binding of the N-terminal H1 helix region of cTnI to cTnC.

Mutations in genes of human cTn subunits have been found to cause HCM (Harada and Morimoto, 2004; Marian, 2005). *In vitro* and *in vivo* studies

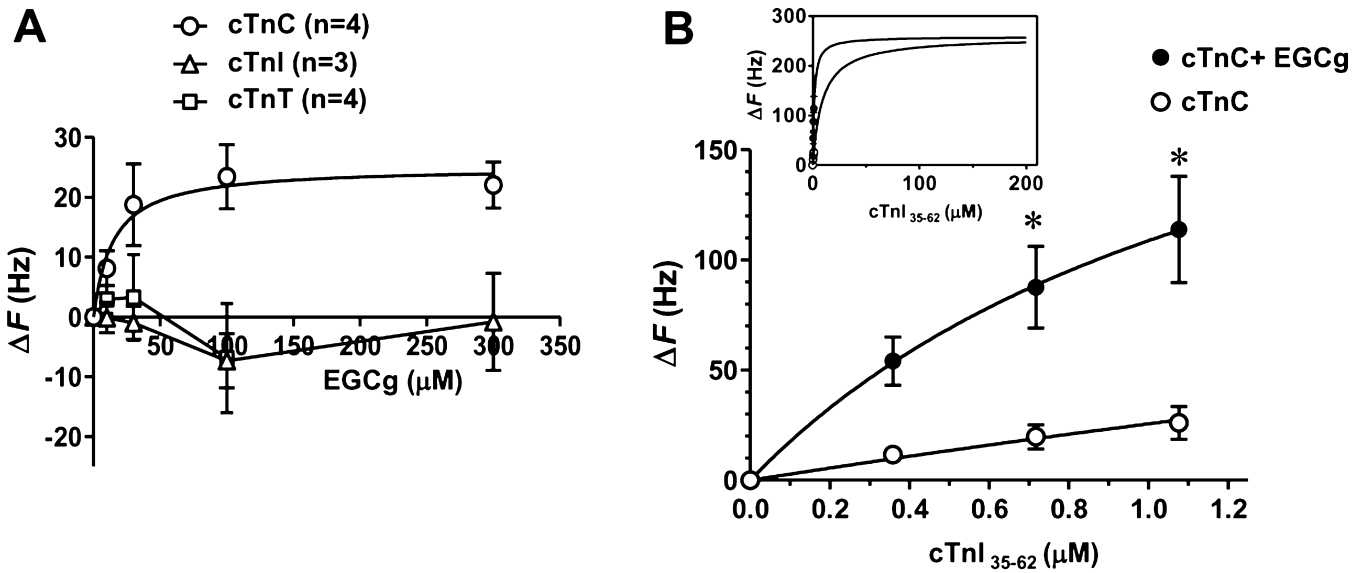

Figure 4

Binding of EGCg to cTn subunits. (A) Binding of EGCg to cTnC, cTnI and cTnT were directly determined from the frequency changes (ΔF) of QCM upon cumulative addition of EGCg. The data represent the means ± SE of 3–4 measurements. The EGCg-cTnC binding data were fitted to a hyperbolic one-site binding equation, and a best-fitted curve was obtained with a K_D of 14.6 μM. (B) Effects of EGCg on the binding of a cTnI N-terminal peptide ($cTnI_{35–62}$) to cTnC. Binding of the cTnI N-terminal peptide ($cTnI_{35–62}$) to cTnC was directly determined from ΔF of QCM upon cumulative addition of the peptide in the absence or presence of 300 μM EGCg. The data represent the means ± SE of 3 measurements. *$P < 0.05$, versus –EGCg (unpaired *t*-test). The data were fitted to a hyperbolic one-site binding equation, and best-fitted curves were obtained with K_D's of 9.1 and 1.4 μM for cTnC and cTnC+EGCg, respectively. EGCg, (-)-Epigallocatechin-3-gallate; QCM, quartz crystal microbalance.

using mutant proteins and transgenic animals indicate that an increased Ca^{2+} sensitivity of cardiac myofilament is the causal functional defect triggering the pathogenesis of HCM associated with the mutations in cTn subunits (Harada and Morimoto, 2004; Ahmad *et al.*, 2005; Morimoto, 2008; Morimoto, 2009). We created a transgenic mouse model of HCM expressing the deletion mutant ΔE160 of cTnT in the heart, which had been found to cause familial HCM in human (Figures 5A–C). The mutant mice showed extensive level of cardiomyocyte disarray (Figure 5D), a hallmark of HCM, and Ca^{2+}-sensitization in the force generation of skinned cardiac muscle fibres (Figure 5E). EGCg reversed the increased myofilament Ca^{2+} sensitivity of mutant mice (Figure 5E). *Ex vivo* analyses of isolated working heart preparations showed normal systolic function with diastolic dysfunction, another hallmark of HCM, as shown by preserved left ventricular dP/dt_{max} and decreased left ventricular $-dP/dt_{min}$ (Figure 6A). Cardiac output from the hearts of ΔE160cTnT-Tg mice was significantly lower than those from WTcTnT-Tg mice or Non-Tg mice due to diastolic dysfunction (Figure 6B). EGCg improved the diastolic dysfunction of the hearts of these mice (Figure 6A) and increased their cardiac output (Figure 6B). EGC had no such beneficial effects on the diastolic function and cardiac output (data not shown). Figure 6C shows Ca^{2+} transients measured in Fura-2-loaded cardiomyocytes. The peak amplitude and the peak rates of increase and decrease in cytoplasmic Ca^{2+} were decreased in ΔE160cTnT-Tg mice as in the other Tg mouse models of HCM caused by mutations of cTnI and cTnT (Wen *et al.*, 2008; Willott *et al.*, 2010); EGCg tended to restore these parameters of Ca^{2+} transient. The resting Ca^{2+} levels of ΔE160cTnT-Tg mice were not significantly different from those of WTcTnT-Tg mice; EGCg had no significant effects on the resting Ca^{2+} levels.

Discussion

In this study, we found that ECg and EGCg, major polyphenols in green tea, are Ca^{2+} desensitizers that directly decrease the Ca^{2+} sensitivity of cardiac myofilaments. We have previously reported that EGCg induced amide chemical shift perturbations on several residues of cTnC in NMR spectroscopy (Figure S1) (Tadano *et al.*, 2005a,b). The residues T124, G125 and I128 of the FG loop and the C-terminal residue E161 in the C-lobe undergo significant chemical shift perturbations. GCg, a diastereomer of EGCg, did not induce the chemical shift perturbations on these residues of cTnC (data not shown).

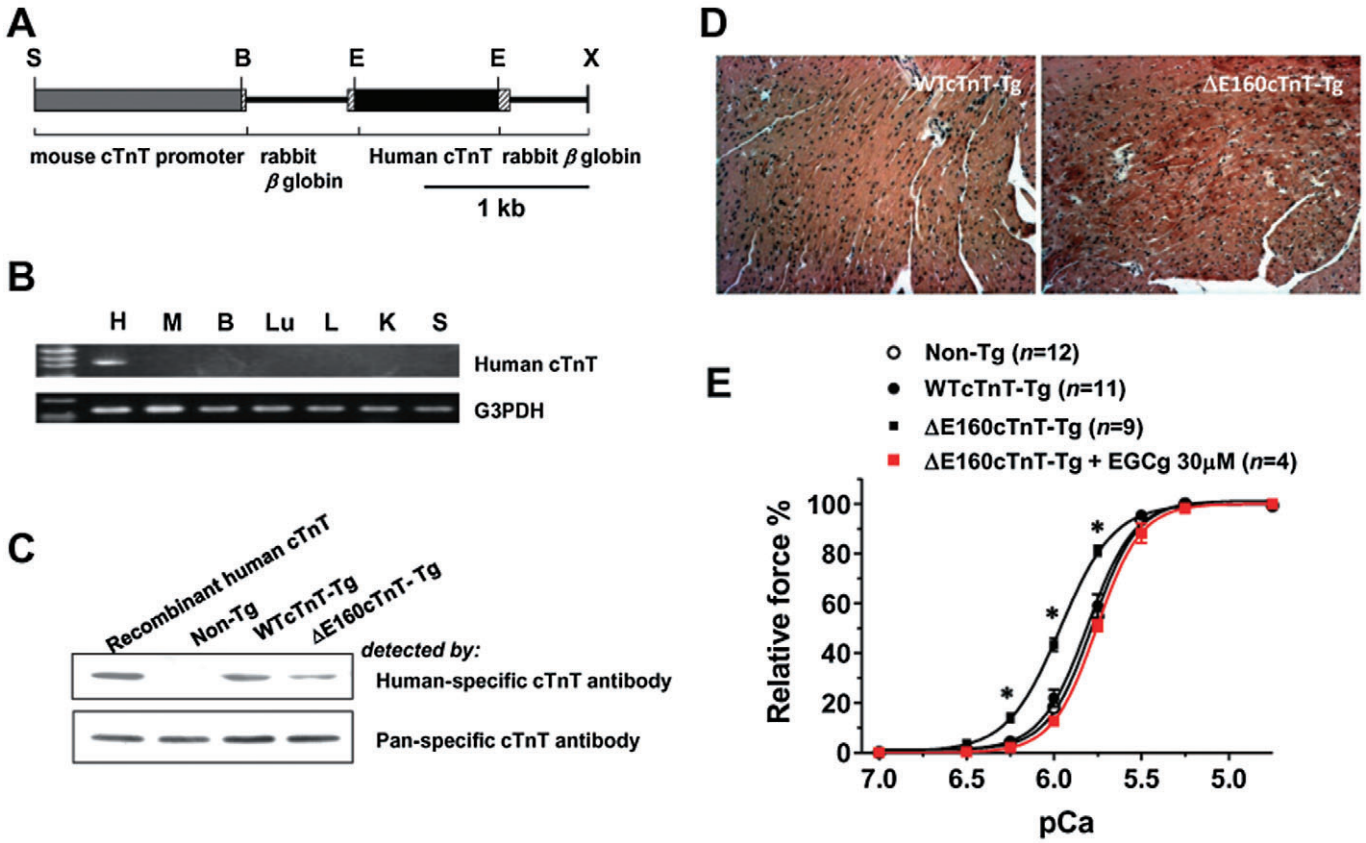

Figure 5

Creation of a transgenic mice model for hypertrophic cardiomyopathy. (A) The transgene used to generate the transgenic mice expressing the ΔE160 mutant cTnT. S, *Spe*I; B, *Bam*HI; E, *Eco*RI; X, *Xho*I. (B) RT-PCR analysis of total RNA extracted from various tissues (H, heart; M, skeletal muscle; B, brain; Lu, lung; L, liver; K, kidney; S, spleen) demonstrating heart-specific expression of the transgene. (C) Immunoblot analyses of the skinned cardiac muscle fibres. Human cTnT and total cTnT were detected using a monoclonal anti-human cTnT specific antibody (upper panel) and a monoclonal anti-pan specific TnT antibody (lower panel), respectively. Expression levels of human cTnT in WTcTnT-Tg and ΔE160cTnT-Tg mice were about 60 and 30%, respectively, as determined by using the density ratio of human cTnT to total cTnT for purified human cTnT as 100%. (D) Histology of hearts (HE staining) excised from 3 months old anesthetized Tg mice. (E) Force-pCa relationships in the skinned cardiac muscle fibres. The data represent the means ± SE of measurements on *n* fibers from different mice. $^*P < 0.05$ versus non- or WTcTnT-Tg mice (Tukey's multiple comparison test).

These results strongly suggest that EGCg binds to a region near the FG loop in the C-lobe, consistent with a recent NMR spectroscopic study using a C-terminal half peptide of cTnC (Robertson *et al.*, 2009), and also binds to a region near the C-terminus of cTnC in a stereospecific manner, both of which lie in a close vicinity to the binding site of the N-terminal H1 helix (residues 43–79) of cTnI (Figure S2). The present study, together with these previous NMR spectroscopic studies, strongly suggest that EGCg causes a Ca^{2+}-desensitization of cardiac myofilaments by enhancing the interaction of the H1 helix of cTnI with the C-lobe of cTnC through its stereospecific binding to the C-termininal regions of cTnC. Interestingly, a Ca^{2+} sensitizer EMD57033, which directly increases the Ca^{2+} sensitivity of cardiac myofilament, has been reported to disrupt the interaction of the N-terminal helix region of cTnI with the C-lobe of cTnC (Wang *et al.*, 2001), strongly suggesting that the stability of this interaction plays a critical role in determining the Ca^{2+} sensitivity of cardiac myofilaments.

A recent NMR spectroscopic study has reported that EGCg binds to the C-terminal half peptide of cTnC very weakly with a K_D of 0.4–1 mM (Robertson *et al.*, 2009). However, aromatic stacking of EGCg occurs in aqueous solution at high concentrations of EGCg (Kitano *et al.*, 1997; Wroblewski *et al.*, 2001), and this additional equilibrium would confound accurate K_D determination in NMR spectroscopy (Robertson *et al.*, 2009). In fact, a fluorescence spectroscopic study using low concentrations of EGCg shows that EGCg strongly binds to cTnC with a K_D of 3–4 μM (Liou *et al.*, 2008). The present QCM study also shows that EGCg binds to cTnC with a high affinity (K_D value 15 μM). A significant

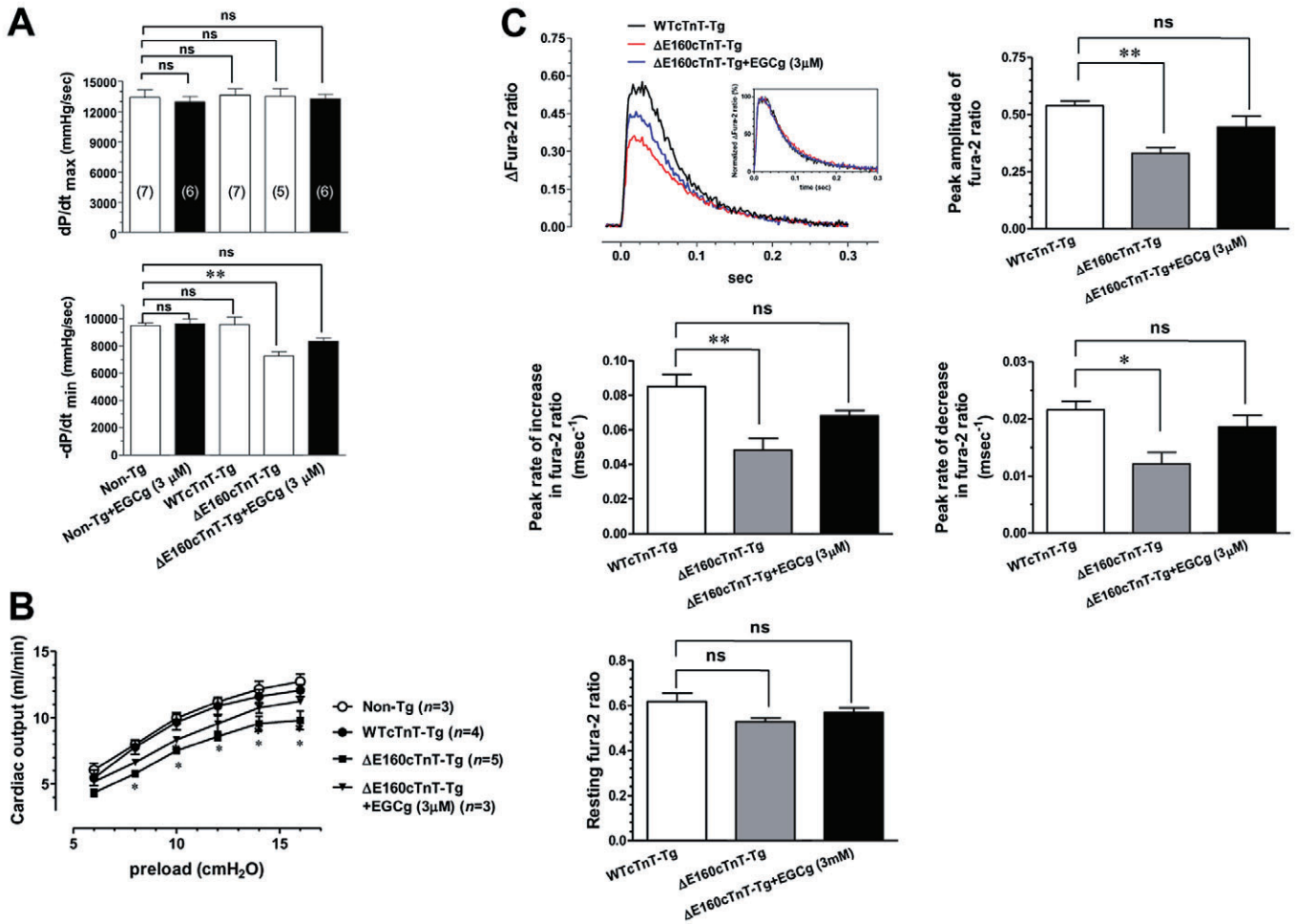

Figure 6

Effects of EGCg on cardiac muscle function of HCM mice. (A) Maximum and minimum derivatives of left ventricular pressure determined on working heart preparations. The data represent the means ± SE for the numbers of hearts indicated in parentheses. (B) Cardiac outputs from isolated working hearts of 2–3 months old mice. The data represent the means ± SE of *n* hearts. (C) Ca^{2+} transients induced by electrical stimulation at 3 Hz in left ventricular cardiomyocytes. The data represent the means ± SE of parameters determined on nine cardiomyocytes from three3 hearts. $^{**}P < 0.01$ versus non-Tg mice in panel A; $^{*}P < 0.05$, $^{**}P < 0.01$ versus WTcTnT-Tg mice in panels B and C (Dunnett's multiple comparison test). EGCg, (-)-Epigallocatechin-3-gallate; HCM, hypertrophic cardiomyopathy.

Ca^{2+}-desensitizing effect of EGCg, however, was only detected at above 30 μM on the skinned cardiac muscle fibres. Although the reason for this discrepancy remains to be elucidated, it should be noted that the effective concentrations of Ca^{2+}-sensitizers pimobendan and EMD57033 in skinned cardiac muscle fibres were also reported to be much higher than those estimated *in vivo* (Fujino *et al.*, 1988; Solaro *et al.*, 1993; Chu *et al.*, 1999), suggesting that drugs generally have much lower potency in skinned cardiac muscle preparations than *in vivo*. In the present study, a low concentration of EGCg (3 μM) improved the diastolic dysfunction of the hearts of ΔE160cTnT-Tg mice and increased their cardiac output. We found that the peak amplitude and the peak rates of increase and decrease in cytoplasmic Ca^{2+} were significantly decreased in the cardiomyocytes of ΔE160cTnT-Tg mice, irrespective of the fact that the hearts of these mice showed no significantly altered myocardial contractility assessed by the left ventricular dP/dt_{max}. These findings suggest that sarcoplasmic reticulum (SR) function in ΔE160cTnT-Tg mice might be reduced to suppress an enhanced contractility expected to be caused by increased myofilament Ca^{2+} sensitivity at the cost of retardation of relaxation, in an opposite manner to the case we have demonstrated in a mouse model of dilated cardiomyopathy caused by decreased myofilament Ca^{2+} sensitivity (Du *et al.*, 2007). We also found that EGCg increased the Ca^{2+} transient in ΔE160cTnT-Tg mice without changing the myocardial contractility assessed by the left ventricular dP/dt_{max} and improved the diastolic dysfunction without changing the resting Ca^{2+} level.

These results suggest that EGCg restores the impaired cardiac pump function due to diastolic dysfunction by reversing the increased Ca^{2+} sensitivity of cardiac myofilaments. Although further studies are needed to see if EGCg has any direct effect on SR function, the enhancement of Ca^{2+} transients by EGCg should be at least partly due to a decrease in sarcomeric Ca^{2+} buffering that could be caused by Ca^{2+} desensitizing effect of EGCg on cTnC.

HCM is an inherited cardiac disease with a high prevalence and is the main cause of sudden death of young adults, with no therapy being currently established. Many mutations in genes for sarcomeric proteins have been found to cause HCM (Harada and Morimoto, 2004; Marian, 2005), and increased Ca^{2+} sensitivity of cardiac myofilaments is found to be a primary cause for the pathogenesis of HCM, at least those forms associated with the mutations in the regulatory proteins cTnT, cTnI, cTnC and α-tropomyosin (Ahmad *et al.*, 2005). To our knowledge, EGCg and ECg are the first chemical compounds that could ameliorate diastolic dysfunction of HCM, at least partially, through their direct Ca^{2+}-desensitizing effects on cardiac myofilament. The present study suggests that EGCg or ECg might be a useful material or lead compound for development of therapeutic agents to treat the inherited HCM caused by increased myofilament Ca^{2+} sensitivity.

Acknowledgements

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Conflict of interest

None declared.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 NMR spectroscopy of Ca^{2+}-cTnC-EGCg complex. Upper panel: Superposition of TROSY-type ^{15}N-^{1}H HSQC spectra of ^{15}N-labeled human Ca^{2+}-cTnC, free and in complex with EGCg. The Ca^{2+}-cTnC/EGCg ratio is 1:5. The cross-peaks of free Ca^{2+}-cTnCare shown in blue, and those of Ca^{2+}-cTnC in complex with EGCg are shown in red. Lower panel: Chemical shift perturbations due to EGCg binding to Ca^{2+}-cTnC. Horizontal solid and dotted lines show the average chemical shift throughout cTnC and standard deviation (SD), respectively. Chemical shift changes outside 2.5 SD are considered significant according to Van Selst and Jolicoeur (1994) and labelled with asterisk.
Figure S2 EGCg binding sites mapped on the crystal structure of troponin core domain complex (PDB: 1J1D) (Van Selst and Jolicoeur, 1994). The cTnIN-teminalhelix (residues 42–62) is colored cyan. The other regions of cTnIare colored blue. $cTnT_2$ is colored red. The figures were generated by PyMOL™, Molecular Graphics System, Version 0.97.

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