Wogonin Attenuates Isoprenaline-Induced Myocardial Hypertrophy in Mice by Suppressing the PI3K/Akt Pathway

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INTRODUCTION

Myocardial hypertrophy is characterized by thickening of the ventricle wall in the heart, an adaptive response to, for example, mechanical and neurohumoral stimulations (Hill and Olson, 2008; Maillet et al., 2013). At the early stage of myocardial hypertrophy, cardiomyocytes grow in length and/or in width, and it is the main reason for ventricular thickening (Francis et al., 1993; Shimizu and Minamino, 2016). However, if the stimuli persist, apoptosis of cardiomyocytes would occur and lead to heart failure, arrhythmia, and sudden death (Lyon et al., 2015). Thus myocardial
hypertrophy is considered as the pathological foundation for multiple cardiac events (Haider et al., 1998).

β-adrenoceptors, locating on the membranes of the three major cardiac cell types (cardiomyocytes, fibroblasts, and endothelial cells), belong to the G protein-coupled receptor superfamily (Kawano et al., 2009). Their stimulation activates downstream signaling pathways that regulate different intracellular, sarcosomal, and myofilamental substrates (Feldman et al., 2005; Siryk-Bathgate et al., 2013). Neurohumoral stimulation or binding of catecholamine to β-adrenoceptors of cardiomyocytes causes the related heterotrimeric G proteins to dissociate into $G_{o/3}/G_{ï/3}$ and $G_{o/3}/G_{ï/3}$ subunits (Nienaber et al., 2003). $G_{o/3}$ activates adenyl cyclase to generate the second messenger $cAMP$, leading to increased heart rate and myocardial contractility (Kamide et al., 2015). The $G_{o/3}$ subunit activates the PI3K/Akt and mitogen-activated protein kinase (MAPK) signaling pathways both promoting myocardial hypertrophy (Esposito et al., 2002; Lohse et al., 2003; Feldman et al., 2005; Heineke and Molkentin, 2006; Go et al., 2014). Therefore, β-adrenoceptor signaling pathway should likely contain some potential targets for myocardial hypertrophy therapy.

Wogonin (5,7-dihydroxy-8-methoxyflavone; Figure 1) is a natural dihydroxy flavonoid compound isolated from the roots of Scutellaria baicalensi Georg. S. amoena C. H. Wright, or S. rivularis Wall (Tai et al., 2005). It has a variety of biological activities, including anti-oxidation, anti-inflammation, neuroprotection, and anti-carcinoma activities (Liu et al., 2011; Chirumbolo, 2013; Ku and Bae, 2015). Wogonin reportedly attenuates diabetic cardiomyopathy (Khan et al., 2016). However, whether and how wogonin attenuates β-adrenoceptor-mediated myocardial hypertrophy is unknown.

In the present study, we confirm the therapeutic effect of wogonin on isoprenaline-induced myocardial hypertrophy and identify Nedd4l as the target of wogonin. Nedd4l is a ubiquitin E3 ligase that promotes the degradation of Pik3ca and thus attenuates the over-activation of the PI3K/Akt pathway stimulated by isoprenaline treatment.

### MATERIALS AND METHODS

#### Materials and Reagents

Wogonin was purchased from Spring & Autumn Biotec Co., Ltd. (Nanjing, China). Isoprenaline was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). All-trans-retinoic acid (RA), DBcAMP, and phorbol 12, 13-dibutyrate (PDBU) were purchased from Sigma-Aldrich LLC. (Shanghai, China). MG132 was obtained from Selleck Chemical (Houston, TX, United States). The vectors pUSEamp(+)/myc-tagged Akt (constitutively active, CA) and pUSEamp(+)-Pik3ca were kindly provided by Liangyou Rui from the University of Michigan. The vectors pcDNA3HA and pGL3-Basic were provided by Dongping Wei from Nanjing First Hospital. The empty vector pAdeno-MCMV-MCS-3Flag and pDONR223 vector carrying a human Nedd4l gene were purchased from Ohio Technology Corp., Ltd. (Shanghai, China) and Public Protein/Plasmid Library (Nanjing, China), respectively. The primers 5′-TCGAGCTCAAGTTTCAATGAGCAGCGACCATGATTACA TTT-3′ and 5′-GTCATCTTGTAGTGCCGACTCCACCC TTCAATCCT-3′ were used to subclone human Nedd4l cDNA from pDONR223-Nedd4l by PCR. The PCR products were recombined with pAdeno-MCMV-MCS-3Flag vector cut by EcoR1/BamH1 to obtain the expression vector pAdeno-MCMV-MCS-3Flag-Nedd4l. Pik3ca cDNA was subcloned from pUSEamp(+)–Pik3ca using the primers 5′-GATCCCCCGGGCGTTCGAGGATCTGCTGAGCGACGCAAG AGCAAG-3′ and 5′-ATAGAATAGGGCCCCCCTCGAGTCTA GTCGAAATCATGCTG-3′. The PCR products were recombined with pcDNA3HA vector cut by EcoR1/Xho1 to obtain the expression vector pcDNA3HA-Pik3ca.

#### Animals and Treatment

Male ICR mice were purchased from Model Animal Research Center of Nanjing University. They were housed in a pathogen-free barrier facility with a 12-h light/dark cycle and given free access to food and water. Eight-week-old mice ($n = 29$) were divided into five groups as indicated (Figure 2). Under inhalation anesthesia by isoflurane, an osmotic minipump (model 2004D; Alzet, Durect Corp, Cupertino, CA, United States) was implanted subcutaneously on the back of the neck (Krenek et al., 2009; Heap et al., 2015) and the delivery of isoprenaline (5 mg/kg/day) was started. Immediately after implantation, wogonin was administered by intraperitoneal injection for 2 weeks at a dose of 1 mg/kg or 10 mg/kg once daily. All animal experiments complied with the guidelines of the Nanjing Medical University Regulations of Animal Experiments and were approved by the Animal Experiment Committee of the Nanjing Medical University.

#### Cell Culture and Treatment

Rat fetal cardiomyocytes (H9c2) were obtained from American Type Collection (ATCC, Manassas, VA, United States). H9c2 cells were cultured in Dulbecco’s modified Eagle’s high-glucose medium (DMEM) supplemented with 10% fetal bovine

![FIGURE 1](image-url) | Chemical structure of wogonin (5,7-dihydroxy-8-methoxyflavone).
FIGURE 2 | Wogonin attenuates isoprenaline-induced myocardial hypertrophy in mice. Male 8-week-old ICR mice were subcutaneously implanted with mini-pumps delivering isoprenaline (ISO, 5 mg/kg/day, \( n = 6 \)) or PBS (Control, \( n = 5 \)), and then treated by wogonin (Wog, 1 mg, 10 mg kg/day, \( n = 6 \)) via intraperitoneal injection for 14 days. (A) Representative echocardiography and H&E staining in a cross-section of the heart. (B) Quantitation of cross-sectional area of cardiomyocytes \( (n = 8) \). (C) The mRNA levels of ANP and BNP in heart were determined using RT-qPCR and normalized to 36/B4. Data are expressed as fold-change relative to the level of control group are presented as the mean ± SEM; *\( p < 0.05 \), versus control or ISO alone treated group.
serum (FBS) and 1% penicillin/streptomycin (Hyclone) in a humidified incubator in an atmosphere of 5% CO₂ at 37°C. When the cell confluence reached to 70–80%, cell culture medium was switched to DMEM containing 1% FBS and 1 µM RA for further 5-day culture before experimental treatments.

**Echocardiographic Assessments**

After the 2-week medication, mice were anesthetized with isoflurane for echocardiographic examination. The images were obtained using a Vevo 2100 system with a 45 MHz probe (Visualsonics, Toronto, ON, Canada) to evaluate the cardiac function and chamber size. M-mode tracings were used to measure interventricular septum diameter (IVSd), left ventricular posterior wall diameter (LVPWd), left ventricular end-diastolic diameter (LVIDd), and left ventricular end-systolic diameter (LVIDs). Ejection fraction (EF, %) and left ventricular (LV) mass were calculated as left ventricular end-systolic volume (LVEDV) = 7.0/(2.4 + LVIDd); left ventricular end-diastolic volume (LVESV) = 7.0/(2.4 + LVIDd); EF = 100*(LVEDV-LVES)/LVEDV; LV Mass = 1.053*(LVIDd+LVPWd+IVSd)-LVESV.

**Histological Analysis**

Mouse hearts were fixed in 4% paraformaldehyde solution for 48 h, dehydrated in ascending grades of ethanol, and embedded in wax. Heart sections were prepared for routine haematoxylin and eosin (H&E) staining. H9c2 cells were grown on the glass coverslips for immunofluorescence staining. Cells were washed twice with PBS, fixed for 10 min in 4% paraformaldehyde solution, and permeabilized with 0.1% Triton X-100 plus 1% bovine serum albumin (BSA) for 1 h. Then the cells were incubated with alpha-smooth muscle actin (α-SMA) antibody (1:100 dilution) for 4 h at 37°C followed by staining with a 1:100 dilution of 4’,6-diamidino-2-phenylindole (DAPI) for 10 min. A model IX72 fluorescence inverted microscope (Olympus, Tokyo, Japan) was used to observe the cells. α-SMA antibody was purchased from Abcam (Cambridge, United Kingdom). Antibody against DAPI was purchased from Sigma-Aldrich (St. Louis, MO, United States).

**Real Time Quantitative Polymerase Chain Reaction (RT-qPCR)**

Total RNA extracted from mouse heart tissues and H9c2 cells were reverse-transcribed as previously described (Chen et al., 2016). qPCR was performed using SYBR Premix Master Mix (Thermo Fisher Scientific Inc., Shanghai, China). Relative mRNA levels of target genes were quantified using comparative threshold (CT) normalized to house-keeping genes [ribosomal protein, large, P0 (36B4) for mouse heart or β-actin for H9c2 cells]. The primer sequences used for qPCR are demonstrated in Table 1.

**Immunoblotting**

As previously described (Chen et al., 2016), extracts from mouse hearts or cells were immunoblotted using primary antibodies against Nedd4l, Pik3ca, p-Akt, Akt, cAMP response elements binding (CREB), phospho (p)-CREB, c-jun N-terminal kinase (JNK), p-JNK, p-p38, p38, extracellular signal-regulated kinase 1/2 (ERK1/2), p-ERK1, forkhead box protein O1 (FOXO1), p-FOXO1, FoxO3a, p-FoxO3a, ubiquitin, G-protein-coupled receptor kinase 2 (GRK2), and β-actin (all from Cell Signaling Technologies, Beverly, MA, United States). Blots were incubated with secondary antibody conjugated with horseradish peroxidase (Cell Signaling Technologies) and visualized using an ECL kit (Millipore, Billerica, MA, United States). The chemiluminescence signals were detected by a model 4200SF device (Tanon Shanghai, China).

**Luciferase Assay**

The −2000 to +1 promoter region of human Nedd4l was amplified by PCR with genomic DNA from Hep2 cells using primers 5’-GGTACGAGCTCCCTACCTAGC-3’ and 5’-CTTAGATCGCGAGCGTGAGGCTGAGG-3’. The PCR products were recombined with pGL3-Basic vector cut by Mlu1/Xho1 to obtain the reporter construct pGL3−2000/+1LUC. H9c2 cells were transfected with luciferase reporter plasmids plus the internal control vector pRL-TK-Renilla for luciferase assays in 24-well plates. After an overnight culture, cells were incubated for 24 h in complete medium supplemented with wogonin (10 µM). Luciferase activity was measured using the

| TABLE 1 | Primer sequences used for quantitative RT-qPCR. |
|---|---|
| **Target gene** | **Forward primers (5’-3’)** | **Reverse primers (5’-3’)** |
| ANP (mouse) | GCTTCACAGGCTATTTGGAG | GGGGGCGATGACCTCACTC |
| BNP (mouse) | GAGTGACTCCTAATCTGGG | GCCATCTCCGCGTTTCTC |
| ANP (rat) | GGCAACATGATGCTGCC | TTGCTCAAATGCGCGTGGG |
| BNP (rat) | TAGTGTCAAGACGGCGCTT | GCGGATCGCTTCATTTCTC |
| Pik3ca (rat) | CCGACACTCATTCTGGG | ACGGAGGACATTCAAGCATCA |
| Nedd4l (rat) | GGGCACTTACGAGGCGTACA | GAGGGGCAAGTCAGCACTG |
| Trnc1 (rat) | CTGTGAGATCCTCGCAT | TCCGTGATGCTCTACCTG |
| Trnt2 (rat) | CCTGAGGGAGAGATGTCGA | TGTGCTCAGTGGGCGTCGG |
| 36B4 (mouse) | AAGCGCGCTCCTGGACATTGCT | CGCGAGGGCGAGAACG |
| β-actin (rat) | GCGCGCGATCACAATTCTC | CGCGCGATACATTGCAATC |

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Statistical Analyses
The data and statistical analyses in this study comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2015). Data are presented as means ± SE. Data between groups were analyzed by Student's t-test or one-way ANOVA followed by LSD–Dunn multiple comparisons. \( p < 0.05 \) was considered statistically significant.

RESULTS
Wogonin Attenuates Isoprenaline-Induced Myocardial Hypertrophy in Mice
To evaluate the anti-hypertrophic effect of wogonin, myocardial hypertrophy in mice was replicated by subcutaneous implantation of mini-pump delivering isoprenaline for 14 days. Wogonin failed to prevent the isoprenaline-mediated reduction in body weight (BW) but did reverse the abnormal increase in heart weight (HW) (Table 2 and Figure 2A). Wogonin alone had no effects on BW or HW. The detailed anatomical changes in the heart were detected by echocardiography and reflected via hypertrophic indexes including HW, HW/BW, IVSd, LVPWd, LV Mass, LVIDd, and LVIDs. EF is to evaluate the left ventricular systolic function. Isoprenaline treatment increased HW, HW/BW, IVSd, LVPWd, and LV Mass but decreased LVIDd and LVIDs. Those changes, showing typical for concentric hypertrophy, were all reversed by wogonin. EF was neither worsened by isoprenaline nor affected by wogonin. Histologic analysis of heart section slices also revealed that wogonin attenuated the enlargement in heart size after isoprenaline treatment (Figures 2A,B). Atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) are two well-known hypertrophic markers. Compared to the dose of 1 \( \mu \)M, wogonin (10 \( \mu \)M) more effectively reduced the cellular mRNA levels of ANP and BNP which had been enhanced by isoprenaline treatment (Figure 3B). In cardiomyocytes, \( \alpha \)-SMA is a major constituent of the cytoskeleton, which determines the cell shape. Thus, staining of \( \alpha \)-SMA was performed to observe the morphology of H9c2 cells. The average area of H9c2 cells was significantly expanded by isoprenaline treatment, while wogonin relieved this effect (Figures 3C,D).

Wogonin Represses Akt Signaling Pathway Initiated by Isoprenaline Treatment
Isoprenaline induces cardiomyocyte hypertrophy via multiple pathways, including PI3K/Akt, cAMP/PKA/CREB, MAPKs (JNK, p38, and ERK), and PKC (Heineke and Molkentin, 2006). The phosphorylation levels of Akt, CREB, JNK, p38, and ERK were significantly increased by isoprenaline. Wogonin reversed the phosphorylation levels of Akt and CREB (Figures 4A,B). CREB phosphorylation was mediated by two pathways, including cAMP/PKA and PI3K/Akt, during isoprenaline treatment

| Parameter                  | Control (n = 5) | Wog (10 mg/kg) (n = 6) | ISO (n = 6) | ISO + Wog (1 mg/kg) (n = 6) | ISO + Wog (10 mg/kg) (n = 6) |
|----------------------------|-----------------|------------------------|-------------|----------------------------|----------------------------|
| BW(g)                      | 35.20 ± 0.67    | 35.25 ± 0.25           | 31.93 ± 0.51* | 32.03 ± 0.31*              | 31.27 ± 0.82*              |
| HW(mg)                     | 128.00 ± 2.65   | 125.6 ± 1.08           | 138.17 ± 2.27* | 133.3 ± 0.80               | 121.50 ± 2.23*             |
| HW/BW                      | 3.64 ± 0.09     | 3.57 ± 0.06            | 4.33 ± 0.04*  | 4.16 ± 0.05                | 3.89 ± 0.09                |
| IVSd (mm)                  | 0.72 ± 0.02     | 0.69 ± 0.01            | 0.89 ± 0.02*  | 0.83 ± 0.02*               | 0.76 ± 0.02*               |
| LVPWd (mm)                 | 0.75 ± 0.01     | 0.76 ± 0.01            | 0.86 ± 0.03*  | 0.83 ± 0.04                | 0.81 ± 0.02*               |
| LVMass (mg)                | 100.22 ± 2.07   | 99.43 ± 1.35           | 111.07 ± 2.13* | 105.67 ± 3.58              | 103.21 ± 3.11*             |
| LVIDd (mm)                 | 3.87 ± 0.05     | 3.90 ± 0.43            | 3.54 ± 0.07*  | 3.63 ± 0.03                | 3.74 ± 0.07                |
| LVIDs (mm)                 | 2.67 ± 0.08     | 2.56 ± 0.02            | 2.35 ± 0.04*  | 2.40 ± 0.03                | 2.53 ± 0.08                |
| EF(%)                      | 59.61 ± 2.30    | 58.35 ± 0.91           | 62.84 ± 1.99  | 60.47 ± 1.10               | 61.22 ± 1.43               |

All data are presented as mean ± SEM. BW, body weight; HW, heart weight; HW/BW, body weight/heart weight; IVSd, interventricular septum diameter; LVPWd, left ventricular posterior wall diameter; LV Mass, left ventricular mass; LVIDd, left ventricular end-diastolic diameter; LVIDs, left ventricular end-systolic diameter; EF (%), Ejection fraction. versus Control \(*P < 0.05\); versus ISO \(**P < 0.05\).
Wogonin Improves Myocardial Hypertrophy by Reducing Pik3ca Expression

FoxO1, FoxO3a, and CREB mediate PI3K/Akt pathway-induced gene expression involved in myocardial hypertrophy (Ronnebaum and Patterson, 2010; Khalilimeybodi et al., 2017). We observed that isoprenaline increased the phosphorylation levels of Akt, and the downstream FoxO1, FoxO3a, and CREB, which were all inhibited by wogonin (Figures 4A,B, 5A,B). Thus, we reasoned that wogonin specifically acts on the PI3K/Akt signaling pathway. The fact that constitutively active Akt [Akt (CA)] transfected into H9c2 cells significantly enhanced the phosphorylation of FoxO1 and FoxO3a, which were not affected by wogonin, implies that the target of wogonin is located in the upstream of Akt (Figures 5C,D). As class IA PI3K, one of three types of PI3Ks, plays an important role in cardiac growth and function (Shioi et al., 2000), we overexpressed Pik3ca, the catalytic subunit of class IA PI3K, into H9c2 cells to initiate the phosphorylation of Akt. Interestingly, wogonin reversed the Akt phosphorylation by reducing the level of Pik3ca protein (Figures 5E,F). Consistently, α-SMA staining showed that Pik3ca overexpression amplified the size of H9c2 cells and

Bullock and Habener, 1998; Khalilimeybodi et al., 2017). Wogonin did not affect CREB phosphorylation induced by dibutyl cyclic adenosine acid (DB-cAMP), the analog of cAMP (Figures 4C,D), implying that wogonin reduces CREB phosphorylation via the PI3K/Akt pathway instead of the cAMP/PKA pathway. PKCs were activated by its selective agonist, PDBU, which increased the mRNA levels of ANP and BNP. However, wogonin did not affect PKC-induced transcription of ANP and BNP, indicating that the anti-hypertrophy effect of wogonin is not involved in the PKC pathway (Figure 4E).

**Wogonin Improves Myocardial Hypertrophy by Reducing Pik3ca Expression**

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wogonin reversed this pathological change (Figures 5G,H). When catecholamine binding to β-adrenoceptors, the G protein–coupled receptor kinase-2 (GRK2) mediates the translocation of PI3K to β-adrenoceptors and then enhances the recruitment of β-arrestin and AP-2, which finally results in the internalization and downregulation of β-adrenoceptors (Naga Prasad et al., 2002). It has reported that disrupts the interaction between PI3K and GRK2 by displacing class I PI3K isoforms blocks agonist-stimulated β-adrenoceptors internalization (Nienaber et al., 2003). To test whether wogonin potentially affects β-adrenoceptor function by modulating the interaction between PI3K and GRK2, precipitation was performed. As shown in Supplementary Figure S1, wogonin treatment reduced the binding of GRK2 to Pik3ca in H9c2 cells, mostly due to wogonin-induced reduction in Pik3ca expression.

**Wogonin Induces Pik3ca Degradation by Promoting Its Ubiquitination**

Pik3ca was significantly increased at the protein and mRNA levels in H9c2 cells with isoprenaline treatment (Figures 6A,B). Wogonin reduced the protein level of Pik3ca but failed to decrease its mRNA level (Figures 6A,B). We reasoned that wogonin accelerates the degradation of Pik3ca protein. The classic protein degradation process depends on the ubiquitination-proteasome system, which includes protein-ubiquitination by ubiquitin ligase and subsequent protein-recognization by proteasomes for retrogradation. Thus, we utilized the proteasome inhibitor MG132 to prevent the ubiquitinated proteins from proteasome-mediated degradation. Wogonin induced an enhancement in the ubiquitination of plenty of proteins, which should include Pik3ca, since MG132 reversed the wogonin-induced downregulation of Pik3ca (Figure 6A). To confirm the specificity of the wogonin-mediated ubiquitination of Pik3ca, hemaglutinin (HA)-tagged Pik3ca was transfected into H9c2 cells. The cells were then treated with wogonin and MG132. The original protein amounts of HA-tagged Pik3ca were equal in each condition and immunoprecipitation was easier to operate with HA-tagged beads. As shown in Figure 6C, the precipitated Pik3ca displayed higher ubiquitination levels after wogonin treatment.
FIGURE 5 | Wogonin improves myocardial hypertrophy by downregulating Pik3ca. (A) Differentiated H9c2 cells were treated with isoprenaline (10 µM) and/or wogonin (10 µM) for 24 h. Cell extracts were blotted by antibodies against p-FoxO1, FoxO1, p-FoxO3a and FoxO3a. H9c2 cells were transfected with pUSEamp(+)-Akt(CA) (C) or pUSEamp(+)-Pik3ca (E) and grew for 48 h. Then wogonin (10 µM) was added 24 h before harvesting. Cell extracts were immunoblotted by indicated antibodies. (B,D,F) Quantitation of indicated protein bands (n = 3). (G) H9c2 cell shape was demonstrated by the immunofluorescence with α-SMA antibody. (H) Quantitation of average cell surface area (n = 8). Data are presented as mean ± SEM; *p < 0.05.
Wogonin Enhancement of Nedd41 Expression

Nedd4 is the specific ubiquitin ligase of Pik3ca, belonging to the Nedd4 (neural precursor cell-expressed developmentally down-regulated gene 4) family (Yang and Kumar, 2010; Wang Z. et al., 2016). Isoprenaline treatment significantly reduced the mRNA and protein levels of Nedd4 in H9c2 cells (Figures 7A–C), which led to the upregulation in protein level of Pik3ca (Figure 6A). Besides, isoprenaline also increased the mRNA level of Pik3ca (Figure 6B). Wogonin reversed the inhibitory effect of isoprenaline on Nedd4 expression at the transcriptional and post-transcriptional levels (Figures 7A–C). We expressed Flag-tagged Nedd4 in H9c2 cells by transient transfection and found that wogonin treatment did not affect the protein levels of exogenous Nedd4 (Figures 7D,E), indicating that wogonin regulates Nedd4 expression only at the transcriptional level. This was confirmed in the luciferase assay, as wogonin significantly enhanced the Nedd4 promoter-driven luciferase activity (Figure 7F). In line with the data from cells, wogonin treatment significantly reversed the downregulation by isoprenaline on protein level of Nedd4 in mouse heart (Figures 7G,H).

DISCUSSION

The present study confirms the attenuation by wogonin in isoprenaline-induced myocardial hypertrophy in vivo and in vitro. It reminds us that wogonin not only shows its therapeutic value but also can function as a probe to search for new targets for myocardial hypertrophy therapy.

Sympathetic activation is a main native factor in the progression of myocardial hypertrophy, which generates catecholamine to activate β-adrenoceptors as well as its downstream signaling and thus induce hypertrophic gene expression (Ozakca et al., 2013; Tank and Lee, 2015). There are three subtypes of β-adrenoceptors: β1, β2 and β3. β1 and β2-adrenoceptors are the two major adrenoceptor types in the heart (Kawano et al., 2009). Once activated, β1 and β2-adrenoceptors both bind to Gαs subunits to activate the classical adenylate cyclase/cAMP/PKA pathway.
Wogonin enhances the transcription of Nedd4l. (A–C) Differentiated H9c2 cells were treated with isoprenaline (10 µM) and/or wogonin (10 µM) for 24 h. The protein and mRNA levels of Nedd4l were determined by immunoblot and RT-qPCR. (D) H9c2 cells were transfected with pAdeno-MCMV-MCS-3Flag-Nedd4l expression vector and grew for 48 h. Cells were treated with wogonin (10 µM) for another 24 h before harvesting. Cell extracts were immunoblotted by antibodies against Flag and β-actin. (F) H9c2 cells were transfected with luciferase construct containing Nedd4l promoter (–2000/+1) with wogonin (10 µM) treatment for 24 h. Nedd4l promoter driven luciferase activity was normalized to Renilla luciferase activity and expressed as fold-change relative to the level of control. (G) Protein level of Nedd4l in heart were determined with immunoblot. (B,E,H) Quantitation of indicated protein bands (n = 3). Data are presented as mean ± SEM; *p < 0.05, versus control.

(Kamide et al., 2015) or bind Gαi to activate the non-classical PI3K/Akt pathway (Kitaura, 2013). As a β-adrenoceptor agonist, isoprenaline was used to replicate myocardial hypertrophic model in present study (Heap et al., 2015; Wang J.J. et al., 2016). Fourteen-day treatment leads to the typical concentric hypertrophy with still compensated left ventricular systolic function. Actually, we chose a hypertrophic model at the early phase of cardiac remodeling in order to explore the therapeutic effect of wogonin on early myocardial hypertrophy, as early intervention is much more effective in the therapy of...
myocardial hypertrophy, which would prevent the heart from apoptosis/fibrosis and further developing into heart failure. Once left ventricular systolic function is uncompensatory, it is too late to return to normal (Bloom et al., 2017). Therefore, early treatment of wogonin has more clinical significance and is hence more worth studying. Notably, early treatment of wogonin attenuated the development of concentric hypertrophy and decreased mRNA expression of the hypertrophic markers, ANP and BNP (Figure 2). We also replicated the hypertrophic model with isoprenaline in differentiated H9c2 cells and found that wogonin reduced the amplified size and hypertrophic mark gene expression in H9c2 cells (Figure 3). Thus, the antihypertrophic effect of wogonin may depend on the amelioration in the abnormal enlargement of cardiomyocytes.

As far as we know, there are several pathways mediating myocardial hypertrophy activated by β-adrenoceptors. They include the PI3K/Akt, adenylate cyclase/cAMP/PKA, MAPK, and PKC signaling pathways (Heineke and Molkentin, 2006; Khalilimeybodi et al., 2017). We tested the effect of wogonin on the signaling transduction and found that wogonin reduced the phosphorylation of Akt and CREB but had no effects on the MAPK or PKC pathways (Figure 4). Phosphorylation of CREB can be induced by Akt or PKA (Kato et al., 2007; Zhang et al., 2014). The fact that wogonin cannot inhibit DBCAMP-induced phosphorylation of CREB suggests that the reduction in CREB phosphorylation is the result of wogonin exerts its antihypertrophic effect by suppressing PI3K/Akt pathway. The expression of ANP and BNP are also regulated by PI3K/Akt pathway through their transcriptional factor, NF-AT3, since activated Akt suppresses the downstream glycogen synthase kinase 3 beta (GSK3β), the kinase of NF-AT3, and thus provokes NF-AT3 by reducing its phosphorylation level (Redondo-Díiguez, 2000). Therefore, wogonin may at least reduce ANP and BNP transcription via PI3K/Akt pathway. However, whether other pathways regulating the expression of ANP and BNP, such as Ca²⁺/calcineurin/NF-AT3 (Molkentin et al., 1998; Redondo-Díiguez, 2000) and STAT3/glycoprotein 130 (Zhang et al., 2008), are affected by wogonin remains to be explored.

The PI3K/Akt pathway has been linked to an extraordinarily diverse group of cellular functions, including cell growth, proliferation, differentiation, motility, survival, and intracellular trafficking (Abebrathma and Su, 2015). Activated Akt induces compensatory myocardial hypertrophy in physiological conditions (Latronico et al., 2010) or leads to uncompensatory myocardial hypertrophy in pathological conditions if the activation of Akt persists (Condorelli et al., 2002; Shiojima, 2005). Since wogonin reduced the phosphorylation level of Akt but could not terminate hypertrophic signaling transduction promoted by constitutively active Akt (Figure 5), we reason that the target of wogonin is located in the upstream of Akt.

Presumably, PI3K is a potential target of wogonin. Mammalian PI3K can be divided into three major classes (class I, II, and III) based on their structure and substrate specificity. The class I PI3K can be further divided into two subtypes, class IA and class IB. The mammalian class IA PI3Ks are heterodimers of a 110 kDa catalytic subunit (p110α, p110β, or p110γ) and a regulatory subunit of 85 kDa (p85/55), whereas the class IB is composed of a p110γ catalytic subunit and a 101 regulatory subunit (Toker and Cantley, 1997; Aoyagi and Matsui, 2011). Class IA PI3Ks plays an important role in cardiac growth and hypertrophy (Crackower et al., 2002). Since the heterodimer, p110α/p85, is the dominant form of class IA PI3K (Yan et al., 2015), we overexpressed p110α (Pik3ca) to enhance the PI3K activity in H9c2 cells, and observed the enhancement in hypertrophic development, including amplification of cell size and activation of Akt (Figure 5). Importantly, wogonin reduced the protein level of Pik3ca and reversed the subsequent effects induced by Pik3ca overexpression, which confirms that wogonin targets PI3K. Interestingly, isoprenaline not only promotes myocardial hypertrophy via β-adrenoceptor-mediated activation of PI3K/Akt pathway, but also induces the hyperfunction of PI3K/Akt pathway through upregulating Pik3ca expression (Figure 6). Besides the contribution to myocardial hypertrophy, PI3K also induces internalization and downregulation of β-adrenoceptors via the interaction with GRK2, which may promote the heart failure (Naga Prasad et al., 2002). Thus the downregulation of Pik3ca and subsequent inhibition in the binding of GRK2 to Pik3ca by wogonin has double significance to the therapy of myocardial hypertrophy.

Wogonin downregulates Pik3ca by accelerating its degradation, since wogonin promotes the ubiquitination of Pik3a (Figure 6C). Nedd4l is a Pik3ca specific ubiquitin E3 ligase (Wang Z. et al., 2016) whose mRNA and protein levels were reduced by isoprenaline treatment (Figure 7), suggesting that isoprenaline mediates the up-regulation of Pik3ca not only by activating its transcription (Figure 6B) but also by reducing
its protein degradation. Wogonin enhances Nedd4l expression at the transcription level (Figures 7C,F), without affecting its protein stability (Figures D,E). It is worth emphasizing that we cannot exclude the possibility that other ubiquitin E3 ligases of Pik3ca are also regulated by wogonin. Additional ubiquitin ligases that potentially act on Pik3ca are still being searched.

As shown in Figure 8, the present investigation has revealed that wogonin targets Nedd4l and elevates its expression in cardiomyocytes, which promotes the ubiquitination and degradation of its substrate, Pik3ca. Thus, wogonin suppresses the signaling transduction in the PI3K/Akt pathway mediating the expression of hypertrophic genes, and ameliorates the myocardial hypertrophy induced by isoprenaline treatment in mice. Nowadays, β-blockers are the main drugs clinically used to treat cardiac hypertrophy and heart failure that feature over-activation of β-adrenoceptors (Khalilimeybodi et al., 2017). In the early stage of β-blocker treatment, hypofunction in the left ventricle often occurs in patients due to the suppression in the β-adrenoceptors/cAMP/PKA pathway, which would lead to intolerance. Therefore, β-blockers cannot be used in the target dose for some patients (Kukin et al., 1999). In that case, drugs to improve cardiac hypertrophy independent of the β-adrenoceptors/cAMP/PKA pathway such as wogonin, targeting the PI3K/Akt pathway, may be beneficially supplemented.

AUTHOR CONTRIBUTIONS
LS, MC, and WQ contributed to experiment design. WQ, DY, JZ, QH, CT, PL, XW, PY, and QL performed the experiments and analyzed the data; WQ and DY wrote the initial draft of manuscript. LS and MC reviewed the manuscript. LS and WQ obtained the funding.

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SUPPLEMENTARY MATERIAL
The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2018.00896/full#supplementary-material

FIGURE S1 | Wogonin reduced the binding of GRK2 to Pik3ca. H9c2 Cells were treated with isoprenaline (10 µM) and/or wogonin (10 µM) as indicated for 24 h. Cell extracts were immunoprecipitated by GRK2 antibody plus protein A beads and immunoblotted with antibodies against Pik3ca and GRK2.

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