

## Chronic Pressure Overload Induces Cardiac Hypertrophy and Fibrosis via Increases in SGLT1 and IL-18 Gene Expression in Mice

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### Summary

Increased gene expression levels of sodium-glucose cotransporter 1 (SGLT1) are associated with hypertrophic and ischemic cardiomyopathy. However, it remains unclear whether chronic pressure overload increases SGLT1 expression, which in turn induces hypertrophic cardiomyopathy. We hypothesized that pressure overload could increase SGLT1 gene expression, leading to the development of hypertrophic cardiomyopathy.

To create pressure overload-induced cardiomyopathy, transverse aortic constriction (TAC) was performed in SGLT1-deficient (SGLT1<sup>-/-</sup>) and wild-type (WT) mice. Six weeks after surgery, all mice were investigated. We observed a reduction of left ventricular fractional shortening and left ventricular dilatation in TAC-operated WT but not in TAC-operated SGLT1<sup>-/-</sup> mice. SGLT1, interleukin 18, connective tissue growth factor, and collagen type 1 gene expression levels were increased in TAC-operated WT mouse hearts compared with that of sham-operated WT mouse hearts. Moreover, heart/body weight ratio and ventricular interstitial fibrosis were increased in TAC-operated WT mice compared with that of sham-operated WT mice. Interestingly, these factors did not increase in TAC-operated SGLT1<sup>-/-</sup> mice compared with that of sham-operated WT and SGLT1<sup>-/-</sup> mice. Phenylephrine, an adrenergic  $\alpha_1$  receptor agonist, caused cardiomyocyte hypertrophy in neonatal WT mouse hearts to a significantly larger extent than in neonatal SGLT1<sup>-/-</sup> mouse hearts.

In conclusion, the results indicate that chronic pressure overload increases SGLT1 and IL-18 gene expressions, leading to the development of hypertrophic cardiomyopathy. These results make SGLT1 a potential candidate for the therapeutic target for hypertension-induced cardiomyopathy.

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**Key words:** Hypertension, Cardiomyopathy

Cardiac hypertrophy is an adaptive response in several cardiac diseases, such as chronic hypertension, whereas sustained hypertrophy, which induces cardiac fibrosis and heart failure, is an independent risk factor for cardiac morbidity and mortality.<sup>1)</sup> Therefore, identification of the mechanisms involved in the development of cardiac hypertrophy is important for the treatment of sustained hypertrophy induced cardiomyopathy. Previous studies have demonstrated several factors influencing the induction of hypertrophic cardiomyopathy. For example, chronic pressure overload-induced cardiomyopathy, induced by transverse aortic constriction (TAC) is associated with the activation of sympathetic nerves and the

renin-angiotensin-aldosterone system, which causes activation of hypertrophic signaling pathways, such as extracellular signal-regulated kinase 1/2 (ERK1/2), p38 mitogen-activated protein kinase (p-38MAPK), GATA binding protein 4 (GATA4), and canonical transient receptor potential (TRPC) channels.<sup>2-5)</sup> However, despite this knowledge, current clinical therapies are not enough to treat hypertrophic cardiomyopathy. As the prevalence of hypertrophic cardiomyopathy increases in an aging society, it will be important to find new therapeutic targets. Recently, expression of a novel cardiac glucose transporter, the sodium-glucose cotransporter 1 (SGLT1) was described, and the SGLT1 gene expression is elevated in hy-

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peritrophic, ischemic, and diabetic cardiomyopathy in humans.<sup>6,7)</sup> Moreover, increases in SGLT1 protein expression cause cardiac remodeling, such as hypertrophy and increased interstitial fibrosis in mice,<sup>8)</sup> but it is not clear whether SGLT1 participates in the development of chronic hypertension-induced hypertrophic cardiomyopathy. We first investigated the effects of SGLT1 on cardiac hypertrophy and fibrosis, which leads to cardiac contractile dysfunction, using a mouse model of TAC-induced hypertrophic cardiomyopathy in SGLT1-deficient (SGLT1<sup>-/-</sup>) mice. Previous studies have demonstrated that chronic pressure overload activates p-38MAPK,<sup>3)</sup> ERK<sup>2)</sup> and adenosine monophosphate-activated kinase (AMPK) signaling pathways.<sup>9)</sup> Moreover, activation of AMPK upregulated SGLT1 gene expression in cardiomyocytes and translocated SGLT1 to the plasma membrane.<sup>10)</sup> Interestingly, AMPK activation also enhanced peroxisome proliferator-activated receptor- $\alpha$  (PPAR $\alpha$ ) to inhibit cardiac hypertrophy via the ERK1/2 signaling pathway.<sup>11)</sup> It is also unknown if the p-38MAPK, ERK, and AMPK-SGLT1 signaling pathways have a role in the development of chronic pressure overload-induced hypertrophic cardiomyopathy. Therefore, we also examined the relationship among the p-38MAPK, ERK, and AMPK-SGLT1 signaling pathways.

## Methods

**Ethics:** The study was conducted following the recommendations stipulated in the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health. This study was approved by the Animal Care Committee of Iwate Medical University. The protocol was approved by the Committee on the Ethics of Animal Experiments of Iwate Medical University (Permit Number: 27-25). All the surgeries were performed under sodium pentobarbital and/or isoflurane anesthesia and all efforts were made to minimize suffering on the animals.

**Experimental animals and transverse aortic constriction (TAC) operation:** SGLT1<sup>-/-</sup> mice were generated as described previously.<sup>12)</sup> The genotypes of SGLT1<sup>-/-</sup> mice were identified by polymerase chain reaction (PCR) with the use of tail genomic DNA as previously described.<sup>12)</sup> In order to create chronic pressure overload-induced cardiomyopathy, thoracic transverse aortic constriction (TAC) was performed as described elsewhere.<sup>5)</sup> Briefly, SGLT1<sup>-/-</sup> and wild-type (WT) mice (10-weeks old) were anesthetized with isoflurane (2%-4%) and intubated with a 22-gauge polyethylene catheter. After the mice were ventilated with a rodent ventilator (Harvard Apparatus, Holliston, MA), the chest was opened at the left second intercostal space and the transverse section of the aorta was freed. A 7-0 nylon suture was passed around the aorta between the right innominate and left common carotid arteries. The aorta was tied with a nylon suture using a 27-gauge needle, and the needle was promptly removed. In sham-operated mice, the same procedure was performed except for the aortic constriction. Finally, the chest wall was closed with negative pressure in the chest cavity. Six weeks after surgery, all experiments were performed on both types of mice. In all the experiments, the mice were

anesthetized by Sodium pentobarbital (30 mg/kg) applied intraperitoneally and/or isoflurane inhalation. The adequacy of anesthesia was monitored by heart rate, the degree of motion of the sternum, and movement of the extremities.

**Diet:** Standard diet (CE-2) was obtained from CLEA Japan Inc., Japan. It contained 3394 kcal/kg metabolizable energy and was composed of 49.6% starch, 25.3% protein, 4.9% fiber, and 4.3% fat, minerals, and vitamins. The glucose-galactose-free diet was prepared by Oriental Yeast Co. Ltd., Japan and contained 3016 kcal/kg metabolizable energy. It was composed of 39.8% protein, 31.7% fiber, and 28.5% fat, minerals, and vitamins.

**Echocardiography:** Cardiac contractile function was assessed with echocardiography (ProSound 70 Hitachi Aroka, Tokyo, Japan) in isoflurane anesthetized sham-operated WT and SGLT1<sup>-/-</sup> mice, and TAC-operated WT and SGLT1<sup>-/-</sup> mice ( $n = 8$  for each) as previously described.<sup>13)</sup> Hearts were viewed at the level of the papillary muscles along the short axis. M-mode tracings with the average of three consecutive beats were used to measure the left ventricular end-diastolic dimension (LVEDd) and end-systolic dimension (LVESd). Fractional shortening (LVFS) was calculated as follows:  $(LVEDd - LVESd) / LVEDd \times 100\%$ .

**Histology:** Sham-operated WT and SGLT1<sup>-/-</sup> mice and TAC-operated WT and SGLT1<sup>-/-</sup> mice ( $n = 8$  for each) were anesthetized with sodium pentobarbital (30 mg/kg i.p.). Hearts were quickly excised after a midline sternal incision. The hearts were fixed with a 30% solution of formalin in phosphate-buffered saline at 24°C for more than 24 hours, embedded in paraffin, and then cut serially from the apex to the base. Six sections were stained with hematoxylin/eosin or Masson's trichrome for histopathological analysis. Transverse sections were captured digitally, and the cross-sectional diameter of at least 20 cardiomyocytes in each section was measured using the image analyzing software MacSCOPE (MITANI Corporation, Tokyo). For each preparation, three sections were measured and averaged. To assess the degree of fibrosis, digital microscopic images were taken from the sections stained with Masson's trichrome stain using light microscopy with a digital camera system. The measurements were performed on 3 images from different parts of the left ventricle in each preparation as described previously.<sup>14)</sup> The fibrosis fraction was obtained by calculating the ratio of total connective area to total myocardial area from 3 images in each preparation.

**Mouse neonatal cardiomyocyte isolation and its surface area measurement:** Ventricular cardiomyocytes were isolated from 1-to-3-day-old WT or SGLT1<sup>-/-</sup> mice and cultured for experiments as described previously.<sup>15,16)</sup> Cultured cardiomyocytes were maintained in Dulbecco's Modified Eagle's medium supplemented with 10% fetal bovine serum, and all experiments were performed in Dulbecco's Modified Eagle's medium supplemented with 0% fetal bovine serum. Immunocytochemical analyzes were performed as described previously.<sup>15,16)</sup> Alexa 568-conjugated anti-mouse antibodies were purchased from Molecular Probes (Eugene, OR) and anti-cardiac Troponin I (anti-cTnI) antibody (MAB1691) from Millipore (Billerica,

MA). After treatment with 20  $\mu$ M phenylephrine for 48 hours, the cardiomyocytes were fixed by 4% paraformaldehyde. The fixed cardiomyocytes were incubated with anti-cTnI antibodies for 2 hours at 4°C, followed by Alexa 568-conjugated anti-mouse antibodies for 2 hours. The cardiomyocyte surface area was measured on cTnI-stained cardiomyocytes.<sup>15,16</sup> ImageJ Version 1.38 was used to estimate cardiomyocyte surface area. We measured 100 cells in each condition, including phenylephrine-treated cardiomyocytes and vehicle (water)-treated cardiomyocytes.

**Quantification of mRNA by real-time PCR:** Total RNA was prepared from the ventricular myocardium of anesthetized sham-operated WT and SGLT1<sup>-/-</sup> mice and TAC-operated WT and SGLT1<sup>-/-</sup> mice ( $n = 8$  for each) with RNeasy<sup>TM</sup> RNA Tissue Miniprep System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Five hundred nanograms of total RNA was used as a template for reverse transcription with the SuperScript<sup>®</sup> III First-Strand synthesis system (Invitrogen, Carlsbad, CA). Real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis was performed with an ABI Step One Real-Time PCR System using Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA) to detect SGLT1, glucose transporter 1 (GLUT1), glucose transporter 4 (GLUT4), glucose transporter 8 (GLUT8), connective tissue growth factor (CTGF), atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), collagen type I (collagen 1), interleukin-1 $\beta$  (IL-1 $\beta$ ), interleukin-18 (IL-18), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The expression of each gene was normalized to that of GAPDH mRNA because the expression of GAPDH mRNA was constant between groups.

**Western blot analysis:** Total protein was extracted from the snap-frozen left ventricle in ice-cold Cell Lysis Buffer (Cell Signaling Technology, Danvers, MA) with Protease Inhibitor Cocktail (BD Biosciences, San Jose, CA). Protein concentration was determined by protein assay (DC protein assay kit, Bio-Rad Laboratories, Inc., Hercules, CA). Equal amounts (20  $\mu$ g) of total protein were separated on each gel lane by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 10%) and transferred onto polyvinylidene difluoride membrane (ATTO Co., Tokyo, Japan). Rabbit polyclonal antibodies for adenosine monophosphate-activated kinase  $\alpha$  (AMPK $\alpha$ ), phosphorylated-AMPK $\alpha$ , extracellular signal-regulated kinase1/2 (ERK1/2), phosphorylated-ERK1/2, p-38 mitogen-activated protein kinase (p-38MAPK), and phosphorylated-p-38MAPK, (Cell Signaling Technology) were used. The secondary antibodies were goat anti-rabbit IgG-horse radish peroxidase (Santa Cruz Biotechnology Inc.). The signals from immunoreactive bands were visualized by an Amersham ECL system (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, UK) and quantified using densitometric analysis.

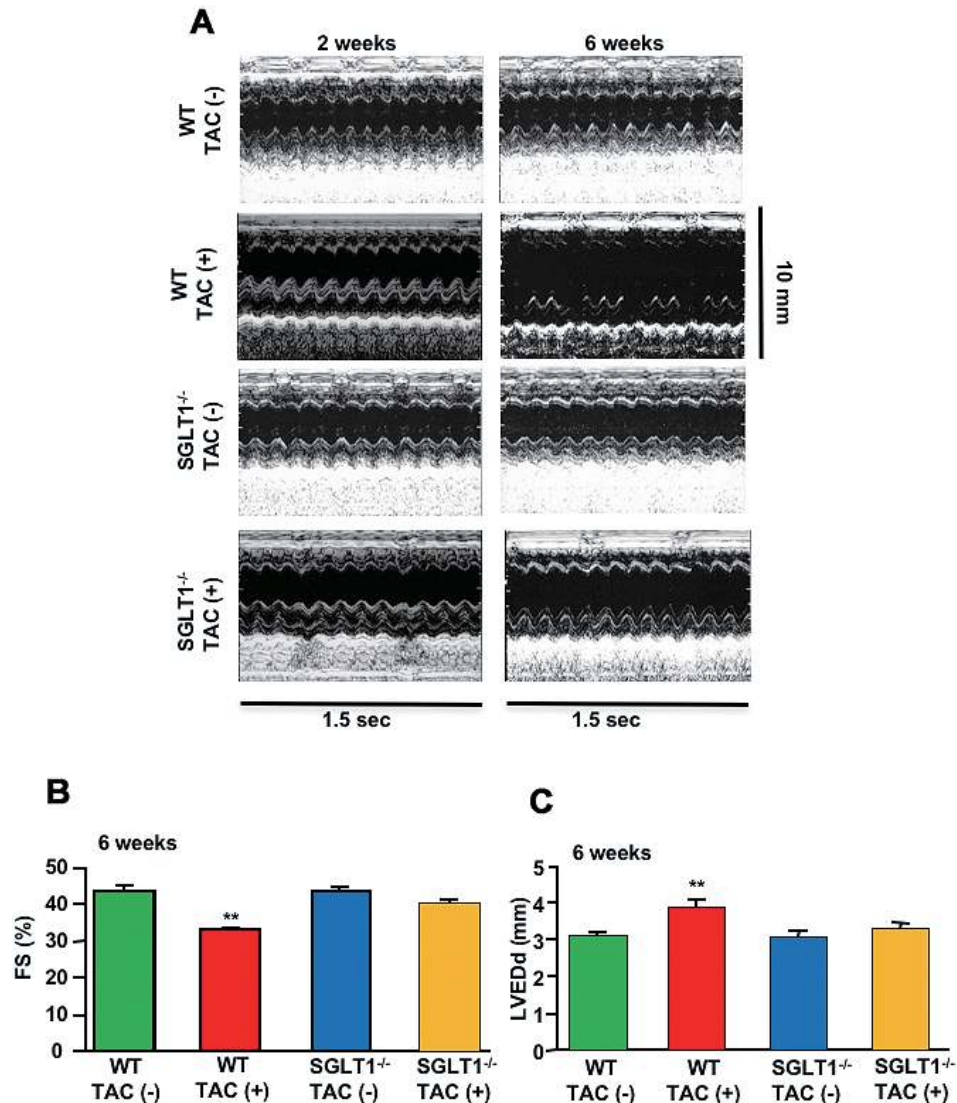
**Data analysis:** All data are shown as the mean  $\pm$  SE. An analysis of variance with Bonferroni's test was used for the statistical analysis of multiple comparisons of data.  $P < 0.05$  was considered significant.

## Results

**SGLT1 induced left ventricular contractile dysfunction and dilatation:** To evaluate whether SGLT1 could play a role in the induction of contractile dysfunction and chamber dilatation, left ventricular FS (LVFS) and LVEDd using echocardiography were examined in TAC-operated WT and SGLT1<sup>-/-</sup> mice. Representative M-mode echocardiograms are shown in Figure 1A. The LVFS and LVEDd were similar among four different groups of a mouse (sham-operated WT, sham-operated SGLT1<sup>-/-</sup>, TAC-operated WT, and TAC-operated SGLT1<sup>-/-</sup> mouse) 2 weeks after TAC operation. In contrast, compared with the sham-operated WT and SGLT1<sup>-/-</sup>, and TAC-operated SGLT1<sup>-/-</sup> mouse, the TAC-operated WT mouse had impaired left ventricular contractility and left ventricular dilatation as demonstrated by the markedly reduced LVFS 6 weeks after TAC operation (Figure 1A, right panel). TAC-operated WT mice had significantly decreased mean LVFS and increased mean LVEDd compared to that of sham-operated WT mice (Figure 1B-C). Interestingly, TAC-operated SGLT1<sup>-/-</sup> mice showed no difference in mean LVFS and LVEDd compared to that of sham-operated WT and SGLT1<sup>-/-</sup> mice (Figure 1B-C). The heart rate (HR) calculated from M-mode echocardiogram did not differ among the four different groups.

**SGLT1 induced cardiac hypertrophy and increased brain natriuretic peptide (BNP) and interleukin 18 (IL-18) gene expression:** To evaluate whether SGLT1 could participate in the induction of cardiac hypertrophy, the heart/body weight ratio and the left ventricular cardiomyocyte diameter in TAC-operated WT and SGLT1<sup>-/-</sup> mice and size of cardiomyocytes in neonatal WT and SGLT1<sup>-/-</sup> mouse hearts were examined. The heart/body weight ratio increased in TAC-operated WT mice compared to sham-operated WT mice (Figure 2A). In addition, the cardiomyocyte diameter significantly increased in TAC-operated WT mouse compared to sham-operated WT mouse left ventricles (Figure 2B). Moreover, the mRNA expression levels of ANP, BNP, and IL-18 but not of IL-1 $\beta$  were significantly increased in TAC-operated WT mouse ventricles compared to sham-operated WT mouse ventricles (Figure 2C-F). In contrast, the heart/body weight ratio and the expression of ANP, BNP, IL-18, and IL-1 $\beta$  in the ventricle were not different among TAC-operated SGLT1<sup>-/-</sup>, sham-operated WT, and SGLT1<sup>-/-</sup> mice (Figure 2A, C-F). Moreover, the cardiomyocyte diameter was significantly reduced in TAC-operated SGLT1<sup>-/-</sup> compared with that of TAC-operated WT mouse left ventricles (Figure 2B). Representative neonatal cardiomyocytes stained with anti-cTnI antibody are shown in Figure 3A. Phenylephrine, an adrenergic  $\alpha_1$  receptor agonist, at a concentration of 20  $\mu$ M increased the surface area of cardiomyocytes in neonatal WT mice compared with that of neonatal SGLT1<sup>-/-</sup> mice. Phenylephrine significantly increased the surface area of cardiomyocytes in a neonatal SGLT1<sup>+/+</sup> mice to a significantly larger extent than in neonatal SGLT1<sup>-/-</sup> mice, which indicates that SGLT1 directly induced cardiomyocyte hypertrophy in WT mouse hearts (Figure 3B).

**SGLT1 increased myocardial fibrosis and fibrotic gene expression:** We examined the participation of SGLT1 in



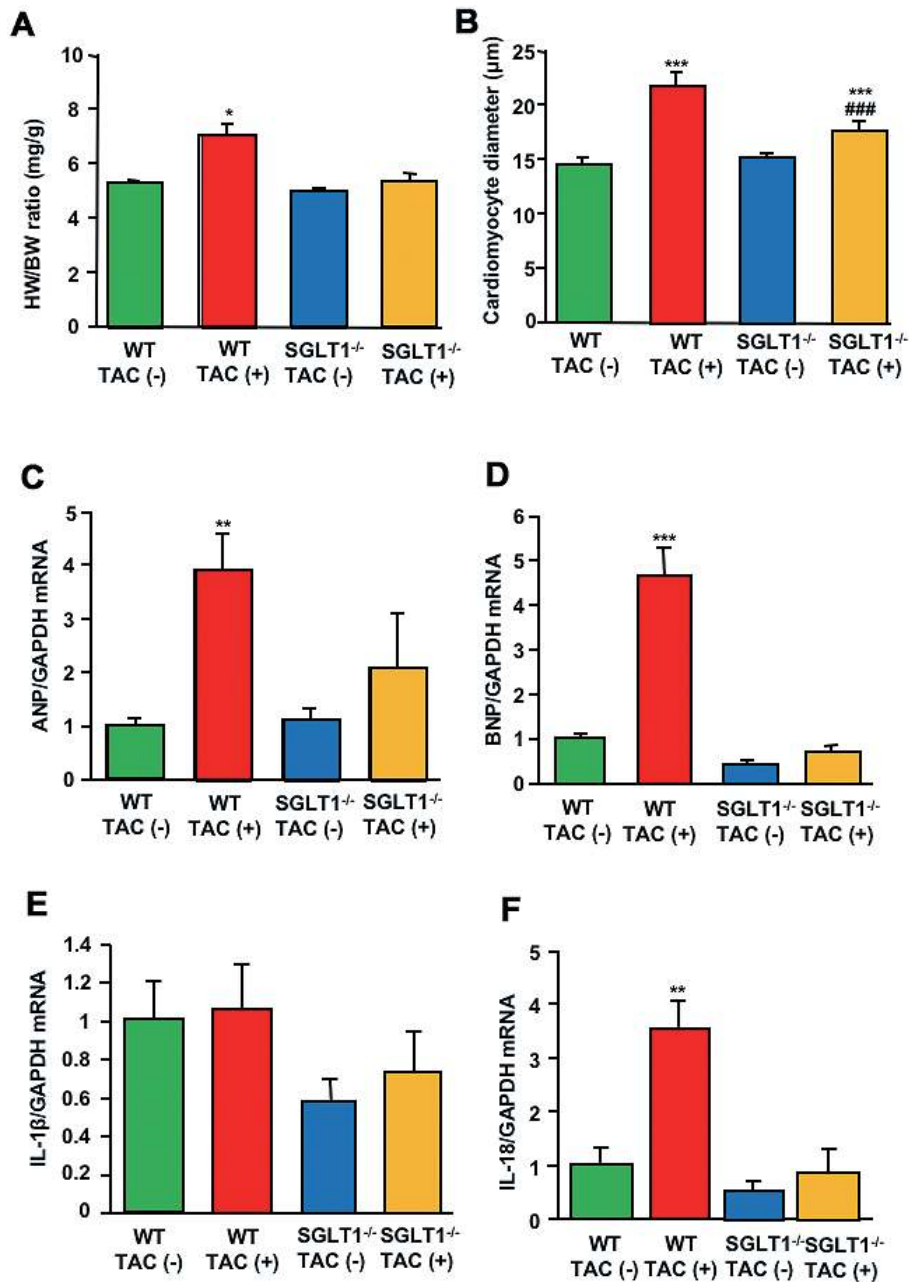
**Figure 1.** The role of SGLT1 in the development of left ventricular contractile dysfunction. Representative M-mode echocardiograms (A) of four different groups of a mouse (sham-operated WT, sham-operated SGLT1<sup>-/-</sup>, TAC-operated WT, and TAC-operated SGLT1<sup>-/-</sup> mouse) at 12 and 16 weeks old. Left ventricular fractional shortening (LVFS) (B) and left ventricular end-diastolic dimension (LVEDd), (C) measured from four different groups of 16-week-old mice. Data are the mean  $\pm$  SE obtained from 68 mice for each group. \*\* $P < 0.01$  versus that of sham-operated WT mice. Sham-operated indicates TAC (-); TAC-operated, TAC (+); 2 weeks, 2 weeks after TAC operation; and 6 weeks, 6 weeks after TAC operation.

the induction of TAC-induced increase in myocardial fibrosis. Extensive interstitial fibrosis in the left ventricle was observed in a TAC-operated WT mouse compared with a sham-operated WT mouse (Figure 4A). The degree of myocardial fibrosis in the left ventricle was significantly greater in TAC-operated WT mice compared to sham-operated WT mice (Figure 4B). In contrast, the degree of myocardial fibrosis in the left ventricle was not increased in TAC-operated SGLT1<sup>-/-</sup> mice compared to sham-operated WT and SGLT1<sup>-/-</sup> mice (Figure 4A, B). Next, we examined the role of SGLT1 in TAC-inducing upregulation of CTGF and collagen 1 profibrotic genes, to investigate whether these morphological observations were

accompanied by alterations in gene expression relevant to fibrotic changes. The expression of CTGF and collagen 1 was significantly upregulated in TAC-operated WT mouse ventricles compared to sham-operated WT mouse ventricles (Figure 4C, D). In contrast, gene expression did not change among TAC-operated SGLT1<sup>-/-</sup>, sham-operated WT, and SGLT1<sup>-/-</sup> mouse ventricles (Figure 4C, D).

**TAC increased gene expression of SGLT1, GLUT1, and GLUT8 in WT mouse ventricles:** We examined the effects of chronic pressure overload-induced by TAC on ventricular mRNA expression of SGLT1, GLUT1, GLUT4, and GLUT8 genes in WT and SGLT1<sup>-/-</sup> mouse hearts. SGLT1 mRNA expression was significantly in-



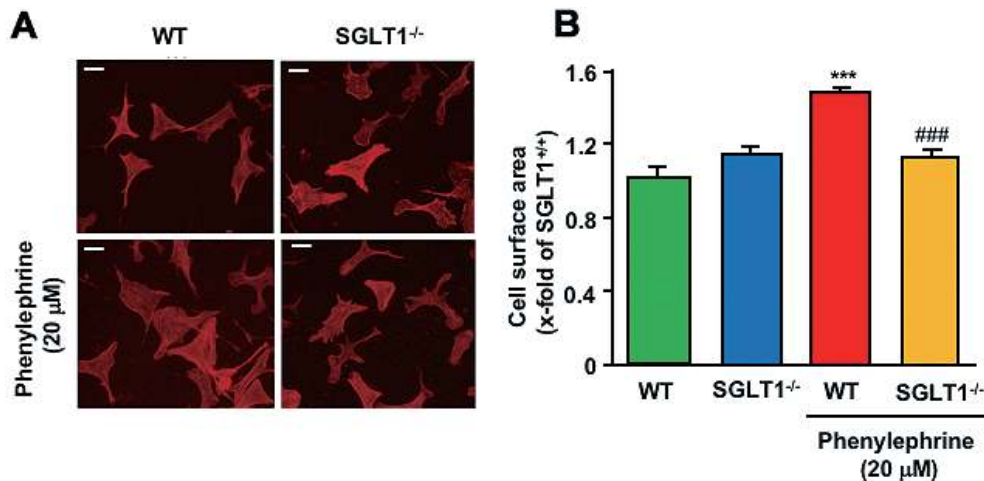


**Figure 2.** The role of SGLT1 in the induction of cardiac hypertrophy and left ventricular gene expression of atrial natriuretic peptide (ANP) brain natriuretic peptide (BNP), interleukin 1 $\beta$  (IL-1 $\beta$ ), and interleukin 18 (IL-18). Heart/body weight ratio (A) and a cross-sectional diameter of left ventricular cardiomyocyte (i.e., cardiomyocyte diameter) (B), and left ventricular ANP (C), BNP (D), IL-1 $\beta$  (E), and IL-18 (F) mRNA expression measured from four different groups of 16-week-old mice. Data are the mean  $\pm$  SE obtained from 6-8 mice for each group.  $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  versus that of sham-operated WT mice. ### $P < 0.001$  versus that of TAC-operated WT mice. Sham-operated indicates TAC (-); and TAC-operated, TAC (+).

creased in TAC-operated WT mouse ventricles compared to sham-operated WT mouse ventricles (Figure 5A). In contrast, SGLT1 mRNA was not detected in SGLT1<sup>-/-</sup> mouse ventricles, regardless of TAC operation (Figure 5A). Moreover, the mRNA expression of GLUT1 and GLUT8, but not GLUT4, were also increased in TAC-operated WT mouse ventricles compared with that of

sham-operated WT mouse ventricles (Figure 5B-D). In contrast, the mRNA expression levels of GLUT1 and GLUT4, but not SGLT1 and GLUT8, increased in both sham-operated and TAC-operated SGLT1<sup>-/-</sup> mouse ventricles compared with that of sham-operated WT mouse ventricles (Figure 5B-D).

**TAC-induced AMPK and ERK but not p38MAPK ac-**



**Figure 3.** Direct effects of SGLT1 on the induction of cardiac hypertrophy in mouse neonatal cardiomyocytes. A representative example of neonatal cardiomyocytes (A) isolated from a WT and SGLT1<sup>-/-</sup> mouse heart in the absence or presence of 20 μM phenylephrine. Scale bar = 50 μm. B: Comparison of the surface area of neonatal cardiomyocytes in WT and SGLT1<sup>-/-</sup> mice in the absence or presence of 20 μM phenylephrine. Data are the mean ± SE obtained from 6 mice for each group. \*\*\**P* < 0.001 versus that of WT mouse neonatal cardiomyocytes in the absence of phenylephrine, ###*P* < 0.001 versus that of neonatal WT mouse cardiomyocytes in the presence of phenylephrine.

**tivation in both WT and SGLT1<sup>-/-</sup> mouse ventricles:** We examined the effects of chronic pressure overload-induced by TAC on ventricular AMPK and ERK protein activation in WT and SGLT1<sup>-/-</sup> mouse hearts. The phosphorylated-AMPKα/total-AMPKα ratio was significantly increased in TAC-operated WT mouse ventricles compared to sham-operated WT mouse ventricles (Figure 6A). Moreover, the phosphorylated-ERK1/2/total ERK1/2 ratio was also significantly increased in TAC-operated WT mouse ventricles compared with that of sham-operated WT mouse ventricles (Figure 6B). Interestingly those ratios were further increased in both sham-operated and TAC-operated SGLT1<sup>-/-</sup> mouse ventricles compared with that of TAC-operated WT mouse ventricles (Figure 6A-B). In contrast, the phosphorylated-p-38MAPK/total p-38MAPK ratio was similar among four different groups of mice tested (Figure 6C).

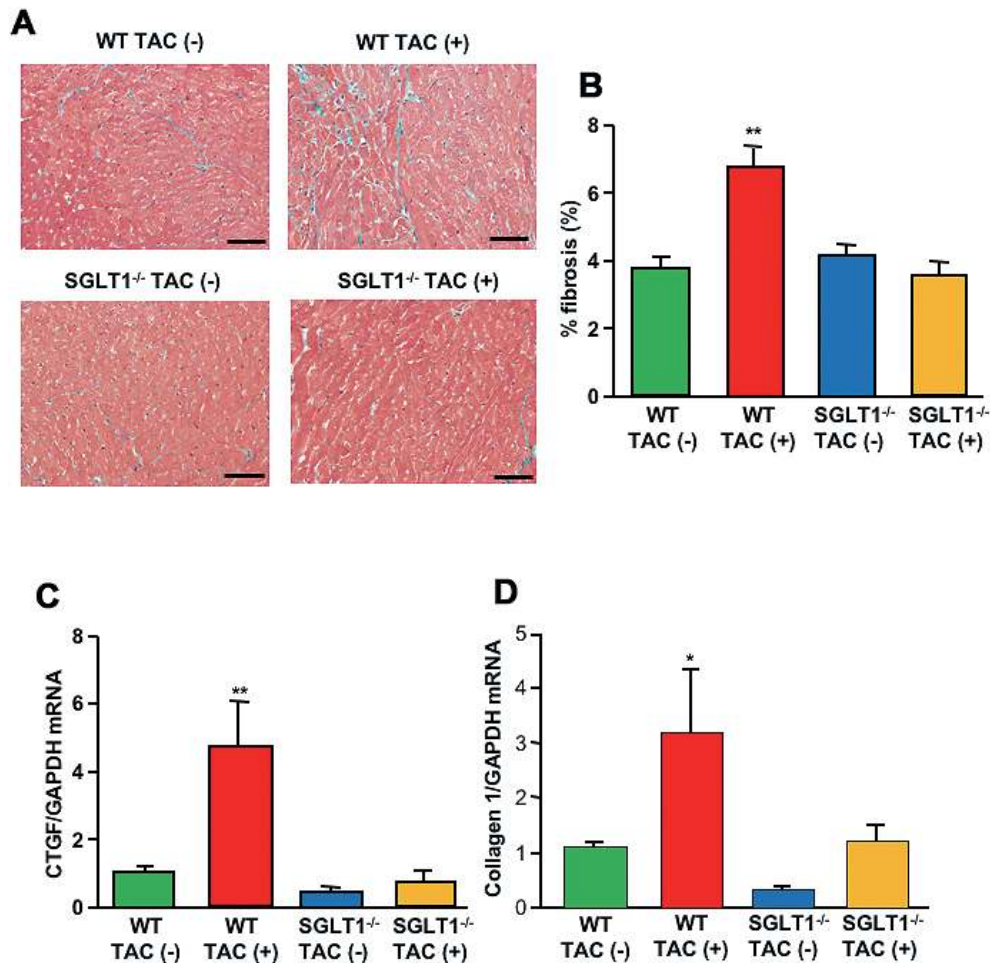
## Discussion

We demonstrated that SGLT1 play a role in the development of cardiac hypertrophy in a mouse model of chronic pressure overload and in phenylephrine-stimulated neonatal mouse cardiomyocytes (Figures 2, 3). However, the mechanism for SGLT1-induced cardiac hypertrophy is still unclear. It is known that angiotensin II activated by chronic pressure overload induces cardiac hypertrophy through increasing intracellular Ca<sup>2+</sup> concentration via TRPC channel activation, suggesting that chronic pressure overload can increase intracellular Ca<sup>2+</sup> concentration.<sup>3,17</sup> In addition, it enhanced Na<sup>+</sup>-glucose co-transport, via SGLT1 elevated intracellular Na<sup>+</sup> concentration in hearts,<sup>18</sup> however, elevated intracellular Na<sup>+</sup> concentration followed by intracellular Ca<sup>2+</sup> overload that was directly linked to a higher propensity for cardiac hypertrophy.<sup>19</sup> This suggests that an increase in SGLT1 expression might elevate intra-

cellular Na<sup>+</sup> concentration followed by intracellular Ca<sup>2+</sup> overload, thus leading to cardiac hypertrophy. Ca<sup>2+</sup>/calmodulin-dependent protein kinase (CaMK) is an important downstream target of Ca<sup>2+</sup> in hypertrophic signaling pathways. Kashiwase, *et al.*<sup>20</sup> have demonstrated that CaMKII induces cardiomyocyte hypertrophy through the nuclear factor (NF)-κB signal transduction pathway.

Chronic pressure overload using TAC induces activation of NF-κB<sup>21</sup> leading to expression of interleukin (IL)-18.<sup>22,23</sup> In addition, it has been reported that TAC-induced cardiac hypertrophy was blunted in IL-18 null mice, suggesting that IL-18 participated in the induction of cardiac hypertrophy.<sup>24</sup> This study demonstrated that chronic pressure overload increased IL-18 gene expression and induced cardiac hypertrophy in WT but not in SGLT1<sup>-/-</sup> mouse hearts (Figure 2A, B, and F). Thus, SGLT1 activation may increase intracellular Ca<sup>2+</sup> concentration and induce cardiac hypertrophy in part via the NF-κB-IL-18 signal transduction pathway. Chronic pressure overload also induces cardiac hypertrophy via p-38MAPK activation.<sup>3</sup> Moreover, p-38MAPK activation induces IL-18 expression.<sup>25</sup> Herein, we demonstrated that p-38MAPK activation did not differ among four different groups of mice, suggesting that p-38MAPK-IL-18 signaling pathway did not participate in cardiac hypertrophy induced by increased expression of SGLT1.

Chronic pressure overload causes AMPK activation in the heart.<sup>9</sup> Moreover, the activation of AMPK upregulated SGLT1 gene expression in cardiomyocytes and translocated it to the plasma membrane.<sup>10</sup> This study demonstrated that chronic pressure overload upregulated phosphorylated-AMPK protein expression and SGLT1 gene expression leading to cardiac remodeling, such as hypertrophy and interstitial fibrosis in WT mice. It also demonstrated that chronic pressure overload further upregulated phosphorylated-AMPK protein expression but

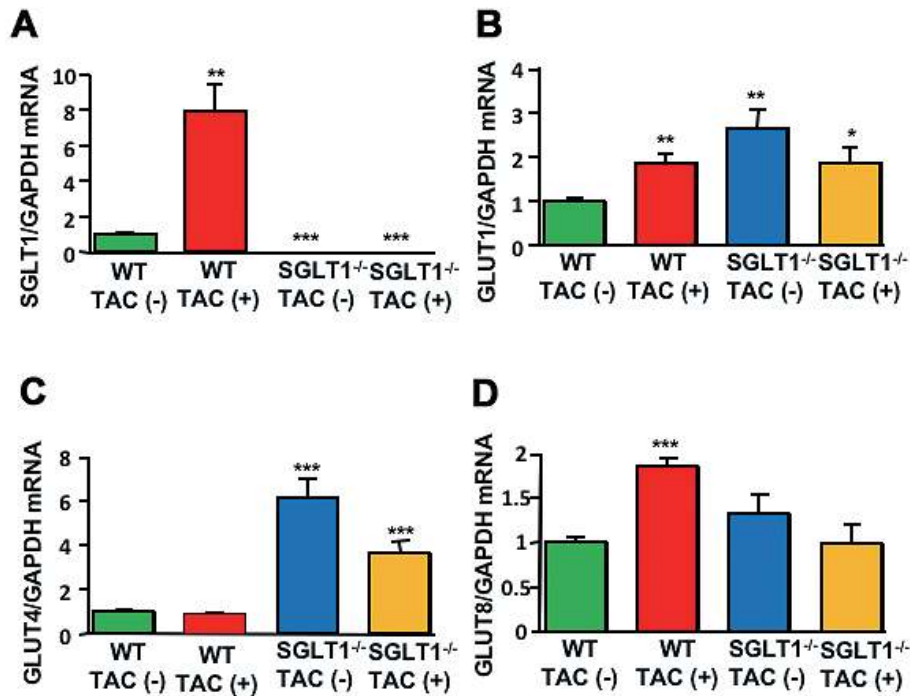


**Figure 4.** The role of SGLT1 in the increase in left ventricular fibrosis, and ventricular connective tissue growth factor (CTGF) and collagen type 1 (collagen 1) gene expression. A representative example of histology of the left ventricle (A) stained with Masson's trichrome in a sham-operated WT, sham-operated SGLT1<sup>-/-</sup>, TAC-operated WT, and TAC-operated SGLT1<sup>-/-</sup> mouse at 16 weeks old. Original magnification: 40 × Scale bar = 50 μm. Comparison of the fibrosis fraction (B) in the left ventricles of four different groups of mice. Quantitative analyses of CTGF (C) and collagen 1 (D) gene expression by real-time reverse transcriptase-polymerase chain reaction (RT-PCR) in four different groups of mouse ventricles at 16 weeks old. Data for CTGF were normalized to those of GAPDH. Data are the mean ± SE obtained from 8 mice for each group. \**P* < 0.05, \*\**P* < 0.01 versus that of sham-operated WT mouse hearts. Sham-operated indicates TAC (-); and TAC-operated, TAC (+).

did not induce SGLT1 gene expression and cardiac remodeling in SGLT1<sup>-/-</sup> mouse hearts. These results suggest that AMPK activation by itself is not sufficient to induce cardiac remodeling when SGLT1 gene expression is inhibited. Chronic pressure overloads upregulated phosphorylated-ERK protein expression and led to cardiac remodeling in WT mice (Figure 6B). In contrast, chronic pressure overload further upregulated phosphorylated-ERK protein expression but did not induce cardiac remodeling in SGLT1<sup>-/-</sup> mice. These results suggest that SGLT1 is more important than ERK activation in the development of chronic pressure overload-induced cardiac hypertrophy and interstitial fibrosis.

The derangement of cardiac energy substrate metabolism has a key role in the pathogenesis of heart disease.<sup>26)</sup> In fact, ATP is predominantly generated by fatty acids in a normal adult heart but generated mainly using glucose un-

der specific pathological conditions, such as ischemia. Recently, Kashiwagi, *et al.*<sup>27)</sup> have shown that enhanced glucose availability through SGLT1 replenishes cardiac ATP stores in the acute phase of ischemia-reperfusion cardiac injury, which protected ischemic cardiac tissues. Moreover, Endo, *et al.*<sup>28)</sup> demonstrated that increased oxidative stress in mitochondria inhibited oxidative phosphorylation in the mitochondria and increased glucose uptake into cells, which protected against acute ischemia-reperfusion-induced cardiac injury. In contrast, SGLT1 caused cardiac remodeling, such as cardiac hypertrophy and fibrosis in chronic pressure overload-induced hypertrophic cardiomyopathy (Figures 2-4) in which glucose availability through SGLT1 might have been enhanced. Previous studies have shown that a normal or increased number of myocardial capillaries were observed in physiological hypertrophy, whereas a reduction in capillary density in



**Figure 5.** Quantitative analyses of ventricular SGLT1 (A), GLUT1 (B), GLUT4 (C), and GLUT8 (D) gene expression by real-time RT-PCR in four different groups of mouse hearts at 16 weeks old. Data for SGLT1, GLUT1, GLUT4, and GLUT8 were normalized to those for GAPDH. Data are the mean  $\pm$  SE obtained from 6 mice for each group. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  versus those of sham-operated WT mouse ventricles. GLUT1 indicates glucose transporter 1; GLUT4, glucose transporter 4; GLUT8, glucose transporter 8; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Sham-operated, TAC (-); and TAC-operated, TAC (+).

pathological hypertrophy induced myocardial hypoxia, which led to the development of hypertrophic cardiomyopathy with contractile dysfunction.<sup>29,30</sup> Moreover, TAC-induced hypertrophy leads to a reduction in myocardial capillary density and accelerated the transition from compensatory hypertrophy to failure in VEGF-deficient mice.<sup>31</sup> These results suggest that myocardial ischemia occurs in the settings of TAC-induced hypertrophic cardiomyopathy. Thus, SGLT1 might have different effects between acute and chronic ischemia in the heart.

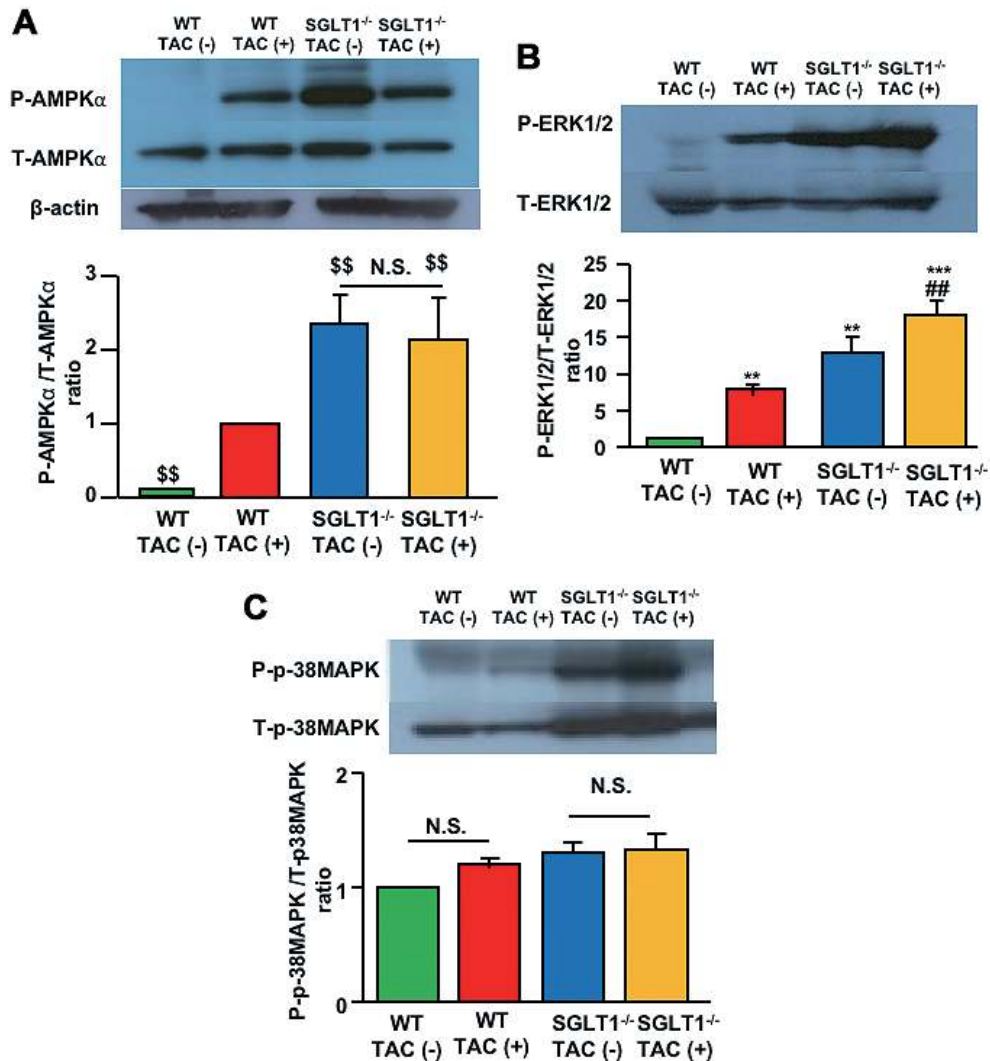
Many studies have demonstrated several factors that contribute to the development of hypertrophic cardiomyopathy induced by chronic pressure overload by demonstrating that increased sympathetic activity and the renin-angiotensin-aldosterone system activation have important roles in the development of hypertrophic cardiomyopathy.<sup>2,3</sup> In fact, adrenaline beta receptor antagonists and inhibitors of the renin-angiotensin-aldosterone system are currently used to treat chronic hypertension in clinical situations. However, because existing therapies are not sufficient to treat hypertrophic cardiomyopathy, new therapeutic targets for hypertrophic cardiomyopathy are needed. For example, ERK1/2, p-38MAPK, GATA4, TRPC channels, and PPAR $\alpha$  are therapeutic targets for hypertrophic cardiomyopathy.<sup>2,5,32</sup> To our knowledge, this study is the first to demonstrate that SGLT1 has a significant role in the development of hypertrophic cardiomyopathy in a mouse model of chronic pressure overload. SGLT1 is expressed in human hearts<sup>6</sup> and increased

SGLT1 gene expression is observed in hypertrophic, ischemic, and diabetic cardiomyopathy in human hearts.<sup>6,7</sup> These results suggest that SGLT1 as a new therapeutic target for hypertrophic cardiomyopathy in the clinical practice.

Ramratnam, *et al.*<sup>8</sup> have demonstrated that cardiac overexpression of SGLT1 increases collagen 1 gene expression and interstitial fibrosis in mouse hearts. We demonstrated that chronic pressure overload increased SGLT1, CTGF, and collagen 1 gene expression and interstitial fibrosis in mouse hearts. These results suggest that chronic pressure overload induces interstitial fibrosis, in part, through increasing SGLT1 gene expression. However, the causative link between SGLT1 and CTGF/fibrosis is still uncertain. Several studies have shown that increased collagen 1 expression and interstitial fibrosis are associated with TGF- $\beta$ <sup>33</sup> and inflammatory cytokine expressions including IL-18 in TAC-induced cardiomyopathy.<sup>34,35</sup> Therefore, SGLT1-induced increase in IL-18 expression may partly participate in the development of interstitial fibrosis in TAC-induced cardiomyopathy.

As described above, enhanced glucose uptake via SGLT1 replenishes cardiac ATP stores and inhibits oxidative phosphorylation in mitochondria, which leads to the protection of ischemic cardiac tissues.<sup>27,28</sup> Moreover, overexpression of GLUT1 prevents mitochondrial dysfunction and attenuates structural remodeling in pressure overload.<sup>36</sup> In contrast, Shimizu, *et al.*<sup>37</sup> recently demonstrated that TAC-induced an inflammatory response of adipose





**Figure 6.** Protein expression of phosphorylated (P) and total (T) AMPK $\alpha$  (A), ERK1/2 (B), and p-38MAPK (C) by western blotting. Relative expression levels are shown in bar graphs as the ventricular P-AMPK $\alpha$ /T-AMPK $\alpha$ , P-ERK1/2/T-ERK1/2, and P-p-38MAPK/T-p-38MAPK ratio in four different groups of 16-week-old mouse hearts. Data are the mean  $\pm$  SE obtained from 6 mice for each group.  $**P < 0.01$ ,  $***P < 0.001$  versus that of sham-operated WT mouse ventricles,  $^{SS}P < 0.01$  versus that of TAC-operated WT mouse ventricles,  $^{##}P < 0.01$  versus that of TAC-operated SGLT1<sup>-/-</sup> mouse ventricles. N.S. indicates not significant; AMPK $\alpha$ , Adenosine monophosphate-activated kinase  $\alpha$ ; Extracellular signal-regulated kinase 1/2, ERK1/2; p-38MAPK, p-38 mitogen-activated protein kinase; Sham-operated, TAC (-); and TAC-operated, TAC (+).

tissue and insulin resistance in the heart, which led to hypertrophic cardiomyopathy. In this study, we demonstrated that gene expression of GLUT1 and GLUT4, an insulin-sensitive GLUT, increased after SGLT1 blockade. These results suggest that SGLT1 blockade by itself inhibits hypertrophic cardiomyopathy induced by chronic pressure overload, and also protects the heart from the development of hypertrophic cardiomyopathy via increases in glucose transporter expression, such as GLUT1 and GLUT4.

This study demonstrated that phenylephrine-induced cardiomyocyte hypertrophy in WT but not SGLT1<sup>-/-</sup> mouse neonatal cardiomyocytes. This result suggests that SGLT1 directly causes cardiomyocyte hypertrophy in cardiac tissue. However, it is still unknown whether or not mecha-

nisms for phenylephrine-induced cardiomyocyte hypertrophy are equal to those of chronic pressure overload-induced cardiac hypertrophy. It has been demonstrated that phenylephrine causes ERK<sup>38)</sup> and AMPK<sup>39)</sup> activation, which induces a hypertrophic response in cardiomyocytes, suggesting that the mechanisms for phenylephrine-induced cardiomyocyte hypertrophy may be similar to those of chronic pressure overload-induced cardiac hypertrophy.

A previous study has shown that SGLT1<sup>-/-</sup> mice need to take in glucose-galactose free diet because they show symptoms of glucose-galactose malabsorption syndrome.<sup>12)</sup> Therefore, effects of diet difference between WT and SGLT1<sup>-/-</sup> mice on chronic pressure overload-induced cardiac hypertrophy cannot be excluded. However, our pre-

liminary experiments demonstrated that WT but not SGLT1<sup>-/-</sup> mice induced the cardiac hypertrophy even if both WT and SGLT1<sup>-/-</sup> mice took in glucose-galactose free diet. Thus, the diet difference might have a low potential for chronic pressure overload-induced cardiac hypertrophy.

### Conclusion

In this study, we first found that chronic pressure overload increased SGLT1 gene expression, which led to cardiomyocyte hypertrophy, interstitial fibrosis, and contractile dysfunction in mice. Moreover, chronic pressure overload increased cardiac gene expression of ANP, BNP, IL-18, CTGF, and collagen type 1. In neonatal WT mouse hearts, phenylephrine, an adrenergic  $\alpha_1$  receptor agonist, caused cardiomyocyte hypertrophy to a significantly larger extent than in neonatal SGLT1<sup>-/-</sup> mouse hearts. These results suggest that SGLT1 has an important role in the development of chronic pressure overload-induced cardiomyopathy.

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### Disclosures

**Conflicts of interest:** None.

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