Celecoxib and 2,5-Dimethyl-Celecoxib Prevent Cardiac Remodeling Inhibiting Akt-Mediated Signal Transduction in an Inherited Dilated Cardiomyopathy Mouse Model

Xue Li Fan, Fumi Takahashi-Yanaga, Sachio Morimoto, Dong-Yun Zhan, Kazunobu Igawa, Katsuhiro Tomooka, and Toshiyuki Sasaguri

Department of Clinical Pharmacology, Faculty of Medical Sciences (X.F., F.T.-Y., S.M., D.-Y.Z, T.S.) and Institute for Materials Chemistry and Engineering (K.I., K.T.), Kyushu University, Fukuoka, Japan

Received January 12, 2011; accepted March 22, 2011

ABSTRACT

Celecoxib, a cyclooxygenase-2 (COX-2)-selective nonsteroidal anti-inflammatory drug, has been shown to inhibit Akt and prevent cardiac remodeling in aortic banding-induced failing heart in mice. However, it may be difficult to use celecoxib for the treatment of heart failure because of thromboembolic adverse reactions. Since 2,5-dimethyl (DM)-celecoxib, a derivative unable to inhibit COX-2, has been also reported to inhibit Akt, we attempted to examine whether DM-celecoxib retains the ability to prevent cardiac remodeling and improve cardiac functions using a mouse model of inherited dilated cardiomyopathy (DCM). DM-celecoxib as well as celecoxib administered daily for 4 weeks inhibited Akt and subsequent phosphorylation of glycogen synthase kinase-3β and mammalian target of rapamycin. Furthermore, both celecoxib and DM-celecoxib inhibited the activities of nuclear factor of activated T cell and β-catenin and the expression of TCF7L2 (T-cell-specific transcriptional factor-7L2) and c-Myc, downstream mediators related to cardiac hypertrophy. Functional and morphological measurements showed that these compounds improved left ventricular systolic functions (ejection fraction: vehicle, 34.7 ± 3.9%; 100 mg/kg celecoxib, 50.3 ± 1.1%, p < 0.01; 100 mg/kg DM-celecoxib, 49.8 ± 0.8%, p < 0.01), which was also evidenced by the decrease in β-myosin heavy chain and B-type natriuretic peptide, and prevented hypertrophic cardiac remodeling (heart/body weight ratio: vehicle, 10.4 ± 0.7 mg/g; 100 mg/kg celecoxib, 8.0 ± 0.3 mg/g, p < 0.01; 100 mg/kg DM-celecoxib, 8.2 ± 0.1 mg/g, p < 0.05). As a consequence, both compounds improved the survival rate (vehicle, 45%; 100 mg/kg celecoxib, 75%, p < 0.05; 100 mg/kg DM-celecoxib, 70%, p < 0.05). These results suggested that not only celecoxib but also DM-celecoxib prevents cardiac remodeling and reduces mortality in DCM through a COX-2-independent mechanism involving Akt and its downstream mediators.

Introduction

Dilated cardiomyopathy (DCM), characterized by cardiac enlargement and systolic dysfunction and often resulting in heart failure and sudden death, is a relatively common but poorly understood group of myocardial disorders caused by inheritable defects of cardiomyocyte-related genes or nongenetic insults, such as ischemia, toxins, metabolic disturbance, and infection (Dec and Fuster, 1994; Morimoto, 2008). Despite routine use of medicines, such as the renin-angiotensin-aldosterone system inhibitors, β-adrenergic receptor antagonists, and diuretics, and general availability of heart transplantation, long-term clinical follow-up results for DCM patients are still unsatisfactory (Arola et al., 1998; Michels et al., 2003). In inherited DCM, patients affected by a deletion mutation (K210 in the cardiac troponin T gene (TNNT2) show a severe early-onset phenotype with a high incidence of sudden death and/or heart failure death (Kamisago et al., 2000; Hanson et al., 2002). Recently, we created a knock-in mouse model carrying this mutation, which closely recapitulated the clinical manifestations of human patients (Du et al., 2007).

Among many signaling molecules in cardiomyocytes, glycogen synthase kinase-3β (GSK-3β) is a crucial regulator for cardiac hypertrophy (Haq et al., 2000; Dorn and Force, 2005). GSK-3β is a serine/threonine kinase that phosphorylates several downstream mediators, such as transcriptional factor-7L2 (TCF7L2) and c-Myc, involved in cardiac hypertrophy. Functional and morphological measurements showed that these compounds improved left ventricular systolic functions (ejection fraction: vehicle, 34.7 ± 3.9%; 100 mg/kg celecoxib, 50.3 ± 1.1%, p < 0.01; 100 mg/kg DM-celecoxib, 49.8 ± 0.8%, p < 0.01), which was also evidenced by the decrease in β-myosin heavy chain and B-type natriuretic peptide, and prevented hypertrophic cardiac remodeling (heart/body weight ratio: vehicle, 10.4 ± 0.7 mg/g; 100 mg/kg celecoxib, 8.0 ± 0.3 mg/g, p < 0.01; 100 mg/kg DM-celecoxib, 8.2 ± 0.1 mg/g, p < 0.05). As a consequence, both compounds improved the survival rate (vehicle, 45%; 100 mg/kg celecoxib, 75%, p < 0.05; 100 mg/kg DM-celecoxib, 70%, p < 0.05). These results suggested that not only celecoxib but also DM-celecoxib prevents cardiac remodeling and reduces mortality in DCM through a COX-2-independent mechanism involving Akt and its downstream mediators.
eral transcription factors to inhibit their functions, such as activator protein-1, cyclic AMP-response element-binding protein, β-catenin, and nuclear factor of activated T cell (NFAT) (Hardt and Sadoshima, 2002; Kerkela et al., 2007). Cardiomyocyte-specific expression of constitutively active GSK-3β inhibited cardiac hypertrophy induced by pressure overload and β-adrenergic receptor stimuli in transgenic mice (Antos et al., 2002). Activated Akt inhibits GSK-3β by phosphorylation and promotes physiological hypertrophic growth of the heart (Morisco et al., 2000). However, prolonged Akt activation results in pathological hypertrophy with a massive increase in heart size and interstitial fibrosis (Shiojima et al., 2005). Therefore, an inhibitor of Akt or an activator of GSK-3β could provide a novel therapeutic strategy against pathological cardiac hypertrophy and heart failure.

Celecoxib, a nonsteroidal anti-inflammatory drug developed as a selective inhibitor of COX-2, is used for the treatment of various forms of arthritis and for the management of acute and chronic pain. This drug has also been used for the treatment of familial adenomatous polyposis patients (Steinbach et al., 2000), and moreover, it is being investigated for its chemotherapeutic potential in other types of cancer (Dannenberg and Subbaramaiah, 2003). The ability of celecoxib to inhibit Akt has been suggested to be one of the mechanisms for the anticancer effect of this drug (Hsu et al., 2000; Yang et al., 2004; Basu et al., 2005). Recently, celecoxib was reported to prevent cardiac

![Graphs and images showing the effects of celecoxib and DM-celecoxib on Akt and its downstream mediators in the LV myocardium of the DCM mice.](http://jpet.aspetjournals.org/pdfs/10.1124/jpet.103.076079/fig1.jpg)

**Fig. 1.** The effects of celecoxib and DM-celecoxib on Akt and its downstream mediators in the LV myocardium of the DCM mice. One-month-old DCM mice were treated with daily administration of celecoxib, DM-celecoxib, or vehicle for 4 weeks. Wild-type mice were treated with vehicle in the same way. LV samples were prepared as described under Materials and Methods. The phosphorylation levels and the total expression levels of Akt (A and B), GSK-3β (C and D), and mTOR (E) were determined by Western blot analysis. The ratios of phosphorylated protein to total protein are shown as the means ± S.E. of six mice in each group. A and C, vehicle versus celecoxib; B and D, vehicle versus DM-celecoxib. Statistical significance was determined by the one-way ANOVA followed by post hoc Tukey's multiple comparison test. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
hypertrophy and dysfunction induced by aortic banding in mice (Jacobshagen et al., 2008). Therefore, celecoxib may have been a promising candidate drug for the treatment of heart failure, unless it was reported that long-term treatment with this drug might be associated with an increase of thromboembolic adverse events (Bresalier et al., 2005). 2,5-Dimethyl (DM)-celecoxib is a celecoxib analog unable to inhibit COX-2. DM-celecoxib has been reported to show antitumor efficacy as strongly as celecoxib in vitro and in vivo by inhibiting Akt and thereby activating GSK-3β (Kulp et al., 2004; Schönthal, 2006). However, the effect of DM-celecoxib on failing heart has not yet been investigated.

Therefore, we attempted to examine whether DM-celecoxib is beneficial for the treatment of DCM using our mouse model with ΔK210 troponin T mutation, comparing its effects on biochemical, morphological, and functional parameters with those of celecoxib.

**Materials and Methods**

**Animal Experiments Using DCM Model Mice.** A knock-in mouse model, in which three base-pairs coding the residue K210 were deleted from the endogenous gene TNNT2 using gene-targeting technology, was created as described previously (Du et al., 2007). The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH Publication 85-23, revised in 1996). The experimental protocol was reviewed by the Committee of Ethics on Animal Experiments at the Faculty of Medical Sciences, Kyushu University, and carried out according to the Guidelines for Animal Experiments, Faculty of Medical Sciences, Kyushu University, and The Law (105) and Notification (6) of the Japanese Government.

**Chemicals.** Celecoxib was kindly provided by Pfizer Inc. (New York, NY). DM-celecoxib was synthesized according to the procedure reported previously (Kardosh et al., 2005), with some modifications as follows: sodium hydride (55 wt% in mineral oil, 7.25 g, 166 mmol); 2,5-dimethylacetophenone (18 g, 122 mmol); and ethyl trifluoroacetate (19.8 ml, 166 mmol) added to methanol (150 ml, 0°C), followed by stirring for 18 h. The reaction was quenched with aqueous HCl (1 N, 180 ml) and then extracted three times with ethyl acetate. The combined organic layer was washed with saturated aqueous sodium bicarbonate (50 ml) and brine (2 x 50 ml), and the solvent was removed under reduced pressure. Thus, obtained crude oil and 4-sulfonylphenylhydrazine hydrochloride (20.5 g, 91.6 mmol) were dissolved in hot ethanol (500 ml), and the mixture was stirred for 24 h. After cooling to room temperature, the mixture was diluted with ethyl acetate (400 ml), washed with water (100 ml) and brine (2 x 100 ml), dried over sodium sulfate and filtered. The solvent was removed under reduced pressure, and obtained crude DM-celecoxib was dissolved in hot toluene (120 ml). The solution was changed to suspension after cooling to room temperature, diluted and filtered.

**Fig. 2.** The effects of celecoxib and DM-celecoxib on NFATc3 and β-catenin in the LV myocardium of the DCM mice. One-month-old DCM mice were treated with daily administration of celecoxib, DM-celecoxib, or vehicle for 4 weeks. Wild-type mice were treated with vehicle in the same way. LV samples were prepared as described under Materials and Methods. A and B, the phosphorylation levels and the total expression levels of NFATc3 (A) and the expression levels of β-catenin and GAPDH (B) were determined by Western blot analysis. The ratios of phosphorylated NFATc3 to total NFATc3 (A) and those of β-catenin to GAPDH (B) were determined by Western blot analysis. The ratios of phosphorylated NFATc3 to total NFATc3 (A) and those of β-catenin to GAPDH (B) are shown as the means ± S.E. of six mice in each group. Statistical significance was determined by the one-way ANOVA followed by post hoc Tukey's multiple comparison test. *, p < 0.05; **, p < 0.01; ***, p < 0.001. C, the LV specimen were stained with an anti-β-catenin antibody. Arrows indicate stained nuclei. Scale bar, 25 μm.
with hexane (250 ml), and filtered. The residue was washed with hexane, which was dried at 50°C under reduced pressure to give 34.9 g of DM-celecoxib (96%).

**Drug Administration.** To 4-week-old DCM mice, celecoxib or DM-celecoxib, pound down using a mortar and suspended in 0.25% methylcellulose solution, was orally administered at doses of 10 to 100 mg/kg once daily for 4 weeks, whereas control mice received only vehicles.

**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, LV was dissected from the heart, blotted on filter paper, and homogenized in Laemmli’s sample buffer. LV homogenerate samples were subjected to Western blot analysis as described previously (Du et al., 2007), using polyclonal anti-phospho-Akt (Ser473) antibody, polyclonal anti-phospho-mammalian target of rapamycin (mTOR) (Ser2448) antibody, polyclonal anti-mTOR antibody, monoclonal anti-c-Myc antibody (Cell Signaling Technology, Danvers, MA), monoclonal anti-GSK-3β antibody, monoclonal anti-β-catenin antibody (BD Biosciences, San Jose, CA), polyclonal anti-NFATc3 antibody, polyclonal anti-cyclin D1 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), polyclonal anti-phospho-NFATc3 (Ser365) antibody, polyclonal anti-B-type natriuretic peptide (BNP) antibody, monoclonal anti-GAPDH antibody (Abcam, Cambridge, MA), or monoclonal anti-T-cell-specific transcriptional factor-7L2 (TCF7L2) antibody (Millipore, Billerica, MA). The immunoreactivity of the bands were visualized by SuperSignal West Femto Maximum sensitivity substrate (Thermo Scientific, Waltham, MA) and Hyperfilm ECL (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK), and an optical densitometric scan using Phoretix gel analysis software (Phoretix International, Newcastle upon Tyne, UK).

**Ultrasound Tomography.** Mice were placed in a supine position and allowed to breathe spontaneously after being anesthetized with sodium pentobarbital (50 mg/kg i.p.). The hair on chest was removed by a hair remover, and acoustic coupling gel was applied. Transthoracic ultrasound tomography (M-mode) was performed using a 14-MHz linear array probe with a diagnostic ultrasound system, Nemio SSA-550A (Toshiba, Tokyo, Japan).

![Fig. 3](https://example.com/fig3.png)

**Fig. 3.** The effects of celecoxib and DM-celecoxib on the expression of the Wnt/β-catenin pathway target gene products in the DCM mouse LV myocardium. One-month-old DCM mice were treated with daily administration of celecoxib, DM-celecoxib, or vehicle for 4 weeks. Wild-type mice were treated with vehicle in the same way. LV samples were prepared as described under **Materials and Methods.** The expression levels of TCF7L2 (A), cyclin D1 (B), c-Myc (C), and GAPDH were determined by Western blot analysis. The ratios of each protein to GAPDH are shown as the means ± S.E. of six mice in each group. Statistical significance was determined by the one-way ANOVA followed by post hoc Tukey’s multiple comparison test. *, p < 0.05; **, p < 0.01.
Histological Analyses. Histological analyses were performed as described previously (Du et al., 2007). In brief, hearts excised from the mice anesthetized with pentobarbital (50 mg/kg i.p.) were fixed and embedded in paraffin. The adequacy of anaesthesia was monitored by a paw pinch of the mouse. The hematoxylin-eosin staining and azan staining were performed. The extent of fibrosis and the widths of cardiomyocytes were quantified using the NIH ImageJ program.

Immunohistochemical Analysis. For immunohistochemical staining of β-catenin, primary 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 and incubated for 1 h. The slides were analyzed with Biozero microscopy (Keyence, Osaka, Japan).

Myosin Isoform Contents. Myosin heavy chain (MyHC) isoforms in the LV myocardium were separated on SDS-polyacrylamide gel electrophoresis according to the method of Rundell et al. (2004). We applied an equal amount of protein in each lane (10 μg/lane). Relative β1-isoform expression levels (percentage of total MyHC) were determined by optical densitometric scan using Phoretix gel analysis software.

Statistical Analyses. Survival curves were generated using the Kaplan-Meier method, and the survival rates were statistically compared by log-rank test. Results are presented as mean ± S.E., and statistical analyses were performed by the one-way ANOVA followed by post-hoc multiple comparison test. A P value < 0.05 was considered as significant.

Results

The Effects of Celecoxib and DM-Celecoxib on Akt and Its Downstream Mediators. We previously reported the activation of Akt in our DCM model mice (Wang et al., 2010), and it has been reported that celecoxib and DM-celecoxib inhibit Akt activity through the suppression of phosphorylation at the Ser473 residue in vitro. Therefore, we examined the effect of oral administration of celecoxib and DM-celecoxib on Akt phosphorylation status in the mouse LV. As shown in Fig. 1, A and B, the phosphorylation level of Akt was significantly increased compared with wild-type mice, indicating that Akt was activated in the LV of DCM mice, and this observation was consistent with our previous report (Wang et al., 2010). Oral administration of celecoxib and DM-celecoxib significantly suppressed the phosphorylation of Akt in a dose-dependent manner, whereas the effect of celecoxib was stronger than DM-celecoxib at the lower doses.

Given that GSK-3β is phosphorylated by Akt at the Ser9 residue for inactivation, we also examined the phosphorylation status of GSK-3β (Fig. 1, C and D). The phosphorylation level of GSK-3β in the LV of DCM mice was higher than that of wild-type mice, and administration of celecoxib or DM-celecoxib significantly suppressed the phosphorylation of GSK-3β in a dose-dependent manner, whereas the effect of celecoxib was stronger than DM-celecoxib at the lower doses.

mTOR, a serine/threonine kinase involved in protein synthesis in hypertrophic cardiac growth, is also regulated by Akt (Dorn and Force, 2005). As shown in Fig. 1E, the phosphorylation level of mTOR in the LV of DCM mice was higher than that of wild-type mice. However, the administration of celecoxib or DM-celecoxib significantly reduced its phosphorylation level.

The Effects of Celecoxib and DM-Celecoxib on Transcription Factors Related with Cardiac Hypertrophy. GSK-3β is known to phosphorylate transcription factors related

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Echocardiography data in DCM or WT mice with vehicle, celecoxib, or DM-celecoxib treatment</th>
<th>One-month-old DCM mice were treated with daily administration of celecoxib, DM-celecoxib, or vehicle for 4 weeks, and transthoracic ultrasound sonography was performed. Wild-type mice administered vehicle were treated in the same way. Data represent the means ± S.E., and statistical analyses were performed by the one-way ANOVA with Tukey's post hoc multiple comparison test. A P value &lt; 0.05 was considered as significant.</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>Vehicle</td>
<td>Celecoxib</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Age (weeks)</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Number of mice</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>HR (b.p.m.)</td>
<td>389 ± 14</td>
<td>387 ± 14</td>
</tr>
<tr>
<td>GR (mm)</td>
<td>0.43 ± 0.02</td>
<td>0.40 ± 0.03</td>
</tr>
<tr>
<td>IVST (mm)</td>
<td>0.43 ± 0.02</td>
<td>0.43 ± 0.03</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>1.92 ± 0.11</td>
<td>1.90 ± 0.12</td>
</tr>
<tr>
<td>LVPWT (mm)</td>
<td>0.45 ± 0.02</td>
<td>0.47 ± 0.03</td>
</tr>
<tr>
<td>FS (%)</td>
<td>30.7 ± 1.0</td>
<td>31.3 ± 1.1</td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>65.2 ± 3.9</td>
<td>65.6 ± 4.0</td>
</tr>
</tbody>
</table>
to cardiac hypertrophy, including NFATs and β-catenin. Among five NFAT isoforms, it has been reported that NFATc3 is important in cardiac hypertrophic machinery (Wilkins et al., 2002). Therefore, the effects of oral administration of celecoxib and DM-celecoxib on NFATc3 and β-catenin were analyzed. As shown in Fig. 2, the phosphorylation level of NFATc3 was lower, and the amount of β-catenin was higher in the heart from the DCM mice compared with wild-type mice, indicating that the activities of both transcription factors in the DCM mice were higher than in wild type. However, oral administration of celecoxib or DM-celecoxib markedly reduced the levels of both transcription factors to the levels comparable with those in wild type. An immunohistochemical analysis showed that the administration of celecoxib or DM-celecoxib reduced β-catenin accumulated in the nuclei of the DCM mouse heart (Fig. 2C). These results indicated that celecoxib and DM-celecoxib activated GSK-3β, resulting in the suppression of transcriptional activities of NFATc3 and β-catenin.

**Fig. 4.** The effects of celecoxib and DM-celecoxib on the morphology of the DCM mouse heart. A, gross morphology. One-month-old DCM mice were treated with daily administration of celecoxib, DM-celecoxib, or vehicle for 4 weeks. Wild-type mice were treated with vehicle in the same way. Photographs of excised hearts are displayed. Scale bar, 2 mm. B, Azan staining. Top panel, low magnification views of the transverse sections at the mid-ventricular level. Scale bar, 2 mm. Bottom panel, high magnification views of the LV myocardium. Scale bar, 100 μm. Azan staining-positive areas quantified with NIH ImageJ are shown in the right graph. Data represent the means ± S.E. obtained using three mice in each group. C, hematoxylin-eosin staining. Scale bar, 50 μm. Short-axis lengths of cardiomyocytes measured with NIH ImageJ are shown as percentage of those of vehicle-treated wild-type mice in the right graph. Data represent the means ± S.E. obtained using three mice. Statistical significance was determined by the one-way ANOVA followed by post hoc Tukey’s multiple comparison test. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
Since we previously reported that celecoxib inhibited the Wnt/β-catenin signaling pathway (Sakoguchi-Okada et al., 2007; Takahashi-Yanaga et al., 2008), and recently it has been reported that this pathway plays an important role in the onset of cardiac hypertrophy development (van de Schans et al., 2007; Malekar et al., 2010), we examined the effects of celecoxib and DM-celecoxib on TCF7L2, c-Myc, and cyclin D1, the target gene products of this pathway (Fig. 3). The expression levels of TCF7L2 and c-Myc proteins in the LV were greater in the DCM mice than wild-type mice, although we could not find a significant difference in the amount of cyclin D1. Both celecoxib and DM-celecoxib significantly reduced TCF7L2 and c-Myc, whereas they had no effect on cyclin D1. Therefore, the activation of the Wnt/β-catenin signaling pathway may play an important role in the process of cardiac hypertrophy in the DCM mice with TnT mutation, although cyclin D1 did not seem to be significantly involved.

The Effects of Celecoxib and DM-Celecoxib on Cardiac Functions and Remodeling. Consistent with our previous report (Du et al., 2007), dysfunction and remodeling of the DCM mouse heart were evident from decreased LV ejection fraction (LVEF) and fractional shortening (FS) and increased LV end-systolic diameter (LVESD) and LV end-diastolic diameter (LVEDD) (Table 1). Oral administration of celecoxib or DM-celecoxib (100 mg/kg per day) significantly increased LVEF and FS and decreased LVEDD and LVESD (Table 1).

As shown in Fig. 4A and Table 2, both celecoxib and DM-celecoxib significantly reduced heart size and the heart-to-body weight ratio in a dose-dependent manner. Both drugs also prevented LV dilatation, and interstitial fibrosis developed in the DCM mouse heart (Fig. 4B). The widths of cardiomyocytes were significantly larger in the DCM mice than wild-type mice, consistent with our previous observation in isolated cardiomyocytes (Wang et al., 2010), whereas both drugs prevented this cellular hypertrophy (Fig. 4C). In addition, although high doses of celecoxib have been reported to induce apoptosis in several cell lines (Maier et al., 2004; Basu et al., 2005; Sakoguchi-Okada et al., 2007), the administration of celecoxib up to 100 mg/kg per day did not cause histological changes in cardiomyocytes.

As shown in Fig. 5, the expressions of β-MyHC isoform and BNP in the ventricular myocardium, known to be up-regulated in heart failure (Lompre et al., 1979; Sagnella, 1998; Miyata et al., 2000), were found to be markedly increased in the DCM mice, as we reported previously (Du et al., 2007). However, both celecoxib and DM-celecoxib markedly decreased the expression levels of β-MyHC isoform and BNP in the LV myocardium of the DCM mice (Fig. 5). Taken together, the treatment with celecoxib and DM-celecoxib at 100 mg/kg per day may effectively prevent cardiac remodeling and fibrosis and improve cardiac functions in the DCM mice.

The Effects of Celecoxib and DM-Celecoxib on the Mortality of the DCM Mice. Because DCM mice show a higher incidence of sudden death during the first 1 to 2 months of their growth period (Du et al., 2007), celecoxib (10, 30, 50, and 100 mg/kg per day) or DM-celecoxib (30, 50, 75, and 100 mg/kg per day) was orally administered during this period to assess their effects on the survival rate of the DCM mice. Figure 6, A and B, shows that both celecoxib and DM-celecoxib had a tendency to extend the survival of the DCM mice in a dose-dependent manner and that the treatment with 100 mg/kg per day of celecoxib and DM-celecoxib
significantly decreased mortality. These results indicated that celecoxib and DM-celecoxib have a beneficial effect in preventing premature or sudden death of the DCM mice.

Discussion

In the present study, we investigated the effects of celecoxib and its derivative DM-celecoxib on our DCM model mice with a deletion mutation ΔK210 in the TNNT2 gene. The results demonstrated that both celecoxib and DM-celecoxib reduced phosphorylation levels of Akt, GSK-3β, and mTOR and inhibited downstream transcription factors in LV myocardium. Both compounds effectively prevented hypertrophic remodeling of the DCM mouse heart, indicated by not only morphological observations but also by reduction of β-MyHC isoform and BNP expressions. These effects may have resulted in the improved LV systolic function and ultimately in the reduced mortality of DCM mice. This is the first report on the therapeutic effects of celecoxib and DM-celecoxib in an inherited DCM model. In particular, DM-celecoxib was revealed for the first time to be able to prevent cardiac remodeling and improve cardiac functions in a failing heart.

The therapeutic effects of celecoxib and DM-celecoxib on DCM seemed to be practically independent of COX-2 inhibition, because DM-celecoxib is unable to inhibit COX-2. However, as celecoxib showed a slightly stronger effect than DM-celecoxib, especially in the lower concentration range, anti-inflammatory activity might encourage the beneficial effect of celecoxib. Unfortunately, however, clinical use of celecoxib in patients with cardiovascular diseases remains controversial because of its thrombogenic potential (Bresalier et al., 2005). Several studies report a dose-related increase in the incidence of cardiovascular events in patients receiving COX-2 inhibitors (Solomon et al., 2005; Antman et al., 2007). Con-

Fig. 5. The effects of celecoxib and DM-celecoxib on β-MyHC and BNP in the LV myocardium of the DCM mice. A and B, expression levels of MyHC isoforms in the LV myocardium. One-month-old DCM mice were treated with daily administration of celecoxib, DM-celecoxib, or vehicle for 4 weeks. Wild-type mice were treated with vehicle in the same way. α-MyHC (top arrows) and β-MyHC (bottom arrows) were separated by electrophoresis as described under Materials and Methods. Percentages of β-isof orm levels in total MyHC levels are shown as the means ± S.E. of six mice in each group. C, the expression levels of BNP were analyzed by Western blot. The ratios of BNP to GAPDH are shown as the means ± S.E. of six mice in each group. Statistical significance was determined by the one-way ANOVA followed by post hoc Tukey’s multiple comparison test. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
Considering the thrombotic adverse effect of celecoxib, DM-celecoxib might be a better candidate as a new drug for prevention of cardiac hypertrophy.

To date, the deletion mutation \( \Delta K^{210} \) in TNNT2, as an inheritable cause of familial dilated cardiomyopathy, has been defined clinically (Kamisago et al., 2000; Hanson et al., 2002), and major clinical manifestations of DCM patients have been successfully reproduced in our knock-in mouse model (Du et al., 2007). Physiologic studies suggest that decreased myofilament \(Ca^{2+} \) sensitivity is a primary functional defect triggering the pathogenesis of this disease (Morimoto et al., 2002; Du et al., 2007), and a decrease of myocardial contractility caused by the TnT mutation in our DCM model mice resulted in up-regulation of type 2 iodothyronine deiodinase (Dio2), which generates tri-iodothyronine (Wang et al., 2010). Recent studies have demonstrated that triiodothyronine induces cardiac growth by activation of Akt (Kenessey and Ojamaa, 2006). Therefore, up-regulation of Dio2 could cause cardiac hypertrophy through Akt activation. Moreover, we observed up-regulation of Dio2 expression not only in the heart of DCM model mice but also in the heart of postmyocardial infarction mice (Wang et al., 2010). Thus, Dio2-mediated increase in Akt activity could occur in various types of cardiac hypertrophy. Therefore, inhibitors of Akt-mediated signal transduction, such as DM-celecoxib, might have a therapeutic value for a wide spectrum of cardiac hypertrophy. Further studies using different types of cardiac hypertrophy models are required to confirm the therapeutic value of DM-celecoxib on cardiac hypertrophy in general.

Since we reported that celecoxib inhibited the Wnt/\( \beta \)-catenin signaling pathway (Sakoguchi-Okada et al., 2007; Takahashi-Yanaga et al., 2008), and since this pathway has been reported to play an important role in the development of cardiac hypertrophy (van de Schans et al., 2007; Malekar et al., 2010), we paid special attention to this signaling pathway. The expression level of \( \beta \)-catenin was greater in the DCM mice than wild type, and, moreover, \( \beta \)-catenin was detected in the nuclei, indicating that the Wnt/\( \beta \)-catenin signaling pathway was persistently activated in the heart of the DCM mice. In fact, the expression levels of TCF7L2 and c-Myc, the target gene products, were elevated in the DCM mouse heart. In contrast, however, we could not observe increased expression of cyclin D1, of which transcription is also regulated by \( \beta \)-catenin. It is noteworthy that van de Schans et al. (2007) also reported inconsistency in the expressions of \( \beta \)-catenin and its downstream genes (c-Myc, c-Fos, c-Jun, and cyclin D1). They found that the amounts of \( \beta \)-catenin, c-Myc, and c-Fos proteins were increased significantly in hypertrophic heart induced by pressure overload, whereas c-Jun and cyclin D1 were down-regulated. Although we could not address this discrepancy at present, other regulatory factors might be involved to regulate the expression of these proteins in heart, considering that matured cardiomyocytes do not proliferate. Further study is required to clarify this point.

The present study not only provided new insight into the application of DM-celecoxib for the treatment of inherited DCM but also elucidated a key role played by the Akt/GSK-3\( \beta \)-catenin signaling pathway in pathological development of DCM, which may contribute to a better understanding of DCM.

**References**


Antos CL, McKinsey TA, Frey N, Kutsche W, McAnally J, Shelton JM, Richardson JA, Hill JA, and Olson EN (2002) Activated glycogen synthase-3\( \beta \)-catenin was greater in the DCM mice than wild type, and, moreover, \( \beta \)-catenin was detected in the nuclei, indicating that the Wnt/\( \beta \)-catenin signaling pathway was persistently activated in the heart of the DCM mice. In fact, the expression levels of TCF7L2 and c-Myc, the target gene products, were elevated in the DCM mouse heart. In contrast, however, we could not observe increased expression of cyclin D1, of which transcription is also regulated by \( \beta \)-catenin. It is noteworthy that van de Schans et al. (2007) also reported inconsistency in the expressions of \( \beta \)-catenin and its downstream genes (c-Myc, c-Fos, c-Jun, and cyclin D1). They found that the amounts of \( \beta \)-catenin, c-Myc, and c-Fos proteins were increased significantly in hypertrophic heart induced by pressure overload, whereas c-Jun and cyclin D1 were down-regulated. Although we could not address this discrepancy at present, other regulatory factors might be involved to regulate the expression of these proteins in heart, considering that matured cardiomyocytes do not proliferate. Further study is required to clarify this point.

The present study not only provided new insight into the application of DM-celecoxib for the treatment of inherited DCM but also elucidated a key role played by the Akt/GSK-3\( \beta \)-catenin signaling pathway in pathological development of DCM, which may contribute to a better understanding of DCM.

**Authorship Contributions**

**Participated in research design:** Takahashi-Yanaga, Morimoto, and Sasaguri.

**Conducted experiments:** Fan, Takahashi-Yanaga, and Zhan.

**Contributed new reagents or analytic tools:** Igawa and Tomooka.

**Performed data analysis:** Fan, Takahashi-Yanaga, Morimoto, and Sasaguri.

**Wrote or contributed to the writing of the manuscript:** Fan, Takahashi-Yanaga, Morimoto, and Sasaguri.

**Other:** Takahashi-Yanaga acquired funding for the research.

**Fig. 6.** The effects of celecoxib and DM-celecoxib on the survival of the DCM mice. From one-month of age, the DCM mice were treated with daily oral administration of celecoxib (A) (10 mg/kg, \( n = 22 \); 30 mg/kg, \( n = 22 \); 50 mg/kg, \( n = 22 \); 100 mg/kg, \( n = 20 \)). DM-celecoxib (B) (30 mg/kg, \( n = 22 \); 50 mg/kg, \( n = 20 \); 75 mg/kg, \( n = 20 \); 100 mg/kg, \( n = 22 \)) as described under Materials and Methods. Results shown in the Kaplan-Meier survival curves were analyzed by log-rank test. Mice treated with celecoxib or DM-celecoxib at 100 mg/kg per day had a significantly longer life span than those treated with vehicle (\( p < 0.05 \)).


Address correspondence to: Fumi Takahashi-Yanaga, Department of Clinical Pharmacology, Faculty of Medical Sciences, Kyushu University, Fukuoka 812-8582, Japan. E-mail: yanaga@clipharm.med.kyushu-u.ac.jp