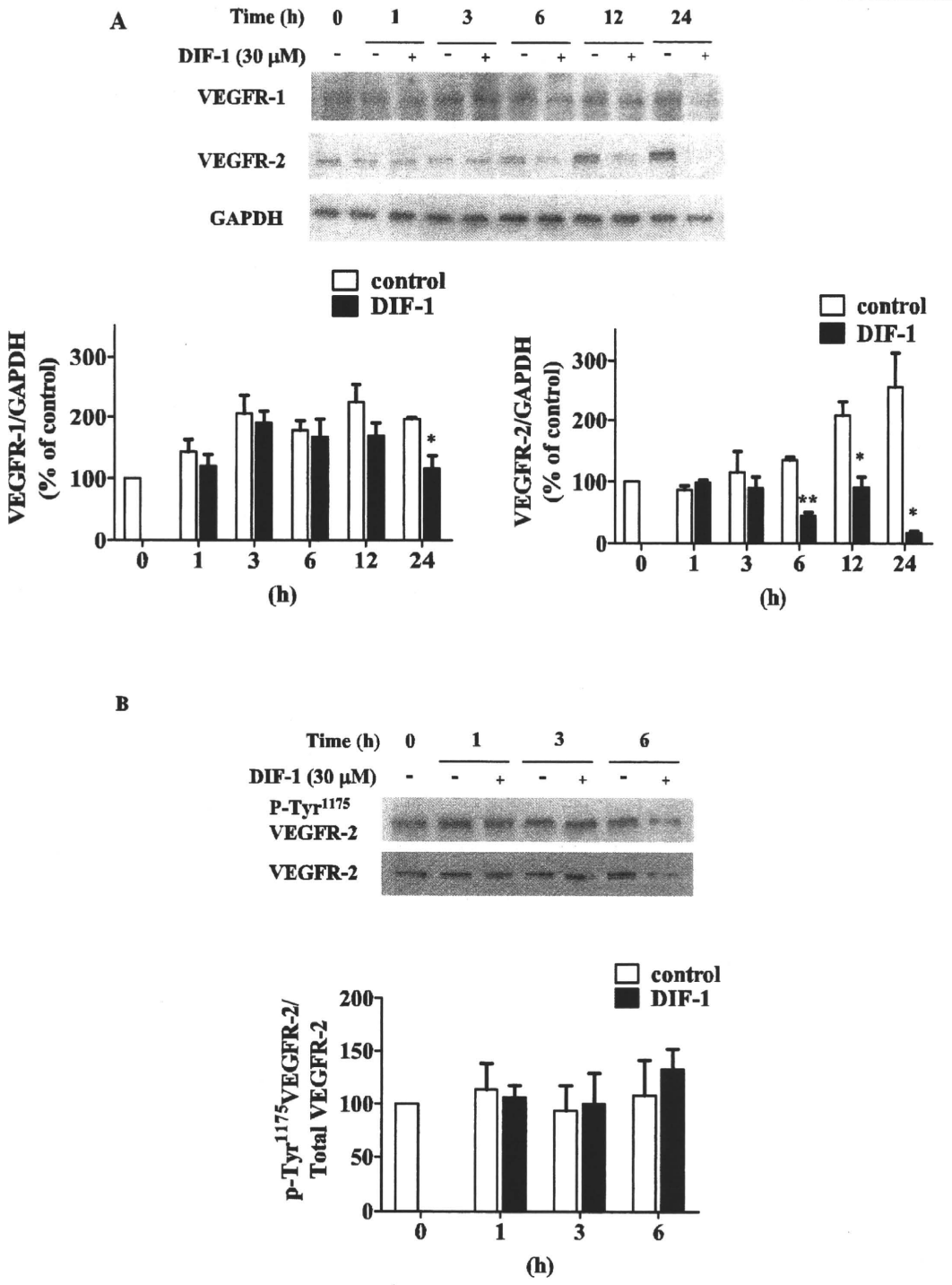

Figure 6 The effects of DIF-1 on VEGF receptors, VEGFR-1 and VEGFR-2. (A) DIF-1 decreased VEGFR-1 and VEGFR-2 expression in HUVECs. HUVECs were incubated with or without DIF-1 (30 μM) for the periods indicated and the samples were subjected to Western blot analysis for VEGFR-1 and VEGFR-2. The levels of protein bands were quantified and are shown as percentage of the control level at time 0. Values are mean ± SE of three independent experiments. The asterisk indicates *P < 0.05 **P < 0.001 versus control. (B) DIF-1 did not affect phosphorylation level of VEGFR-2. HUVECs were incubated with or without DIF-1 (30 μM) for the periods indicated and the samples were subjected to Western blot analysis using anti-phospho-VEGFR-2 (Tyr^{1175}) antibody. The membrane was re-probed with anti-VEGFR-2 antibody. The levels of protein bands were quantified and are shown as percentage of the control level at time 0. Values are mean ± SE of three independent experiments.

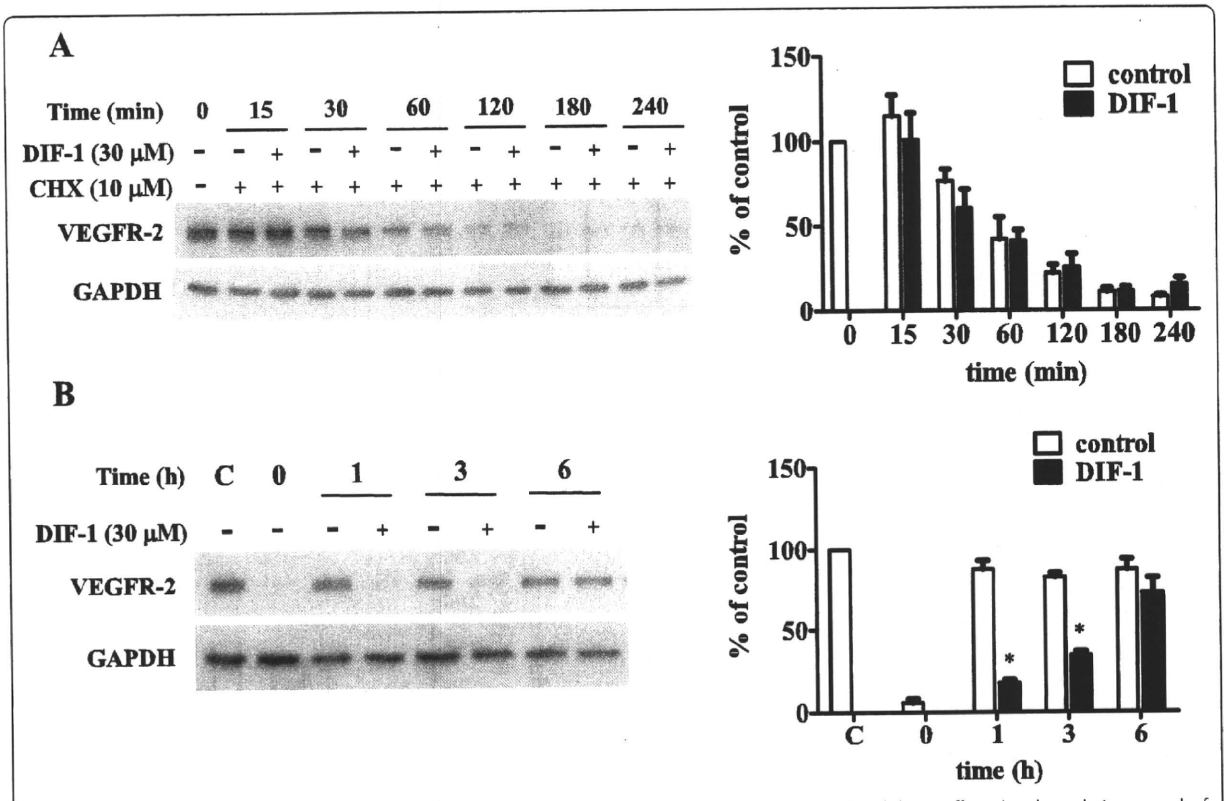

Figure 7 The effects of DIF-1 on the proteolysis and the proteosynthesis of VEGFR-2. (A) DIF-1 did not affect the degradation speed of VEGFR-2. HUVECs were incubated with or without DIF-1 (30 μM) for the given periods in the presence of cycloheximide (10 μM). Protein samples were subjected to Western blot analysis for VEGFR-2. The membrane was re-probed with anti-GAPDH antibody. The levels of protein bands were quantified and are shown as percentage of the control level at time 0. Values are mean ± SE of three independent experiments. (B) DIF-1 attenuated the protein synthesis of VEGFR-2. After HUVECs were pretreated with cycloheximide (10 μM) for 3 h, the medium was changed to fresh medium and cells were incubated with or without DIF-1 (30 μM) for the given periods. Protein samples were subjected to Western blot analysis for VEGFR-2. The membrane was re-probed with anti-GAPDH antibody. The levels of protein bands were quantified and are shown as percentage of the control cells which were not treated with cycloheximide (lane 1). Values are mean ± SE of three independent experiments. The asterisk indicates *P < 0.001 versus control.

protein quantity suppression). Although we could not explain this difference at present, the short half-life of VEGFR-2 protein of about 1 h [35] could be associated with this phenomenon. In other words, as the proteolysis of VEGFR-2 is quick and rapid synthesis is required to restore VEGFR-2, even weak inhibition of promoter activity may significantly affect the quantity of VEGFR-2 protein.

Since we have shown that DIF-1 inhibits the Wnt/β-catenin signaling pathway in various cells, the effects of DIF-1 on the Wnt/β-catenin signaling pathway in HUVECs were examined. We found that DIF-1 also inhibited this signaling pathway via GSK-3β activation in HUVECs. Although the Wnt/β-catenin signaling pathway has been reported to be important to promote angiogenesis *in vitro* [24-28], the role of Wnt/β-catenin signaling pathway in endothelial cells and angiogenesis is controversial. Cheng *et al.* reported that Wnt1 signaling inhibits HUVEC proliferation [36]. On the other hand, it has been reported that Wnt1 and 3a mediated induction of VEGFR-2 (Quek-1) expression during avian somite development [37]. In this study, we showed that Wnt3a slightly but significantly reduced promoter activity and VEGFR-2 protein expression. Therefore, suppression of VEGFR-2 expression induced by DIF-1 may not be due to suppression of the Wnt/β-catenin signaling pathway. Our results might suggest that activation of the Wnt/β-catenin signaling pathway suppressed the promotion of angiogenesis. However, Samarzija *et al.* showed that although Wnt3a stimulated HUVEC proliferation and migration independent of VEGFR signaling [38]. Therefore, further studies are needed to elucidate the relationship between the Wnt/β-catenin signaling pathway and angiogenesis.

Cyclin D1 plays a key role in the initiation and progression of the G_1 phase [39]. We previously showed

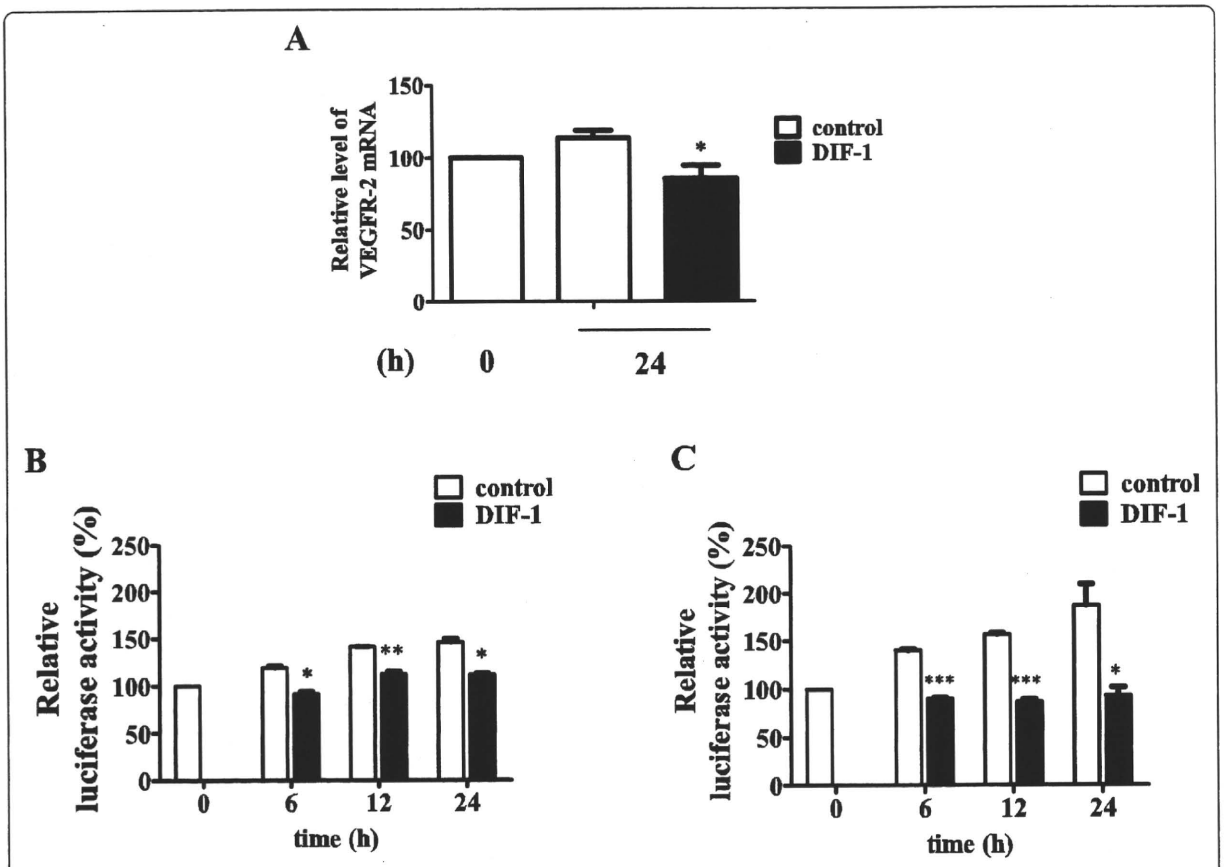

Figure 8 DIF-1 reduced VEGFR-2 mRNA level and promoter activity. (A) The effects of DIF-1 on the VEGFR-2 mRNA levels. Total RNAs were extracted from HUVECs treated with or without DIF-1 (30 μM) for 24 h. The VEGFR-2 mRNA levels were determined by TaqMan quantitative real-time RT-PCR. Values are mean ± SE of four independent experiments. The asterisk indicates *P < 0.05 versus control. (B and C) The effects of DIF-1 on the human VEGFR-2 gene promoter activity on HUVECs (B) or BAECs (C). HUVECs or BAECs were co-transfected with VEGFR-2 pGL-3 and pRL-SV40. After 24 h incubation, HUVECs and BAECs were stimulated with or without DIF-1 (30 μM) for the given periods. Luciferase activity is shown as percentages of the control level at time 0. Values are mean ± SE of three independent experiments. The asterisk indicates *P < 0.01 **P < 0.001 ***P < 0.0001 versus control.

that DIF-1 and DIF-3 reduced cyclin D1 quantity and induced cell cycle arrest in G_0/G_1 phase using various mammalian cells [19,21,22]. In this study, we also demonstrated that DIF-1 inhibited HUVECs proliferation and induced restriction of cell cycle in the G_0/G_1 phase by degrading cyclin D1. This result is consistent with that published in our previous reports, and indicates that cyclin D1 also plays an important role in HUVEC proliferation. Furthermore, it has been reported that antisense to cyclin D1 inhibited tumor-associated neovascularization [40]. As such, suppression of cyclin D1 expression may be one of the anti-angiogenesis mechanisms induced by DIF-1.

Conclusions

In summary, we found that DIF-1 reduced the expression of cyclin D1 and VEGFR-2 in HUVECs. The reduction of cyclin D1 and VEGFR-2 expression may inhibit proliferation, and reduction of VEGFR-2 may cause inhibition of migration and tube formation. These effects may explain the powerful anti-angiogenic properties of DIF-1.

We previously reported that DIF-1 showed anti-tumor activity by inhibiting cyclin D1 expression and the Wnt/β-catenin signaling pathway. In addition to these effects, this study demonstrated that DIF-1 also exhibited anti-angiogenic effects independent of the Wnt/β-catenin signaling pathway. Elucidation of the target molecule of DIF-1 will facilitate the development of potent novel anti-tumor agents which suppresses not only the Wnt/β-catenin signaling pathway but also angiogenesis.

Methods

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were purchased from DS Pharma Biomedical (Osaka, Japan).

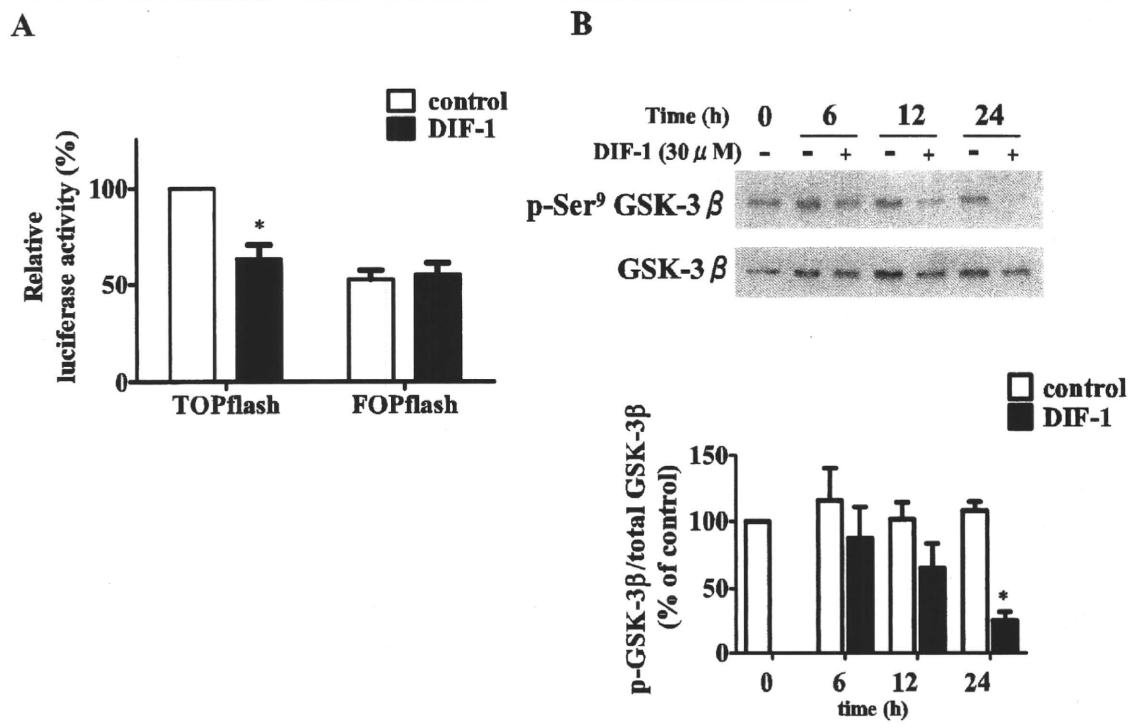

Figure 9 DIF-1 suppressed Wnt/β-catenin signaling pathway in HUVECs. (A) Effect of DIF-1 on TCF transcriptional activity. TOPflash or FOPflash was co-transfected with pRL-SV40 into HUVECs. After 24 h incubation, HUVECs were stimulated with or without DIF-1 (30 μM) for 24 h. Luciferase activity is shown as percentages of the control level. Values are mean ± SE of three independent experiments. The asterisk indicates *P < 0.01 versus control. (B) DIF-1 activated GSK-3β. HUVECs were incubated with or without DIF-1 (30 μM) for given periods. Protein samples were subjected to Western blot analysis for phosphorylation level of Ser^9 on GSK-3β. The membrane was reprobed with the anti-GSK-3β antibody. The levels of protein bands were quantified and are shown as percentage of the control level at time 0. Values are mean ± SE of three independent experiments. The asterisk indicates *P < 0.0001 versus control.

The cells were grown in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St Louis, Mo, USA) supplemented with 20% fetal bovine serum, 5 ng/ml (0.29 nM) recombinant human basic fibroblast growth factor (PeproTech, Rocky Hill, NJ, USA), 100 units/ml penicillin G, and 100 μg/ml streptomycin using 0.1% gelatin coated dishes. HeLa cells (human cervical carcinoma cell line) and bovine aortic endothelial cells (BAECs) were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich) supplemented with 10% fetal bovine serum, 100 units/ml penicillin G and 100 μg/ml streptomycin.

Reagents and antibodies

DIF-1 (1-(3,5-dichloro-2, 6-dihydroxy-4-methoxyphenyl)-1-hexanone) was synthesized as described previously [41]. MG132 was obtained from the Peptide Institute (Osaka, Japan). Cycloheximide was obtained from Sigma-Aldrich. Polyclonal anti-cyclin D1 antibody, polyclonal anti-PECAM-1 (CD31) antibody and the polyclonal anti-VEGFR-1/Flt-1 antibody were purchased from Santa Cruz Biotechnology (CA, USA). Monoclonal anti-VEGFR-2 antibody and the monoclonal anti-phospho-VEGFR-2 (Tyr^{1175}) antibody were from Cell Signaling Technology (Danvers, MA, USA). The monoclonal GAPDH antibody was obtained from Abcam (Cambridge, MA, USA). Growth factor reduced Matrigel was obtained from BD Biosciences (San Jose, CA, USA). TOPflash (TCF reporter plasmid) and FOPflash (negative control of TOPflash) were purchased from Upstate Biotechnology (Lake Placid, NY, USA). Human Wnt3a was from R&D Systems (Minneapolis, MN, USA).

Cell proliferation assay

The cells were plated on 24-well plates (1.0×10^4 cells/well) and treated with or without various amounts of DIF-1 for defined periods. Cells were harvested by trypsin/EDTA treatment and enumerated using Coulter Counter (Beckman Coulter, Brea, CA, USA).

Flow Cytometry

Cells harvested by trypsin/EDTA treatment were suspended in hypotonic fluorochrome solution containing 50 μg/ml of propidium iodide, 0.1% sodium citrate, and 0.1% Triton X-100. Cells (5×10^3) for each sample were

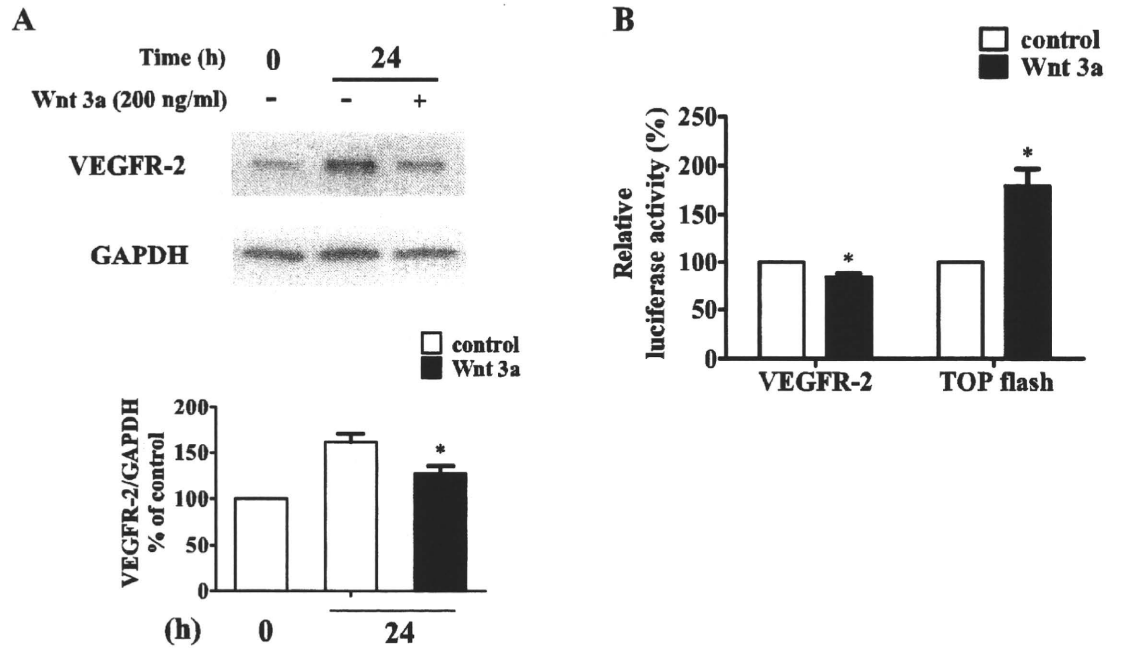

Figure 10 Effects of Wnt3a on VEGFR-2. (A) Wnt3a suppressed VEGFR-2 expression. HUVECs were incubated with or without Wnt3a (200 ng/ml = 5.35 nM) for 24 h and the samples were subjected to Western blot analysis for VEGFR-2. The levels of protein bands were quantified and are shown as percentage of the control level at time 0. Values are mean ± SE of three independent experiments. The asterisk indicates *P < 0.05 versus control. (B) Wnt3a suppressed the VEGFR-2 promoter activity. HUVECs were co-transfected with VEGFR-2 pGL-3 or TOPflash and pRL-SV40. After 24 h incubation, cells were stimulated with or without Wnt3a (200 ng/ml) for 12 h. Luciferase activity is shown as percentages of the control. Values are mean ± SE of three independent experiments. The asterisk indicates *P < 0.05 versus control.

analyzed for fluorescence by a Becton-Dickinson FACScalibur (Franklin Lakes, NJ, USA).

Western blotting

Samples were separated by 10 or 12% SDS-PAGE and transferred to a polyvinylidene difluoride membrane using a semidry transfer system (1 h, 12 V). After blocking with 5% skim milk, the membrane was probed with a first antibody. Incubation was carried out overnight at 4°C. The membrane was then washed three times and incubated with horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG (Bio-Rad, Hercules, CA, USA) for 1 h. Immunoreactive proteins on the membrane were visualized by treatment with a detection reagent (LumiGLO, Cell Signaling Technology). Optical densitometric scan was performed using NIH Image J software.

Tube formation assay

Tube formation assay was performed as previously described [42] with slight modification. Briefly, Matrigel was thawed at 4°C and 250 μl of the solution were added to each well in a 24-well plate and formed a gel at 37°C for 30 min. HUVECs were suspended at 3×10^4 cells in 500 μl of 3% FBS with or without 30 μM DIF-1, and then added to each well. After 8 h-incubation, the degree of tube formation was determined by counting the number of areas surrounded by tubes contained in 10 random fields, and expressed as mean ± SE.

Cell migration assay

The effect of DIF-1 treatment on *in vitro* migration of HUVECs was determined using a Boyden Chamber [43]. The PET membrane (8 μm pore size, Greiner Bio-One, Frickenhausen, Germany) was pre-coated with 10 μg of Matrigel. HUVECs were suspended at 5×10^4 cells in 100 μl of serum free DMEM with or without 30 μM DIF-1 and seeded into the upper part of each chamber, whereas the lower compartments were filled with 600 μl of DMEM supplemented with 0.1% bovine serum albumin and 20 ng/ml (0.52 nM) VEGF. After incubation for 10 h at 37°C, non-migrated cells were scraped off with a cotton swab. Migrated cells on the lower surface of the membrane were fixed with 1% glutaraldehyde for 10 min and stained with 4% crystal violet for 30 min. HUVEC migration was quantified by counting the number of cells in ten random fields per membrane. Data are expressed as mean ± SE of cells/fields.

In vivo mouse Matrigel-plug assay

In vivo angiogenesis was assayed as growth of blood vessels from mouse subcutaneous tissue into the exogenous Matrigel plug induced by VEGF or tumor cells [43]. For the analysis of VEGF-induced angiogenesis, Matrigel was prepared with 100 ng/ml (2.62 nM) VEGF, 20 units/ml heparin in the presence or absence of 30 μM DIF-1 at 4°C. The liquid Matrigel was injected (final volume; 500 μl) into the flanks of C57BL/6 mice (5~7 weeks, n = 7 for each group) using a cold syringe and allowed to polymerize into a solid gel by body temperature. Seven days later, Matrigel plugs were extracted and samples were prepared for immunohistochemical analysis. To analysis for tumor-induced angiogenesis, 1×10^6 HeLa cells were mixed with liquid Matrigel in the presence or absence of 30 μM DIF-1 (final volume; 500 μl). The mixture was injected subcutaneously in the flanks of 6 week-old nude mice (Kyudo, Saga, Japan). Two weeks later, the tumors were removed and samples were prepared for Western blot and immunohistochemical analyses. The handling and sacrificing of all animals were carried out in accordance with nationally prescribed guidelines, and ethical approval for studies was granted by the Animal Care and Use Committee of Kyushu University.

Immunohistochemical analysis

The removed Matrigel plugs and tumors were fixed in 10% buffered formalin followed by embedding in paraffin. Sections were then stained with hematoxylin-eosin staining and immunofluorescence staining. For immunofluorescence staining, primary PECAM-1/CD31 antibody (1:50 dilution) was applied to the sections and the slides were incubated overnight at 4°C. The secondary antibody (Histofine, Nichirei, Tokyo, Japan) was applied to the sections and incubated for 1 h. The slides were subsequently incubated with streptavidin-FITC (Invitrogen, Carlsbad, CA, USA) and the fluorescence strength was analyzed with Biozero fluorescence microscopy (Keyence, Osaka, Japan).

Real-time quantitative reverse transcriptase-polymerase chain reaction

Total RNAs were extracted from HUVECs using TRIzol (Invitrogen) and SV total RNA isolation system (Promega, Madison, WI, USA). First-strand cDNAs were synthesized from 2 μg of total RNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA, USA). The 100 ng cDNA products were used for quantitative real-time PCR performed using TaqMan Universal PCR Master Mix (Applied Biosystems) and TaqMan MGB primers [VEGFR-2 (Hs00911700_m1) and GAPDH (Hs99999905_m1)] with an ABI Prism 7500 (Applied Biosystems). The following PCR conditions were used: 50°C for 2 minutes, then 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. Cycle threshold (C_T) values for each gene were obtained for each sample. Differences in C_T values between VEGFR-2 gene and endogenous control (GAPDH) were calculated and used for statistical analyses.

Construction of reporter plasmid

The 5'-flanking region of human VEGFR-2 [44] was amplified and cloned into PCR 2.1 (Invitrogen) for DNA sequencing. After confirming the sequence, DNA fragments (-1003/-48 bp relative to the transcription start site) were excised with *Sac*I and *Bgl*II and cloned into pGL3-Basic vectors (Promega).

Luciferase reporter gene assay

Cells were transiently transfected with plasmid DNA (TOPflash, FOPflash orVEGFR-2/pGL-3) and pRL-SV40, a Renilla luciferase expression plasmid (Promega) to control transfection efficacy, using Superfect reagent (Qiagen, Hilden, Germany). To measure luciferase activities, Dual-luciferase Reporter Assay (Promega) and a luminometer (Lumat LB 9507; Berthold Technologies, Bad Wildbad, Germany) were used. Firefly luciferase activities were normalized to that of Renilla luciferase.

Statistics

The results are expressed as mean ± SE. Statistical analysis of the differences between values were conducted using the Student's *t*-test or the one-way ANOVA with Bonferroni post-hoc tests (GraphPad Prism 5.0, GraphPad Software, La Jolla, CA, USA). A P value < 0.05 was considered statistically significant.

Abbreviations

HUVEC: human umbilical vein endothelial cell; BAEC: bovine aortic endothelial cell; VEGF: vascular endothelial growth factor; VEGFR: vascular endothelial growth factor receptor; DIF: differentiation-inducing factor; GSK-3β: glycogen synthase kinase-3β

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Authors' contributions
TY contributed to the major part of experimental work, analyzed and interpreted data, performed the statistics and drafted the manuscript. FT conceived the study, participated in its design and data analysis, and contributed with scientific discussion and manuscript preparation. FS contributed the production of reporter plasmid. YW provided DIF-1. SM, MH and SH interpreted data and contributed with scientific discussion. TS supervised the project and helped draft the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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Therapeutic effect of β-adrenoceptor blockers using a mouse model of dilated cardiomyopathy with a troponin mutation

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Dilated cardiomyopathy;
β-Blocker;
Ventricular fibrillation;
Sudden death;
Survival

Aims Extensive clinical studies have demonstrated that β-adrenoceptor blocking agents (β-blockers) are beneficial in the treatment of chronic heart failure, which is due to various aetiologies, including idiopathic dilated cardiomyopathy (DCM) and ischaemic heart disease. However, little is known about the therapeutic efficacy of β-blockers in the treatment of the inherited form of DCM, of which causative mutations have recently been identified in various genes, including those encoding cardiac sarcomeric proteins. Using a mouse model of inherited DCM with a troponin mutation, we aim to study the treatment benefits of β-blockers.

Methods and results Three different types of β-blockers, carvedilol, metoprolol, and atenolol, were orally administered to a knock-in mouse model of inherited DCM with a deletion mutation ΔK210 in the cardiac troponin T gene (*TNNT2*). Therapeutic effects were examined on the basis of survival and myocardial remodelling. The lipophilic β_1-selective β-blocker metoprolol was found to prevent cardiac dysfunction and remodelling and extend the survival of knock-in mice. Conversely, both the non-selective β-blocker carvedilol and the hydrophilic β_1-selective β-blocker atenolol had no beneficial effects on survival and myocardial remodelling in this mouse model of inherited DCM.

Conclusion The highly lipophilic β_1-selective β-blocker metoprolol, known to prevent ventricular fibrillation via central nervous system-mediated vagal activation, may be especially beneficial to DCM patients showing a family history of frequent sudden cardiac death, such as those with a deletion mutation ΔK210 in the *TNNT2* gene.

1. Introduction

Dilated cardiomyopathy (DCM) is a cardiac muscle disorder characterized by ventricular chamber dilation and systolic dysfunction, which often leads to sudden death and heart failure.[1–3] Although DCM had been known to result from non-genetic insults, such as viruses, alcohol, toxins, and immunological injury, recent studies have revealed that mutations in genes for sarcolemmal transmembrane proteins, cytoskeletal proteins, nuclear envelope proteins, and sarcomeric proteins are also important causes of DCM.[4,5]

Patients affected by a deletion mutation ΔK210 in the cardiac troponin T gene (*TNNT2*), which has so far been identified in four unrelated families,[6–8] show a severe early-onset phenotype with a high incidence of sudden death and/or heart failure death. Recently, we created a knock-in mouse model of DCM using this mutation.[9] The knock-in mice developed markedly enlarged hearts with left ventricular (LV) systolic dysfunction and frequent sudden cardiac death, closely recapitulating the clinical phenotypes of human patients.

Several uncontrolled clinical studies provided the first evidence that metoprolol, a β_1-selective β-adrenoceptor blocking agent (β-blocker), had a beneficial clinical and haemodynamic effect in patients with heart failure due to idiopathic DCM.[10–13] Subsequent controlled clinical studies showed that metoprolol[14–20] and carvedilol, a non-selective β-blocker,[21–23] were both consistently beneficial to patients with symptomatic chronic heart failure due to idiopathic DCM or ischaemic heart disease. Comparisons between the effects of metoprolol and carvedilol therapy on cardiac performance reported that these two agents had parallel

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beneficial effects in patients with chronic heart failure mostly due to ischaemic heart disease or idiopathic DCM.[24] Carvedilol was also later reported to have more beneficial effects in patients exclusively with idiopathic DCM.[25] The COMET study showed that carvedilol had a greater beneficial effect on survival than metoprolol when used to treat chronic heart failure mostly due to ischaemic heart disease or idiopathic DCM.[26]

In the present study, we sought to explore the therapeutic effects of metoprolol and carvedilol, as well as a low-lipophilic β-blocker, atenolol, on inherited DCM caused by a deletion mutation ΔK210 in the *TNNT2* gene, using a knock-in mouse model. We found that the highly lipophilic β_1-selective β-blocker metoprolol, but not the lipophilic non-selective β-blocker carvedilol or the hydrophilic β_1-selective β-blocker atenolol, was able to extend survival in DCM mice and prevent cardiac remodelling and dysfunction. This may be due to the blockade of central nervous β_1-adrenoceptors modulating vagal nervous outflow.

2. Methods

2.1 Animal model

A knock-in mouse model, in which three base-pairs coding for K210 in cTnT were deleted from the endogenous gene *TNNT2*, was created as described previously.[9] Homozygous mutant mice were used for the DCM model. The investigation conforms with 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.

2.2 Drug administration

Carvedilol was supplied by Nippon DAIICHI SANKYO Co., Ltd (Japan). (±)-Metoprolol (+)-tartrate salt was purchased from Sigma (USA). Atenolol was purchased from Wako Pure Chemical Industries, Ltd (Japan). From 30 days of age, metoprolol, carvedilol, or atenolol, suspended in 0.25% methylcellulose solution, was administered orally once daily to DCM mice; control mice received vehicles only. The doses for each of the drugs were selected on the basis of a comparable suppression of heart rate.

2.3 Electrocardiography, echocardiography, and blood pressure measurements

Surface electrocardiography (ECG) (standard limb lead II) and transthoracic echocardiography (M-mode) were measured after sodium pentobarbital administration (50 mg/kg, i.p.) using, respectively, an ECG processor SP-2000 (Softron, Japan) and a 14 MHz linear array probe with a diagnostic ultrasound system, Nemio SSA-550A (Toshiba, Japan). Blood pressure was measured in conscious mice using a computerized non-invasive tail-cuff system BP-98A (Softron, Japan).

2.4 Histochemistry

The hearts, excised from mice anaesthetized with pentobarbital (50 mg/kg, i.p.), were fixed in a 10% formalin neutral buffered solution after perfusion with oxygenated Krebs-Henseleit solution (118 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4, 1.2 mM KH2PO4, 25 mM NaHCO3, 2.5 mM CaCl2, 0.5 mM EDTA-Na2, 10 mM HEPES, 11 mM D-glucose) containing 50 mM 2,3-butanedione monoxime in a Langendorff mode at 37°C. Fixed hearts were cut transversely at the mid-ventricular level, embedded in paraffin, sectioned at 5 μm, and stained with azan. The extent of fibrosis in LV myocardium was quantified using the ImageJ program from NIH for three cardiac sections from each mouse.

2.5 Respiratory sinus arrhythmia

The trachea was cannulated under light anaesthesia with ether, and the mice were mechanically ventilated with 100% room air (tidal volume 0.5 mL, 120 inflations/min). At 10 min after the administration of D-tubocurarine (3 mg/kg, i.p.), surface ECG (standard limb lead II) was recorded three times for 20 s every 5 min under unanaesthetized conditions. The standard deviation of the R-R intervals per respiratory cycle (SD R-R), as an index of vagal activity,[27,28] was calculated on the basis of the mean of 120 respiratory cycles in each mouse.

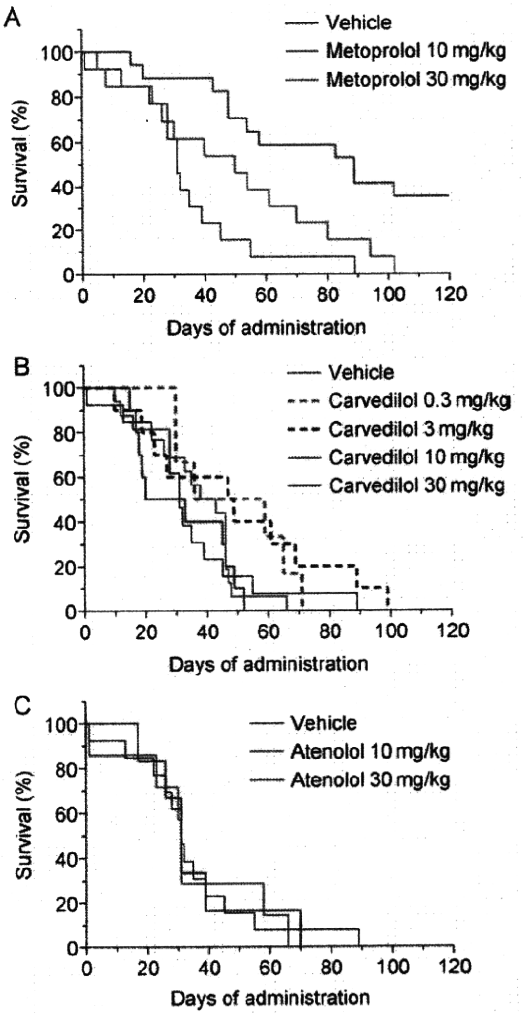

Figure 1 Effects of β-blockers on the survival of a mouse model of inherited DCM caused by a deletion mutation ΔK210 in the *TNNT2* gene. From 30 days of age, metoprolol (10 mg/kg, $n = 13$; 30 mg/kg, $n = 17$) (*A*), carvedilol (0.3 mg/kg, $n = 6$; 3 mg/kg, $n = 10$; 10 mg/kg, $n = 16$; 30 mg/kg, $n = 10$) (*B*) and atenolol (10 mg/kg, $n = 7$; 30 mg/kg, $n = 6$) (*C*), and vehicle only (methylcellulose, $n = 13$) were administered to DCM mice orally once daily. Kaplan–Meier survival curves indicate that mice treated with metoprolol at 30 mg/kg per day have significantly longer life spans than mice treated with vehicle only (log-rank test, $P = 0.0002$).

2.6 Western blot analysis

After a brief perfusion of the isolated heart with oxygenated Krebs-Henseleit solution at 37°C in a Langendorff mode to remove blood from the myocardium, ventricles were dissected from the heart, blotted on filter paper, and homogenized in Laemmli's sample buffer. LV homogenate samples were subjected to western blot analysis as described previously.[29] Expression levels of brain natriuretic peptide (BNP) were determined using an anti-proBNP polyclonal antibody (ab32842; Abcam) and an anti-GAPDH monoclonal antibody (ab9484; Abcam). Signals were visualized using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) and Hyperfilm ECL (GE Healthcare) and were quantified using GAPDH signal as a protein loading control. Similarly, relative phosphorylation levels of phospholamban (PLB) were obtained by normalizing the signals of the PLB band, probed with anti-phospho-PLB-Ser16 polyclonal antibody (ab15000; Abcam), to that probed with anti-PLB monoclonal antibody (ab2865; Abcam).

2.7 Myosin isoform contents

Myosin heavy chain (MyHC) isoforms in the LV myocardium were separated on SDS-PAGE according to the method outlined in Rundell *et al.*,[30] and relative β-isoform expression (percentage of total MyHC) was determined by an optical densitometric scan using Phoretix gel analysis software (Phoretix International, UK).

3. Results

DCM mice with a deletion mutation ΔK210 in the *TNNT2* gene show a very high mortality due to sudden cardiac death without showing overt heart failure symptoms, such as decreased spontaneous movement activity and dyspnoea, up to, at least, a day before their death.[9] Oral administration of the β_1-selective β-blocker metoprolol (10–30 mg/kg per day) was found to extend the survival of

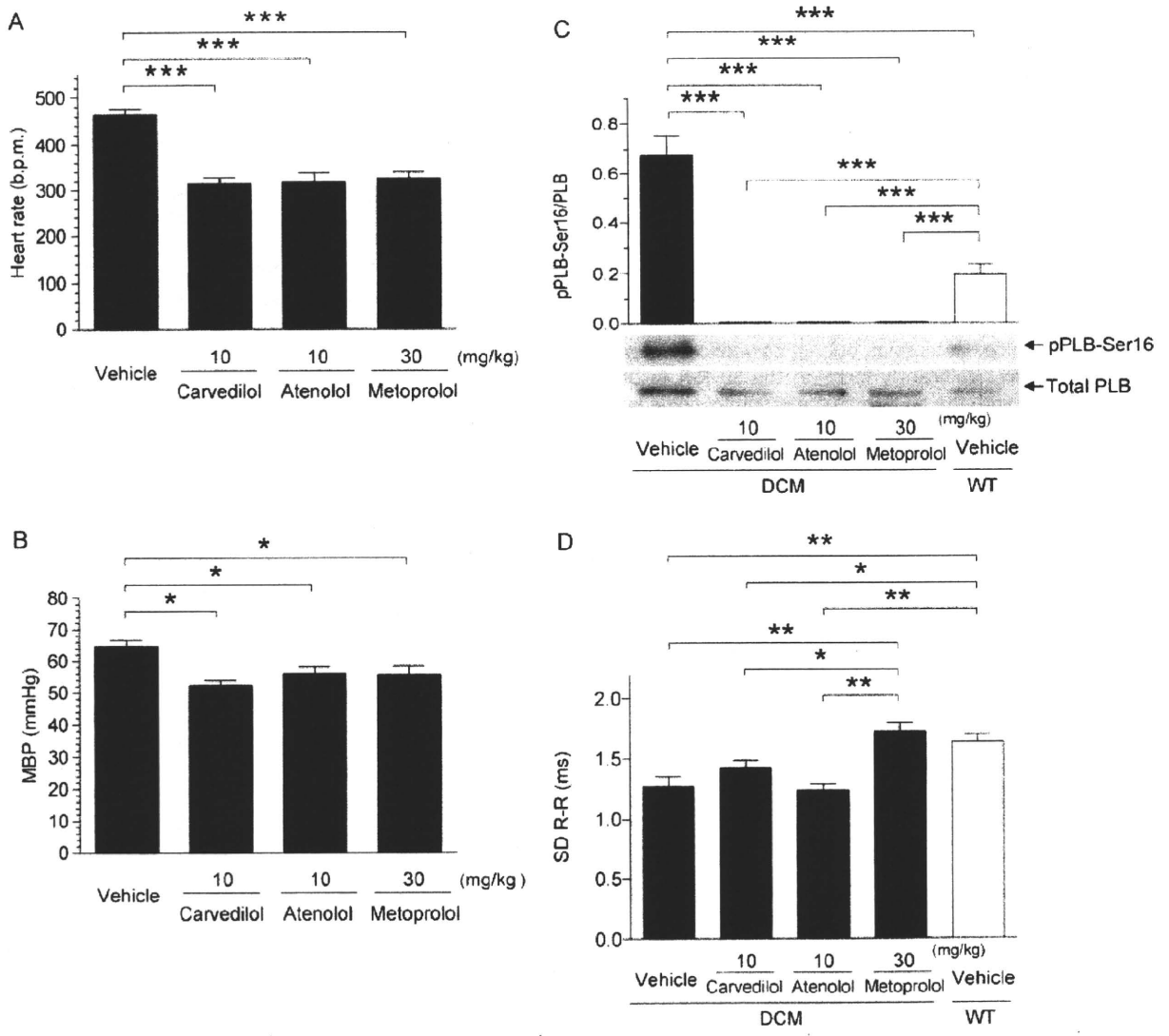

Figure 2 Effects of β-blockers on cardiac autonomic nervous activity in DCM mice with a deletion mutation ΔK210 in the *TNNT2* gene. (*A*) Chronotropic effects of β-blockers on DCM mice. Heart rates were determined by echocardiography under anaesthesia (30 mg/kg of pentobarbital, i.p.). Data represent the means ± SE for five mice. (*B*) Hypotensive effects of β-blockers on DCM mice. Mean blood pressures (MBP) were determined under conscious conditions. Data represent the means ± SE for four mice. (*C*) Effects of β-blockers on the phosphorylation level of phospholamban (PLB) in the LV myocardium of DCM mice. The phosphorylation level of PLB was determined by western blot analysis of the hearts. Data represent the means ± SE for three mice. (*D*) Effects of β-blockers on the standard deviation of the mean of the R-R intervals (SD R-R). Data represent the means ± SE for three mice. All data were obtained at 2 h after a single oral administration of β-blocker or vehicle only to 2-month-old untreated mice. Statistical significance was determined by ANOVA followed by *post hoc* Newman-Keuls multiple comparison test. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Note that pairs with no significant differences are not indicated.

DCM mice in a dose-dependent manner (*Figure 1A*). In contrast, oral administration of the non-selective β-blocker carvedilol had no significant effects on the survival of DCM mice at a dose showing a similar time-course of pharmacodynamic effect on heart rate as the effective dose of metoprolol (*Figure 1B*) (see also Supplementary materials online, *Figure S1*). Although lower doses of carvedilol showed some trend to slightly extend the survival of DCM mice, no statistically significant differences were demonstrated against the control vehicle. These results suggest that β_1-selectivity may be beneficial in extending survival. However, another β_1-selective β-blocker, atenolol, also had no significant effect on the survival of DCM mice at a dose showing a similar time-course of pharmacodynamic effect on heart rate as the effective dose of metoprolol (*Figure 1C*). Carvedilol and atenolol had significant negative chronotropic and hypotensive effects on DCM mice even at non-beneficial doses, which were comparable to those exerted by a beneficial dose of metoprolol (*Figure 2A* and *B*). Phosphorylation of Ser16 of PLB in the heart was significantly increased in DCM mice, and it was completely inhibited by any of the three β-blockers (*Figure 2C*). These results indicate that the three β-blockers exert equivalent levels of sympathetic blockade, independent of their effects on the survival of DCM mice. The respiratory sinus arrhythmia in artificially ventilated DCM mice was measured by determining the variability of R-R interval of the ECG per respiratory cycle (SD R-R), which has been shown to reflect the tonic activation of the efferent cardiac vagal nerves (*Figure 2D*).[27,28] DCM mice had significantly smaller SD R-R than wild-type mice. Metoprolol, but not carvedilol or atenolol, was found to significantly increase the SD R-R in DCM mice, consistent with the findings that metoprolol could activate the vagal nervous outflow to the heart, possibly due to the inhibition of β_1-adrenoceptor in the central nervous system.[31]

DCM mice commonly have electrophysiological abnormality in the heart with long QT, which might be involved in their frequent sudden death.[9] Metoprolol was found to shorten the QT interval significantly at 30 mg/kg per day, whereas carvedilol and atenolol had no significant effects on the QT interval of DCM mice (*Table 1*). DCM mice also commonly showed decreased amplitude of S-wave and a widened QRS complex in the ECG results. Metoprolol, but not carvedilol or atenolol, reduced these ECG abnormalities (*Table 1*).

DCM mice developed markedly enlarged hearts with ventricular dilation and significant interstitial fibrosis in the myocardium (*Figure 3*). Oral administration of metoprolol prevented the cardiac remodelling and fibrosis in a dose-dependent manner (*Figure 3*), with the heart-to-body weight ratio (*Table 2*) and fibrosis area (*Figure 3B*) showing a significant decrease at 30 mg/kg per day. Carvedilol and atenolol had no anti-remodelling and anti-fibrotic effects on the hearts of DCM mice.

DCM mice had LV systolic dysfunction and dilation, as were evident from reduced LV ejection fraction (EF) and increased LV end-diastolic dimension (LVEDD), respectively (*Table 3*). Metoprolol improved LV systolic function of DCM mice in a dose-dependent manner while reducing LV dilation (*Table 3*); metoprolol increased EF significantly at 30 mg/kg per day while significantly reducing LVEDD. Carvedilol and atenolol had no beneficial effects on LV systolic function and structure in DCM mice. DCM mice had significantly lower blood pressures than wild-type mice, and only metoprolol restored the blood pressure to normal levels (*Table 3*). Metoprolol, but not carvedilol or atenolol, decreased the myocardial expression of BNP and β-myosin heavy chain—biomarkers of heart failure—in a dose-dependent manner (*Figure 4*), indicating that metoprolol has a beneficial effect on cardiac function in DCM mice.

Table 1 ECG (standard limb lead II) data in DCM or WT mice with vehicle or β-blocker treatment

	DCM						WT (vehicle)
	Vehicle	Carvedilol		Atenolol (10 mg/kg)	Metoprolol		
		10 mg/kg	30 mg/kg		10 mg/kg	30 mg/kg	
Age, weeks	8	8	8	8	8	8	8
Number of mice	5	5	5	4	5	5	5
HR, b.p.m.	338 ± 6	337 ± 8	321 ± 8	341 ± 22	349 ± 6	334 ± 7	341 ± 10
P, mV/100	0.17 ± 0.02	0.18 ± 0.02	0.16 ± 0.02	0.14 ± 0.05	0.16 ± 0.02	0.18 ± 0.02	0.17 ± 0.02
R, mV/100	1.64 ± 0.12	1.60 ± 0.12	1.35 ± 0.16	1.63 ± 0.17	1.46 ± 0.09	1.43 ± 0.17	1.53 ± 0.09
S, mV/100	−0.04 ± 0.01***	−0.04 ± 0.01***	−0.04 ± 0.02***	−0.11 ± 0.01***	−0.17 ± 0.02***	−0.22 ± 0.01***,†	−0.51 ± 0.09
T, mV/100	0.03 ± 0.01***	0.02 ± 0.01***	0.02 ± 0.01***	0.03 ± 0.02***	0.05 ± 0.01***	0.07 ± 0.01***,†	0.17 ± 0.01
QRS, ms	19.6 ± 0.5***	19.2 ± 0.7***	20.6 ± 0.5***	20.6 ± 0.8***	18.2 ± 0.9***	15.0 ± 0.7**,†††	12.2 ± 0.6
PR, ms	46 ± 2	46 ± 2	47 ± 2	45 ± 2	44 ± 1	41 ± 3	43 ± 2
QT, ms	59 ± 4**	55 ± 4*	57 ± 5**	61 ± 4**	52 ± 4*	39 ± 3†	36 ± 4

Vehicle only (methylcellulose) or β-blockers were administered to mice orally for 28 days from 30 days of age. Surface ECG was measured at ~24 h after the last administration. Data represent the means ± SE. Statistical significance was determined by ANOVA followed by *post hoc* Newman–Keuls multiple comparison test.

WT, wild-type; DCM, DCM mice; HR, heart rate; b.p.m., beats per minute.

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. vehicle-treated WT mice.

†$P < 0.05$, †††$P < 0.001$ vs. vehicle-treated DCM mice.

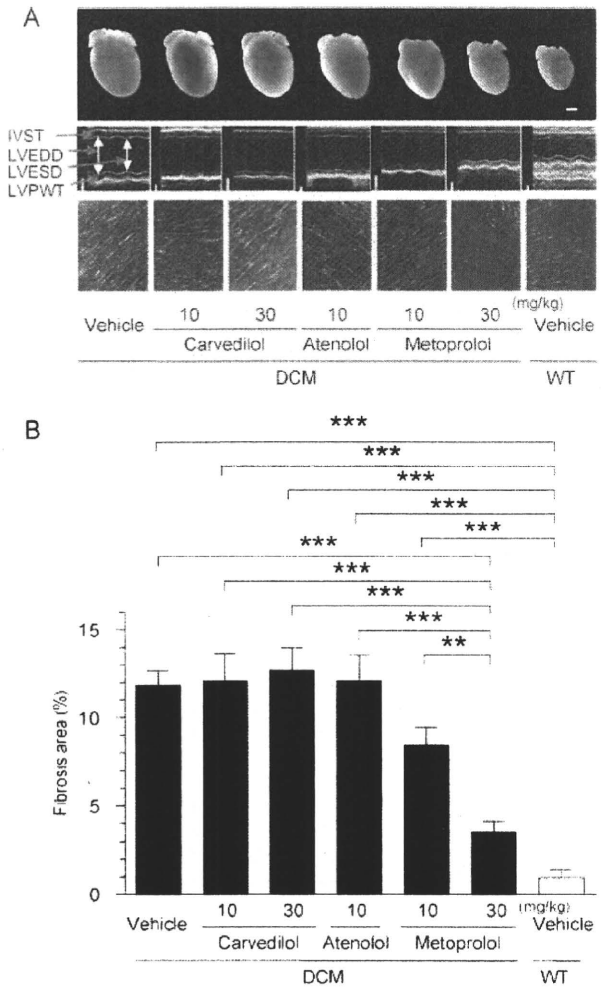

Figure 3 Effects of β-blockers on cardiac remodelling and function in DCM mice with a deletion mutation ΔK210 in the *TNNT2* gene. From 30 days of age, β-blocker or vehicle only was administered to mice orally once daily for 28 days. (*A*) Transthoracic echocardiography images (middle images) were measured at ~24 h after the last administration, and the hearts were then excised to analyse their gross morphology (top images; scale bar 2 mm) and the histology of the LV myocardium (bottom images; connective tissues were stained blue with azan). (*B*) Quantitative analysis of the fibrosis area in the LV myocardium. Data represent the means ± SE for three mice. Statistical significance was determined by ANOVA followed by *post hoc* Newman–Keuls multiple comparison test. $^{**}P < 0.01$, $^{***}P < 0.001$. Note that pairs with no significant differences are not indicated.

4. Discussion

Metoprolol, a second-generation β-blocker with β_1-selective blockade action, has been demonstrated to improve haemodynamic status and symptoms and reduce cardiac transplantation and all causes of mortality in patients with idiopathic DCM.[10–13,17–20,32] In the present study, we found that metoprolol improved cardiac systolic function, prevented cardiac remodelling and sudden death, and extended the survival of a mouse model of DCM with a deletion mutation ΔK210 in the *TNNT2* gene. These findings strongly suggest that a common pathogenic mechanism targeted by metoprolol may exist between this genetic model of DCM and idiopathic DCM. Carvedilol, a third-generation β-blocker with non-selective β-blockade, α1-blockade, and antioxidant actions, has also been shown to improve LV systolic function,

Table 2 Heart weight data in DCM or WT mice with vehicle or β-blocker treatment

	DCM								WT (vehicle)
	Vehicle	Carvedilol		Atenolol		Metoprolol			
		10 mg/kg	30 mg/kg	10 mg/kg	30 mg/kg	10 mg/kg	30 mg/kg		
Age, weeks	8	8	8	8	8	8	8		8
Number of mice	5	6	7	4	4	7	5		5
BW, g	21.2 ± 0.7	20.7 ± 1.1	20.4 ± 0.5	21.6 ± 1.0	19.8 ± 0.3	21.9 ± 0.5	21.8 ± 1.3		20.3 ± 0.6
HW, mg	243.2 ± 14.9***	246.2 ± 11.1***	250.5 ± 11.1***	240.1 ± 19.0***	228.3 ± 16.9***	206.8 ± 10.7***	176.7 ± 12.2***,††		111.1 ± 3.3
HW/BW, mg/g	11.5 ± 0.6***	12.3 ± 1.1***	12.3 ± 0.3***	11.3 ± 1.3***	11.5 ± 0.8***	9.5 ± 0.6***	8.1 ± 0.4*,†		5.5 ± 0.1

Vehicle only (methylcellulose) or β-blockers were administered to mice orally for 28 days from 30 days of age. Body weight and heart weight were determined at ~24 h after the last administration. Data represent the means ± SE. Statistical significance was determined by ANOVA followed by *post hoc* Newman–Keuls Multiple Comparison Test.
BW, body weight; HW, heart weight; HW/BW, heart weight-to-body weight ratio.
$^{*}p < 0.05$, $^{***}p < 0.001$ vs. vehicle-treated WT mice.
$^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ vs. vehicle-treated DCM mice.

Table 3 Echocardiography and blood pressure data in DCM or WT mice with vehicle or β-blocker treatment

	DCM						WT (vehicle)
	Vehicle	Carvedilol		Atenolol (10 mg/kg)	Metoprolol		
		10 mg/kg	30 mg/kg		10 mg/kg	30 mg/kg	
Age, weeks	8	8	8	8	8	8	8
Echocardiography							
Number of mice	5	6	6	4	6	8	7
HR, b.p.m.	383 ± 4	377 ± 8	370 ± 7	396 ± 9	393 ± 4	379 ± 8	393 ± 4
IVST, mm	0.45 ± 0.02	0.41 ± 0.01	0.40 ± 0.02	0.45 ± 0.03	0.42 ± 0.02	0.40 ± 0.03	0.48 ± 0.03
LVESD, mm	4.95 ± 0.18***	5.00 ± 0.20***	5.39 ± 0.25***	4.63 ± 0.16***	4.25 ± 0.23***	3.88 ± 0.16***,††	2.32 ± 0.22
LVEDD, mm	5.80 ± 0.19***	5.57 ± 0.18***	6.11 ± 0.20***	5.45 ± 0.12***	5.17 ± 0.14***	4.87 ± 0.15***,††	3.62 ± 0.23
LVPWT, mm	0.46 ± 0.02	0.41 ± 0.01	0.40 ± 0.02	0.43 ± 0.03	0.42 ± 0.02	0.42 ± 0.01	0.45 ± 0.02
FS, %	14.8 ± 1.6***	12.0 ± 1.0***	11.7 ± 1.4***	14.8 ± 2.1***	17.8 ± 2.3***	21.8 ± 1.1***,†	37.9 ± 2.2
EF, %	38.6 ± 2.7***	30.0 ± 2.3***	28.8 ± 3.1***	36.8 ± 4.1***	42.3 ± 4.7***	49.4 ± 2.1***,†	74.3 ± 2.6
Blood pressure							
Number of mice	4	4	ND	4	ND	4	4
MBP	64.75 ± 2.02**	63.75 ± 1.80**	ND	66.00 ± 1.41*	ND	71.50 ± 1.04†	73.75 ± 2.06

Vehicle only (methylcellulose) or β-blockers were administered to mice orally for 28 days from 30 days of age, and transthoracic echocardiography and blood pressure were measured at ~24 h after the last administration. Data represent the means ± SE. Statistical significance was determined by ANOVA followed by *post hoc* Newman–Keuls multiple comparison test.

IVST, interventricular septal wall thickness; LVPWT, LV posterior wall thickness; LV, left ventricular; LVESD, LV end-systolic dimension; LVEDD, LV end-diastolic dimension; FS, fractional shortening; EF, ejection fraction; MBP, mean blood pressure. ND, not determined.

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. vehicle-treated WT mice.

†$P < 0.05$, ††$P < 0.01$ vs. vehicle-treated DCM mice.

heart failure symptoms, endothelium-dependent vasodilation, and coronary microvascular function in patients with exclusively idiopathic DCM.[22,23,33–35] Furthermore, carvedilol has been shown to have a higher beneficial effect on survival than metoprolol in patients with chronic heart failure, which is mostly due to ischaemic heart disease or idiopathic DCM (reported in the COMET study[26]). In the present study, however, carvedilol had no beneficial effects on cardiac systolic function, cardiac remodelling, sudden death, and survival in the mouse model of DCM. Although it is very difficult to directly compare the results from clinical study and animal model study, this somewhat surprising result might be related to the differences in the study design. In the clinical studies, carvedilol is added to the standard therapy with diuretics, angiotensin-converting enzyme inhibitors, and/or digoxin, with its dose being gradually increased to the target dose, although this was not the case in our animal model study. Carvedilol, in fact, may not have had such a beneficial effect on non-ischaemic cardiomyopathy, as an analysis of the COMET study shows that vascular protection by carvedilol contributes to its superior effects in the treatment of heart failure compared with metoprolol.[36] Of note is that carvedilol, but not metoprolol, blocks HERG potassium channels and should have a QT prolonging effect.[37,38] This might be responsible for the lack of beneficial effect of carvedilol on our mouse model, since this model already has a significantly prolonged QT interval. Future studies are required to explore these possibilities.

The present study also showed that in contrast to metoprolol, another β_1-selective β-blocker, atenolol, had no beneficial effects on cardiac systolic function, cardiac remodelling, and survival in the mouse model of DCM. Metoprolol and atenolol have equivalent potency and selectivity for β_1-blockade with similar pharmacokinetics. However, atenolol is not recommended for the treatment of heart failure and is currently approved as an antihypertensive and antiischaemic drug. Atenolol is hydrophilic, however, β-blockers, which have a beneficial effect in heart failure treatment, have in common some degree of lipophilicity.[39] Highly lipophilic β-blockers, including metoprolol, can easily cross the blood–brain barrier,[40] so they could block the β-adrenoceptors in the brain and decrease sympathetic outflow to the heart, which has been shown to play an important role in the progression of heart failure.[41,42] In the present study, however, carvedilol, which has a moderate lipophilicity,[39] was not able to prevent cardiac remodelling and dysfunction and extend survival in the mouse model of DCM. The three β-blockers tested in this study can cause equivalent levels of sympathetic blockade in DCM mice, making it, furthermore, unlikely that the decreased sympathetic outflow to the heart could be a relevant mechanism for the beneficial effect of metoprolol. Of note is that this mouse model of DCM shows almost no overt congestive heart failure symptoms but develops a frequent sudden death due to ventricular fibrillation (VF).[9] Interestingly, a recent animal model study has shown that metoprolol can reduce the incidence of VF after coronary artery occlusion by a better maintained vagal activation, compared with atenolol. This suggests that the blockade of central nervous β_1-adrenoceptors modulating vagal nervous outflow is of importance for the prevention of VF and sudden cardiac death.[31] In the present study, metoprolol, but not atenolol or carvedilol, was found to induce a tonic activation of the efferent cardiac vagal nerves in DCM mice, probably due to β_1-blockade in the central nervous system, and prevent sudden cardiac death. Mechanisms by which long-term metoprolol treatment improves the cardiac systolic function and prevents cardiac remodelling in DCM mice remain unclear. However, since it is unlikely that metoprolol can directly increase myocardial contractility, a reduction in preload due to the decreased end-diastolic LV volume may,

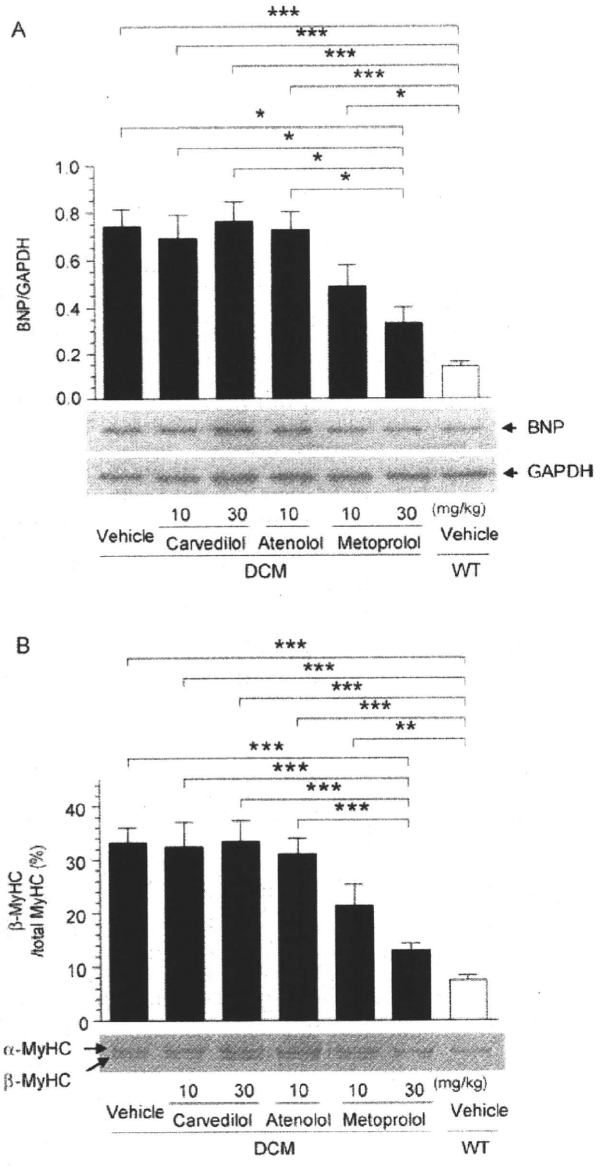

Figure 4 Effects of β-blockers on the expression levels of heart failure markers in the LV myocardium of DCM mice with a deletion mutation ΔK210 in the *TNNT2* gene. From 30 days of age, β-blocker or vehicle only was administered to mice orally once daily for 28 days. The hearts were excised at ~24 h after the last administration to determine the expression levels of pro-BNP (*A*) and β-MyHC (*B*). Data represent the means ± SE for four and five mice in panels A and B, respectively. Statistical significance was determined by ANOVA followed by *post hoc* Newman–Keuls multiple comparison test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Note that pairs with no significant differences are not indicated.

at least in part, contribute to the moderately increased EF after long-term metoprolol treatment. Prevention of the cardiac remodelling could also contribute to the lack of progressive electrophysiological deterioration observed in treated mice. Finally, the present study suggests that the β_1-selective lipophilic β-blocker metoprolol, which could prevent VF via central nervous system-mediated vagal activation, might have an especially important benefit to inherited DCM patients showing frequent sudden death, such as those affected by a deletion mutation ΔK210 in the *TNNT2* gene.[6–8]

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Conflict of interest: none declared.

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Short Communication

Propyl Gallate, a Strong Antioxidant, Increases the Ca^{2+} Sensitivity of Cardiac Myofilament

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Abstract. Ca^{2+} sensitizers are cardiotonic agents that directly increase the Ca^{2+} sensitivity of cardiac myofilament. To find a novel Ca^{2+} sensitizer, we have screened a group of phenolic compounds by examining their effects on the Ca^{2+}-dependent force generation in cardiac muscle fibers. We found that propyl gallate, a strong antioxidant, increased the Ca^{2+} sensitivity of cardiac myofilament in a dose-dependent and reversible manner. The present study indicates that propyl gallate is a novel type of Ca^{2+} sensitizer with antioxidant activity, which might be more beneficial for the treatment of congestive heart failure associated with oxidative stress than existing Ca^{2+} sensitizers.

Keywords: propyl gallate, Ca^{2+} sensitizer, antioxidant

Ca^{2+} sensitizers are cardiotonic agents that elicit a positive inotropic effect via an increase in the sensitivity of cardiac myofilaments to Ca^{2+} (1, 2). These agents have advantages of avoiding Ca^{2+} overloading and oxidative stress, which could be caused by other cardiotonic agents such as β-receptor agonists through increased myocardial oxygen consumption, in the treatment of congestive heart failure (CHF) (3, 4). Ca^{2+} sensitizers have also been shown to be beneficial for the treatment of dilated cardiomyopathy associated with a decrease in the myofilament Ca^{2+} sensitivity (5). Oxidative stress is involved in myocardial ischemia/reperfusion injury, and antioxidants have been shown to attenuate cardiac dysfunction during post ischemic myocardial stunning (6) and oxidative stress–induced myocardial injury (7) by preventing the accumulation of oxygen free radicals. In this study, we screened a group of phenolic compounds to find a novel type of Ca^{2+} sensitizer.

Membrane permeabilized (skinned) cardiac muscle fibers were prepared from the left ventricular trabeculae of young male albino rabbits (2 – 2.5 kg), and force measurements were performed as described previously (8). Briefly, small bundles (0.5 – 1-mm-wide and 5 – 7-mm-long) of trabeculae tied to glass capillary tubes were skinned with relaxing solution containing 50% glycerol. A small fiber (about 200 μm in diameter) dissected from the stock-skinned trabeculae was mounted in a thermostatically controlled chamber with a capacity of 0.2 ml. The fiber length between hooks was about 1 mm, and the resting sarcomere length was set to 2.3 μm by using laser diffraction. The force generated by skinned muscle fibers was measured at 25°C with a strain gauge (UL-2GR; Minebea, Nagano). The relaxing solution consisted of 50 mM MOPS/KOH (pH 7.0), 100 mM KCl, 6 mM MgCl2, 5 mM ATP, 4 mM EGTA, 0.5 mM DTT, 10 mM creatinine phosphate, and 35 units/ml creatine kinase.

Trolox (water-soluble derivative of tocopherol) was purchased from Sigma (St. Louis, MO, USA). Curcumin, quercetin, chrologenate, gallate monohydrate, dodecyl gallate, and propyl gallate were purchased from Wako Pure Chemical Industries (Osaka). All phenolic compounds were dissolved in dimethylsulfoxide (DMSO) and used at the final concentration of 0.1% DMSO.

Antioxidant activities of phenolic compounds were

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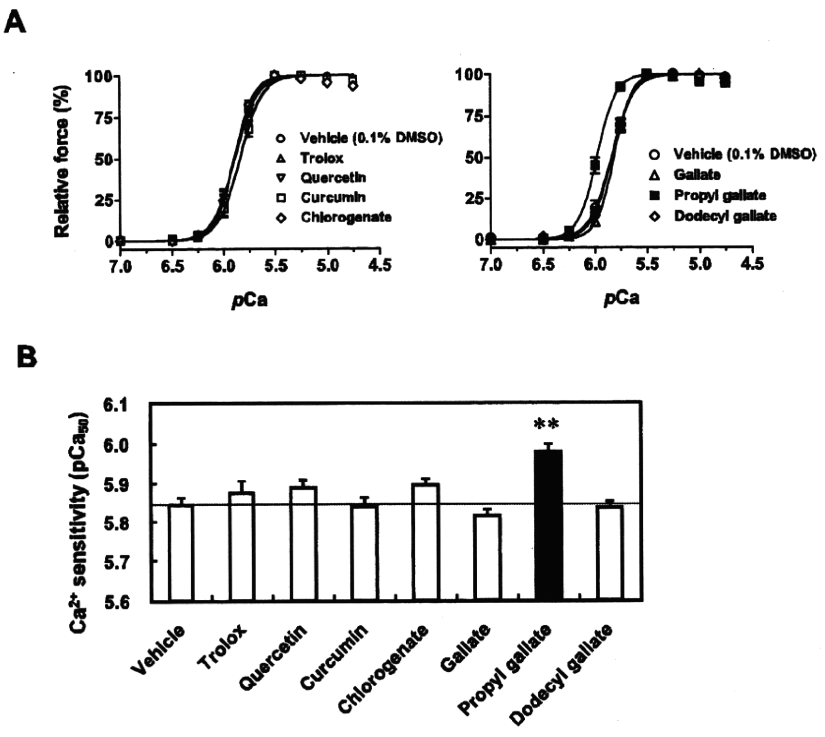

Fig. 1. Effects of phenolic compounds on the force generation in skinned cardiac muscle fibers. A: Force–*p*Ca relationships determined in the presence of 100 μM phenolic compounds. B: Effects of phenolic compounds (100 μM) on the Ca^{2+} sensitivity (pCa_{50}) of force generation. The force–*p*Ca relationship was determined only once for a given muscle fiber by cumulatively increasing [Ca^{2+}] from *p*Ca7 to *p*Ca4.75 in the presence of phenolic compound or vehicle only without pretreatment. Forces were normalized to the maximum force developed by each fiber. Data represent the means ± S.E.M. for 5 – 8 muscle fibers. $^{**}P<0.01$ vs. vehicle (Student's *t*-test).

evaluated by their effects of scavenging stable free radical, 1,1-diphenyl-2-picryl hydrazyl (DPPH), according to the method described by Shirwaikar et al. (9). Briefly, the reaction mixtures containing 10 μM DPPH and various concentrations of phenolic compounds were incubated for 10 min at room temperature in the dark, and then the antioxidant activity was calculated as a percent reduction of the light absorbance of DPPH at 517 nm.

Effects of phenolic antioxidant compounds, trolox, curcumin, quercetin, chrologenate, gallate, dodecyl gallate, and propyl gallate, were examined at 100 μM on the Ca^{2+}-dependent force generation in skinned cardiac muscle fibers (Fig. 1). Propyl gallate was found to shift the force–pCa relationship leftward with a significant increase in the half-maximally activating pCa (pCa_{50}, an index of Ca^{2+} sensitivity), while the other compounds had no significant effects on the force–pCa relationship.

Propyl gallate increased the Ca^{2+} sensitivity of force generation in skinned cardiac muscle fibers in a dose-dependent manner (Fig. 2: A and B), with statistically significant increase in Ca^{2+} sensitivity being detected above 100 μM (Fig. 2C). The Ca^{2+}-sensitizing effect of propyl gallate was reversible and lost immediately after washout (data not shown), and its potency appears to be lower than that of the commercially launched Ca^{2+}-sensitizer pimobendan (cf. supplementary data in ref. 5). All the phenolic antioxidant compounds examined had no significant effects on the maximum force and the slope of force–pCa relationship (i.e., Hill coefficient) in skinned cardiac muscle fibers (data not shown). Propyl gallate had a strong antioxidant activity, which was similar to those of other phenolic compounds, trolox, curcumin, and gallate, when evaluated by their free radical (DPPH) scavenging effects (Fig. 3).

Free radical injury is involved in the pathology of congestive heart failure (CHF); CHF patients have an increased level of plasma lipid peroxide, a marker of oxidative stress, and a decreased activity of glutathione peroxidase, an antioxidant enzyme. Antioxidant supplementation has been shown to improve the myocardial function and survival of CHF patients (10). Clinical therapeutic trials have shown that carvedilol, a nonselective β-adrenergic receptor antagonist with antioxidant activity, reduces the mortality among patients with severe heart failure (11), whereas bucindolol, a nonselective β-adrenergic receptor antagonist with no antioxidant activity, has no favorable effects (12). Propyl gallate is a phenolic antioxidant compound, and its chronic oral administration has been reported to offer significant protection against myocardial oxidative stress–induced injury (7). The present study revealed that this compound also has a Ca^{2+}-sensitizing effect on the cardiac

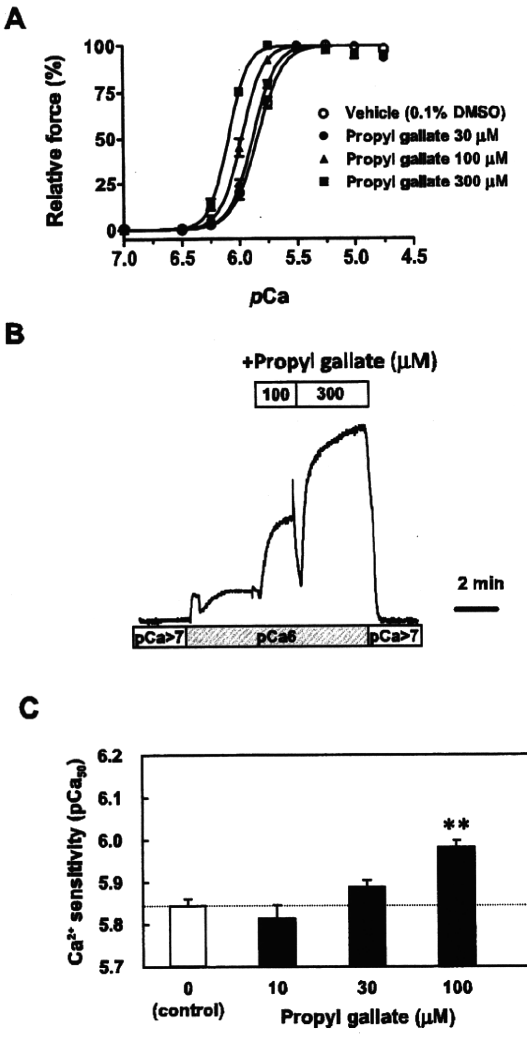

Fig. 2. Effects of propyl gallate on the force generation in skinned cardiac muscle fibers. A: Force–pCa relationships determined in the presence of 0, 10, 30, and 100 μM propyl gallate. B: Force recording showing dose-dependent Ca^{2+}-sensitizing effects of propyl gallate. C: Dose-dependent effects of propyl gallate on the Ca^{2+} sensitivity (pCa$_{50}$) of force generation. The force–pCa relationship was determined only once for a given muscle fiber by cumulatively increasing [Ca^{2+}] from pCa7 to pCa4.75 in the presence of propyl gallate or vehicle only without pretreatment. Forces were normalized to the maximum force developed by each fiber. Data represent the means ± S.E.M. for 5 – 8 muscle fibers. Statistical significance was determined by ANOVA followed by the post hoc Dunnett's multiple comparison test. **P<0.01 vs. control.

myofilament, through which an inotropic effect would be exerted on the heart. Propyl gallate, a Ca^{2+} sensitizer with strong antioxidant activity, thus might be more beneficial for the treatment of CHF than existing Ca^{2+} sensitizers with no antioxidant activities. Further studies would be required to test this possibility.

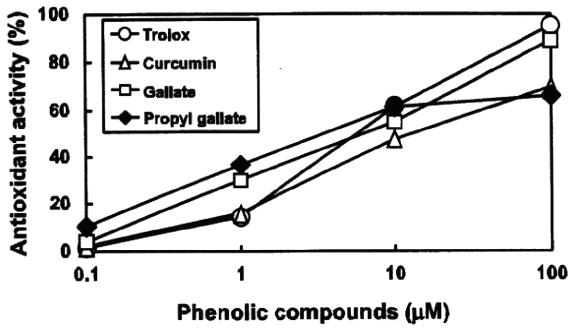

Fig. 3. Antioxidant activities of phenolic compounds. EC_{50} values of free radical (DPPH)-scavenging effects of trolox, curcumin, gallate, and propyl gallate were 8.4, 10.4, 9.0, and 1.3 μM, respectively. Data represent the means ± S.E.M. of 3 determinations.

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Forum Minireview

Drug Development Targeting the Glycogen Synthase Kinase-3β (GSK-3β)-Mediated Signal Transduction Pathway: Inhibitors of the Wnt/β-Catenin Signaling Pathway as Novel Anticancer Drugs

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Abstract. Accumulating evidence suggests that the Wnt/β-catenin signaling pathway is often involved in oncogenesis and cancer development. Accordingly, a novel anticancer drug can be developed using inhibitors of this pathway. However, at present, there is no selective inhibitor of this pathway available as a therapeutic agent. Although all the components of the Wnt/β-catenin signaling pathway can be a target for drug development, glycogen synthase kinase-3β (GSK-3β), in particular, may be a good target because GSK-3β is an essential component of the pathway, and activation of this kinase results in the inhibition of the Wnt signaling pathway. We found that the differentiation-inducing factors (DIFs), putative morphogens for *Dictyostelium discoideum*, inhibit the Wnt/β-catenin signaling pathway via the activation of GSK-3β, resulting in the cell-cycle arrest of human cancer cell lines. In this review, we summarize our recent findings on the antiproliferative effect of DIFs and show the possibility for development of a novel anticancer drug from DIFs and their derivatives.

Keywords: Wnt/β-catenin signaling, cancer, glycogen synthase kinase-3β (GSK-3β), drug development, differentiation-inducing factor

The Wnt/β-catenin signaling pathway and GSK-3β

Cell signaling cascades activated by Wnt proteins (i.e., the Wnt signaling pathways) are well conserved through evolutionary processes across a variety of species. As well as regulating cellular processes such as proliferation, differentiation, motility, and survival /apoptosis, the Wnt signaling pathways play key roles in embryonic development and maintenance of homeostasis in mature tissues. Of four known Wnt signaling pathways, [the Wnt/β-catenin (canonical) pathway, the planar cell polarity (PCP) pathway, the Wnt/Ca^{2+} pathway, the protein kinase A pathway], the Wnt/β-catenin signaling pathway is best characterized (1 – 6).

The activity of the Wnt/β-catenin signaling pathway is dependent on the amount of β-catenin in the cytoplasm. Normally, the cytoplasmic β-catenin level is kept low through continuous ubiquitin-proteasome system–mediated degradation, which is regulated by a multiprotein complex containing axin, adenomatous polyposis coli (APC), and glycogen synthase kinase-3β (GSK-3β).

GSK-3β is a cytoplasmic serine/threonine protein kinase that is known to play central roles in a variety of biological processes including a number of signaling pathways such as the Wnt/β-catenin, Hedgehog, Notch, and insulin signaling pathways (7). The activity of GSK-3β is decreased by the phosphorylation of Ser^9 and several studies have shown that Ser^9 in GSK-3β is phosphorylated by Akt, a serine/threonine kinase that is activated by phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinase-activated protein kinase-1 (MAPKAP-K1), a protein kinase downstream of the mitogen-activated protein kinase (MAPK) cascade, and p70 ribosomal S6 kinase-1 (7 – 9).

In the Wnt/β-catenin signaling pathway, GSK-3β mediates the degradation of β-catenin molecules by phosphorylating specific amino acid residues, which marks the protein to trigger its degradation by the 26S

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