Puerarin attenuates isoproterenol-induced myocardial hypertrophy via inhibition of the Wnt/β-catenin signaling pathway

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Received March 23, 2022; Accepted July 20, 2022

DOI: 10.3892/mmr.2022.12822

Abstract. Myocardial hypertrophy (MH) is an independent risk factor for cardiovascular disease, which in turn lead to arrhythmia or heart failure. Therefore, attention must be paid to formulation of therapeutic strategies for MH. Puerarin is a key bioactive ingredient isolated from *Pueraria* genera of plants that is beneficial for the treatment of MH. However, its molecular mechanism of action has not been fully determined. In the present study, 40 μM puerarin was demonstrated to be a safe dose for human AC16 cells using Cell Counting Kit-8 assay. The protective effects of puerarin against MH were demonstrated in AC16 cells stimulated with isoproterenol (ISO). These effects were characterized by a significant decrease in surface area of cells (assessed using fluorescence staining) and mRNA and protein expression levels of MH-associated biomarkers, including atrial and brain natriuretic peptide, assessed using reverse transcription-quantitative PCR and western blotting, as well as β-myosin heavy chain mRNA expression levels. Mechanistically, western blotting demonstrated that puerarin inhibited activation of the Wnt signaling pathway. Puerarin also significantly decreased phosphorylation of p65; this was mediated via crosstalk between the Wnt and NF-κB signaling pathways. An inhibitor (Dickkopf-1) and activator (IM-12) of the Wnt signaling pathway were used to demonstrate that puerarin-mediated effects alleviated ISO-induced MH via the Wnt signaling pathway. The results of the present study demonstrated that puerarin pre-treatment may be a potential therapeutic strategy for preventing ISO-induced MH and managing MH in the future.

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Key words: puerarin, isoproterenol, cardiac hypertrophy, Wnt/β-catenin signaling pathway, phosphorylation of NF-κB, p65

Introduction

Cardiovascular disease (CVD) is the leading cause of human mortality worldwide accounted for 32.9% of all deaths with a steadily reaching of the number of CVD deaths from 12.1 million in 1990 to 18.6 million in 2019 (1), and places a notable burden on human health (2,3). At present, management of patients with CVD is challenging due to the high mortality, disability rates and rising health care costs (4). According to the World Health Organization, three-quarters of CVD deaths could be prevented through early detection and lifestyle interventions (5). As a result, CVD has attracted the attention of research communities and healthcare professionals to ensure that measures, including developing the care of cardiovascular disease in the ambulatory setting, improving approaches to the prevention of cardiovascular disease, expanding digital health interventions and universal health coverage (6), have been taken. Pharmaceutical developments and technological improvements are used in the Western world to decrease CVD-associated mortality, alongside interventions to control risk factors for CVD (7). Among risk factors for CVD, pathological myocardial hypertrophy (MH) caused by long-term overload is an independent risk factor for adverse cardiovascular events (8). Therefore, reversing MH may minimize the risk of cardiovascular events.

MH refers to an adaptive and compensatory change in heart structure that maintains cardiac output during adverse stimulation (9). The hypertrophic process is categorized as physiological or pathological. Physiological hypertrophy is key to preservation of normal heart structure and function; it is a tightly regulated but reversible phenomenon, such as the hypertrophic process that occurs in the heart following continuous exercise (10). This is a compliant change (an adaptive response to stimuli) in the heart and causes negligible effects in the body. However, pathological hypertrophy is associated with fibrosis, increased proinflammatory cytokine production and cellular dysfunction (11). This type of maladaptive cardiac hypertrophy, typically caused by chronically high, continuous hemodynamic pressure, is irreversible and leads to heart failure in severe cases (12).

Angiotensin-converting enzyme inhibitors (13,14), angiotensin II receptor blockers (15,16), calcium channel antagonists, β-receptor blockers and diuretics (17,18) are currently the primary treatments for MH. They have all been reported to improve the clinical symptoms of patients to a certain extent; however, the single-target therapeutic efficacy of these agents remains unsatisfactory as none of them significantly decrease mortality rate (19). Therefore, MH needs to be addressed in the CVD research. Traditional Chinese medicine (TCM) has been reported to improve the symptoms and quality of life of patients with heart failure (20). It has been reported that TCM confers therapeutic benefits using natural, biologically active ingredients that have multiple targets (21). Furthermore, agents such as Radixastragali (Huangqi) and Ginseng (Renshen) (22,23) have gained attention due to their stable therapeutic effects by targeting multiple targets underlying the development of MH. Astragaloside IV(AS-IV) which is the main component of Radixastragali, improves cardiac function and ameliorate MH, which is mediated by attenuating inflammatory reaction (24) and inactivating TANK-binding kinase 1 (TBK1)/PI3K/AKT signaling pathway (25). The pharmacological properties of ginseng are mainly attributed to ginsenosides which plays a robust antihypertrophic role via inhibition of NHE-1-dependent calcineurin activation (26) and the suppression of MCT-activated calcineurin and ERK signaling pathways (27).

Based on clinical experience and systematic theory, advances have been achieved in the research of TCM formulas, extracts and compounds (28). For example, research and development of 'compound Danshen dropping pill' has contributed to treatment of coronary heart disease and angina pectoris (29). Numerous compounds isolated from TCM products have important properties, such as the antimalarial effects of artemisinin (30) and the anti-inflammatory properties of licorice extract, which contains 3 triterpenes and 13 flavonoids (31). Furthermore, the curative effects of novel TCM compounds that exert specific pharmacological effects may be enhanced by elucidating their mechanism individually whilst also characterizing their target profile. Ultimately, treatment strategies for MH may be developed using the multi-faceted profiles of TCM compounds combined with metabolomics and traditional pharmacological methods (32).

Puerarin (PU) is an isoflavone chemical component (33) found within the *Pueraria* genera of plants (34). PU has been reported to exert numerous therapeutic effects, including anti-cancer, vasodilatory and heart protective effects (35). Furthermore, it has been previously reported that PU ameliorates pressure overload-induced MH in ovariectomized rats via activation of the peroxisome proliferator-activated receptor-α/peroxisome proliferator-activated receptor γ-1 signaling pathway and regulation of energy metabolism remodeling (36). However, the multi-target characteristics of TCM monomers suggest that PU has multiple therapeutic targets (37). Numerous signaling pathways are associated with the pathophysiology of MH, including the 5'AMP-activated protein kinase/mTOR (38), PI3K/AKT/mTOR (39) and insulin-like growth factor 1/PI3K/AKT (40) signaling pathways. Therefore, the exploration of the mechanism of PU on MH to achieve the effect of precise treatment is urgent. Numerous studies have reported that the Wnt signaling pathway, which is activated in early embryonic development to promote cardiac development and silenced in adulthood to maintain normal cardiac function, serves an important role in CVD (41,42). As experimental data on the effects of PU on the Wnt signaling pathway in the context of MH remain incomplete, the present study was preformed to address this.

Materials and methods

Chemicals and reagents. PU (purity, 99.00%) was purchased from TargetMol Chemicals, Inc. Isoproterenol hydrochloride (ISO), Dickkopf-1 (DKK1) and IM-12 were purchased from MedChemExpress. Cell Counting Kit-8 (CCK-8) assay was purchased from Dojindo Molecular Technologies, Inc. PVDF membranes, RIPA buffer, Actin-Tracker Green (microfilament green fluorescent probe), DAPI Staining Solution and BCA Protein Assay kit were purchased from Beyotime Institute of Biotechnology. TransScript® Green Two-Step qRT-PCR SuperMix kit was purchased from TransGen Biotech Co., Ltd. Antibodies against low-density lipoprotein receptor-related protein 6 (LRP6; cat. no. 3395), phosphorylated (p-)p65 (cat. no. 3033), NF-κB p65 (cat. no. C22B4), Tubulin (cat. no. 2128S), β-catenin (cat. no. 8480), Wnt5a/b (cat. no. 2530), c-Myc (cat. no. 5605), glycogen synthase kinase-3β (GSK3β; cat. no. 12456) and p-GSK3β (cat. no. 9322) were purchased from Cell Signaling Technology, Inc. Atrial natriuretic peptide (ANP; cat. no. ab189921) and brain natriuretic peptide (BNP, cat. no. ab236101) were purchased from Abcam. GAPDH (cat. no. BM1623) and the secondary antibodies including HRP Conjugated AffiniPure Goat Anti-Rabbit IgG (cat. No. BA1054) and HRP Conjugated AffiniPure Mouse Anti-Human IgG (cat. No. BM2002) was from Boster.

Cells. The human cardiomyocyte AC16 cell line (MingzhouBio Inc., cat. no. MZ-4038) was used. Cells were cultured in culture flasks with DMEM (BasalMedia Inc.) at 37˚C under 5% CO_2 in a humidified atmosphere. When cells reached the logarithmic growth phase, they were evenly distributed into six-well plates. Experimental treatments were performed after 24 h.

In vitro cardiac hypertrophy model and drug treatment. Cells were randomly divided as follows: i) Control, cells treated with DMEM; ii) ISO, cells treated with 10 μM ISO for 24 h; iii) PU, cells treated with 40 μM PU for 6 h followed by 10 μM ISO for 24 h; iv) DKK1, cells treated with 780 nM DKK1 for 6 h followed by 10 μM ISO for 24 h; v) IM12, cells treated with 30 nM IM-12 for 24 h; vi) PU + IM12, cells treated with 40 μM PU for 6 h followed by 30 nM IM-12 for 24 h and vi) PU + ISO + IM12, cells treated with 40 μM PU for 6 h followed by 10 μM ISO for 24 h and 30 nM IM-12 for 6 h. A total of $1.5x10^5$ cells were plated in culture flasks with DMEM at 37˚C under 5% CO_2 in a humidified atmosphere. The cells were washed three times with PBS before addition of new drugs to avoid mixing of different drug components.

CCK-8 assay. PU was dissolved in DMSO and DMEM to produce a range of concentrations (5, 10, 20, 40 and 80 μM) for subsequent use. The final DMSO content was 0.2%. Cells were evenly dispersed into six groups containing different concentrations of PU (0, 5, 10, 20, 40 and 80 μM) and inoculated into a 96-well plate (~5,000 cells/well). PU incubation was

performed for 6 h at 37˚C under 5% CO_2 before 100 μl CCK-8 reagent was added, followed by incubation for 2 h at 37˚C under 5% CO_2 in a humidified atmosphere. The 96-well plate was assessed using a PT-3502PC microplate reader (Beijing Potenov Technology Co. Ltd.) to quantify the absorption value of each well at 450 nm. A line chart was constructed plotting optical density (OD) values against drug concentrations to assess the effect of PU on cell viability.

Cytoskeletal staining. The surface area of AC16 cardiomyocytes in the different treatment groups was assessed using cytoskeletal staining. Control, ISO and PU), cells were washed with PBS twice and fixed using 3.7% formaldehyde solution at room temperature for 10 min in the dark according to the manufacturer's protocol for the Actin-Tracker Green. PBS containing 0.1% Triton X-100 was added to permeabilize the fixed cells for 10 min at room temperature. The cells were stained with Actin-Tracker Green working solution diluted with PBS containing 1-5% BSA and 0.1% Triton X-100 at a ratio of 1:50 for 30 min at room temperature. Following rinsing with PBS, 5 mg/ml DAPI (sufficient to cover each section) was added to each well for 3-5 min at room temperature before imaging using a fluorescence microscope (Olympus Corporation). The magnification was 400x. A total of five non-overlapping fields located at the upper and lower left and right and center of each well was selected. The cells in each field were numbered and sampled by a random number generator. A total of four cells was randomly extracted from each field. Finally, the size of cells was quantified using ImageJ2 (version 1.53; National Institutes of Health). The mean area of the selected cells was determined for each well and normalized to the control.

Reverse transcription-quantitative (RT-q)PCR. RNA was extracted from AC16 cells (Control, ISO and PU) using TRIzol® (Thermo Fisher Scientific, Inc.). The concentration of RNA was detected using a NanoDrop® spectrophotometer (Thermo Fisher Scientific, Inc.). Complementary DNA synthesis, using incubation at 42˚C for 15 min and heating at 85˚C for 5 sec, was performed according to the manufacturer's protocols of the TransScript® Green Two-Step qRT-PCR SuperMix kit. The primer sequences were as follows: ANP forward (F), 5'-CAACGCAGACCTGATGGATTT-3' and reverse (R), 5'-AGCCCCCGCTTCTTCATTC-3'; BNP F, 5'-TGGAAACGTCCGGGTTACAG-3' and R, 5'-CTGATCCGGTCCATCTTCCT-3'; β-myosin heavy chain (β-MHC) F, 5'-TCACCAACAACCCCTACGATT-3' and R, 5'-CTCCTCAGCGTCATCAATGGA-3' and GAPDH F, 5'-CTGGGCTACACTGAGCACC-3' and R, 5'-AAGTGGTCGTTGAGGGCAATG-3'. The reaction systems of each primer sequence were added into a 96-well fluorescent qPCR plate and CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.). The thermocycling conditions were as follows: 94˚C initial denaturation for 30 sec, followed by 40 cycles of 5 sec at 94˚C and 30 sec at 60˚C. The $2^{-\Delta\Delta Cq}$ method (43) was used to assess mRNA expression levels.

Western blotting. The cells in each group (Control, ISO, PU, DKK1, IM12, PU + IM12, PU + ISO + IM12) were digested with 0.25% trypsin for 25 sec at 37˚C under 5% CO_2 in a humidified atmosphere, washed with PBS three times and lysed with RIPA lysis buffer containing protease and phosphate inhibitors at 4˚C for 30 min. The supernatant was collected following centrifugation (12,000 x g at 4˚C for 10 min), before protein concentration was quantified via BCA assay. An equal amount of protein (15 μg protein/lane) was separated by 10% SDS-PAGE and transferred onto PVDF membranes. PVDF membranes were blocked with 5% skimmed milk powder solution at room temperature for 1 h before being incubated at 4˚C overnight with specific primary antibodies. The antibodies were as follows: LRP6 (1:1,000), p-p65 (1:1,000), p65 (1:1,000), Tubulin (1:10,000), β-catenin (1:1,000), Wnt5a/b (1:1,000), GAPDH (1:5,000), c-Myc (1:1,000), GSK3β (1:1,000), p-GSK3β (1:1,000), ANP (1:1,000) and BNP (1:1,000). After washing with TBST (0.1% Tween-20), membranes were incubated with anti-rabbit (1:5,000) and anti-mouse secondary antibodies (1:5,000) at room temperature for 1 h. The membranes were rinsed again and treated with BeyoECL Moon (cat. no. P0018FS, Beyotime Institute of Biotechnology) before bands were visualized using the Tanon-4600 Chemiluminescence Imaging System. Finally, blots were analyzed using ImageJ2 (version 1.53; National Institutes of Health) to semi-quantify the protein expression levels.

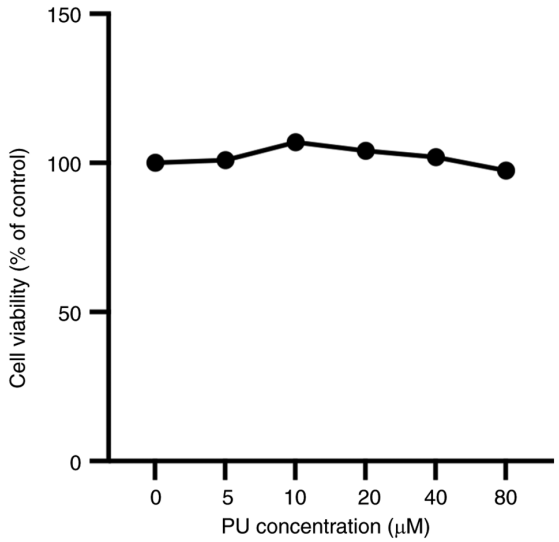

Figure 1. Effect of PU (5-80 μM) on myocardial AC16 cell viability. Association between PU concentration and cell viability. PU, puerarin.

Statistical analysis. All statistical analysis was performed using GraphPad Prism (version, 9.1.1; GraphPad Software, Inc.). Comparisons between groups were performed using one-way ANOVA with Dunnett's (Figs. 1-7) or Tukey's post hoc test (Fig. 8). Data are presented as the mean ± SEM of ≥3 independent experimental repeats. $P<0.05$ was considered to indicate a statistically significant difference.

Results

PU is not toxic to cardiomyocytes. CCK-8 assay OD values were used to assess the effects of PU on AC16 cell viability. The results demonstrated no significant difference in cell viability between treatment groups (Fig. 1). These data demonstrated that the highest concentration of PU (80 μM) had little effect on cell viability after 6 h.

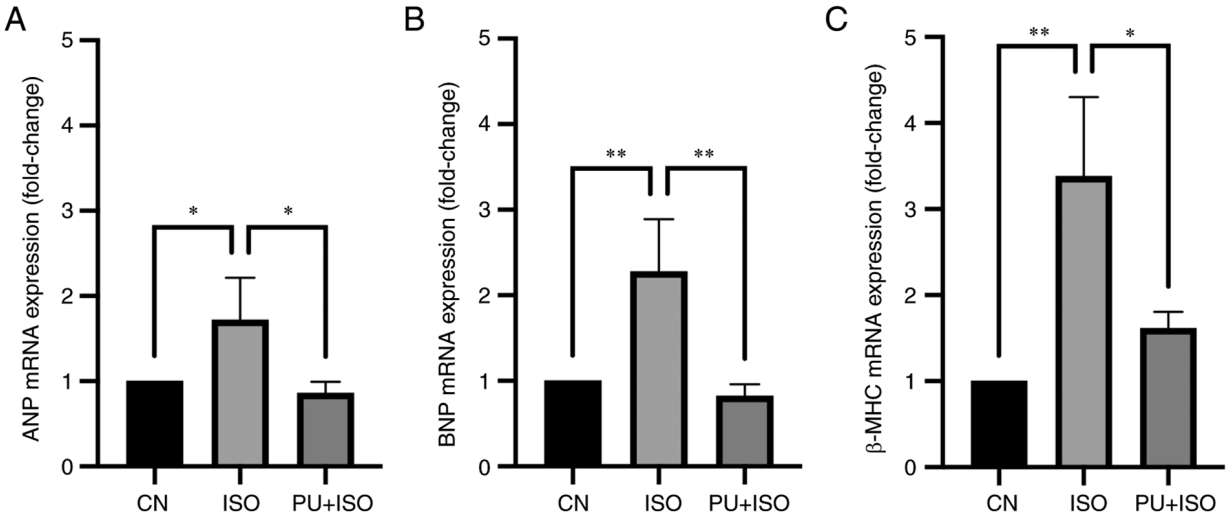

Figure 2. Effect of PU on transcription of ANP, BNP and β-MHC. mRNA expression levels of (A) ANP, (B) BNP and (C) β-MHC in ISO- and PU + ISO-treated AC16 cells were semi-quantified using reverse transcription-quantitative PCR. *P<0.05 and **P<0.01. CN, control; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; β-MHC, β-myosin heavy chain; ISO, isoproterenol; PU, puerarin.

PU decreases ANP, BNP and β-MHC transcription. The mRNA expression levels of MH markers ANP, BNP and β-MHC were quantified using RT-qPCR. The results demonstrated significant increases in mRNA expression levels of ANP (P<0.05; Fig. 2A), BNP (P<0.01; Fig. 2B) and β-MHC (P<0.01; Fig. 2C) in the ISO group compared with the control. By contrast, treatment with PU significantly decreased mRNA expression levels of ANP (P<0.05), BNP (P<0.01) and β-MHC (P<0.05) compared with the ISO group. These results suggested that the ISO-induced MH model was successfully established and the effect of ISO was reversed by PU.

PU decreases protein expression levels of ANP and BNP. To assess the effect of PU on ISO-induced MH, western blotting was performed to determine protein expression levels of ANP and BNP. Following 24 h induction with 10 μM ISO, protein expression levels of ANP and BNP in the AC16 cardiomyocytes were significantly higher compared with those in the control (P<0.01; Fig. 3). However, expression levels of ANP and BNP protein in AC16 cardiomyocytes pretreated with PU were significantly lower compared with those in the ISO group (P<0.01). These results demonstrated that cells pretreated with PU were resistant to ISO-induced MH.

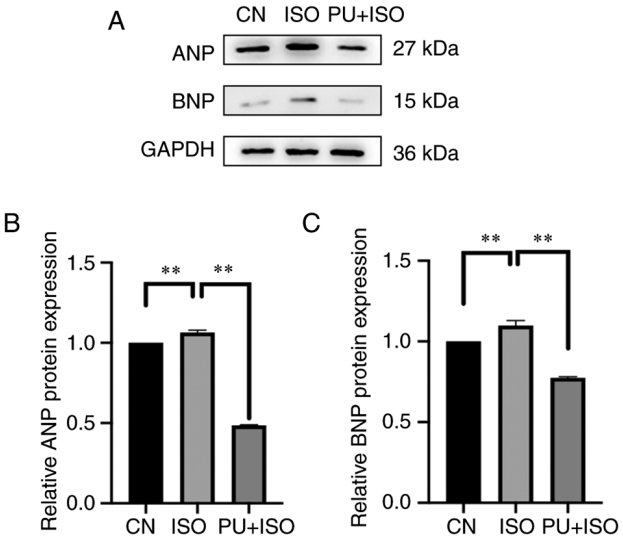

Figure 3. Effect of PU on ANP and BNP protein expression levels. (A) Representative western blotting images of ANP and BNP protein expression. (B) ANP and (C) BNP protein expression levels were semi-quantified using ImageJ. **P<0.01. CN, control; ISO, isoproterenol; PU, puerarin; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide.

ISO-treated AC16 cell surface area decreases significantly following PU pretreatment. Changes in AC16 myocardial cell area were observed using fluorescence microscopy. The surface area of AC16 cells in the ISO group significantly increased compared with those in the control (P<0.01; Fig. 4A, B and D). However, PU pretreatment significantly decreased surface area of AC16 cells compared with those in the ISO group (P<0.05; Fig. 4C). These results suggested that MH was prevented by PU pretreatment.

PU inhibits the Wnt/β-catenin signaling pathway. To determine the potential anti-MH mechanism of PU, core components in the Wnt/β-catenin and NF-κB signaling pathways were assessed using western blotting. The protein expression levels of LRP6, β-catenin, Wnt-5a/b and c-Myc and phosphorylation levels of p65 and GSK3β, significantly increased in the ISO group compared with the control group (P<0.01; Fig. 5A-F and H-K). However, the expression of GSK3β was significantly decreased by ISO compared with the control group (P<0.01; Fig. 5G). There was a significant decrease in the expression of LRP6 (P<0.05), β-catenin (P<0.01), Wnt-5a/b (P<0.01) and c-Myc (P<0.01) as well as phosphorylation levels of p65 (P<0.01) and GSK3β (P<0.01) in the AC16 cells pretreated with PU compared with the ISO group. Furthermore, expression of GSK3β (P<0.01) was significantly increased by PU pretreatment compared with the ISO group. These results suggested that the protective effects of PU on MH may be mediated by Wnt and NF-κB signaling pathway activity.

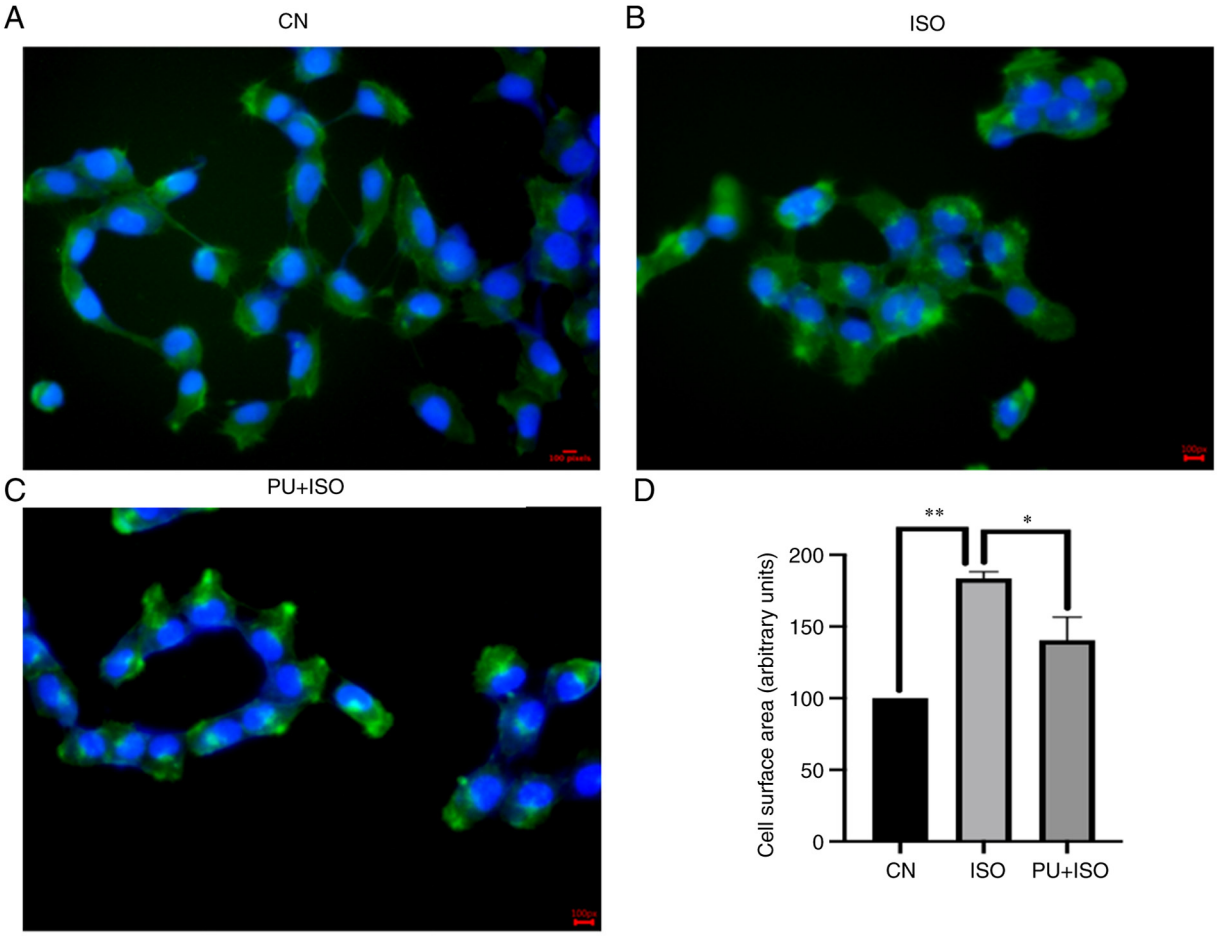

Figure 4. Effect of PU on surface area of AC16 cells. Fluorescent images of (A) control and cells treated with (B) ISO or (C) PU and ISO. (D) Cell surface area was quantified using ImageJ software. Magnification, 400x. *P<0.05 and **P<0.01. CN, control; ISO, isoproterenol; PU, puerarin.

PU serves as an inhibitor of the Wnt signaling pathway by antagonizing its activator. A Wnt inhibitor and activator were used to verify if PU regulated the Wnt signaling pathway. DKK1 is an inhibitor of the Wnt signaling pathway that has been reported to regulate embryonic development (44). Wnt signaling transduction is blocked by DKK1 binding, which induces LRP6 internalization (45).

After cells were pretreated with DKK1, protein expression levels of components in the Wnt signaling pathway, such as LRP6, β-catenin, Wnt5a/b and c-Myc, were significantly suppressed compared with ISO group (P<0.01; Fig. 6A-G); this effect was similar to the aforementioned effect mediated by PU. Furthermore, expression of ANP, a cardiac hypertrophy marker, was significantly decreased by DKK1 pretreatment (P<0.01; Fig. 6G), which suggested that inhibition of the Wnt signaling pathway may be beneficial for the treatment of MH.

IM-12 enhances Wnt signaling, primarily by inhibiting GSK-3β (46). In the present study, IM-12 significantly decreased GSK3β expression compared with the control (P<0.01; Fig. 7G). The protein expression levels of LRP6, β-catenin, Wnt-5a/b and c-Myc were significantly increased by IM-12 compared with the control (P<0.01; Fig. 7A-F). However, protein expression levels of LRP6 (P<0.05), β-catenin (P<0.01), Wnt-5a/b (P<0.01) and c-Myc (P<0.01) were significantly decreased by PU pretreatment. These results suggested that PU suppressed IM-12-induced activation of the Wnt/β-catenin signaling pathway.

The effect of IM-12 on expression of markers in the Wnt signaling pathway and ISO-induced MH in the presence of PU pretreatment were assessed (Fig. 8). Following treatment with IM-12, PU-induced inhibition of Wnt signaling pathway protein expression was partially reversed. With the exception of protein expression of GSK3β, which was increased by PU but significantly reversed by IM-12 compared with the PU + ISO group, expression levels of all proteins in the PU + ISO + IM12 group were significantly increased compared with PU group (P<0.01). Moreover, the inhibitory effects of PU on the MH marker ANP was partially but significantly reversed by IM-12 treatment compared with the PU + ISO group (P<0.01). These results demonstrated that the protective effects of PU on MH may be mediated by inhibition of the Wnt signaling pathway.

Discussion

Despite therapeutic advances, there is a lack of effective therapeutic treatment strategies for MH. Pathological cardiac hypertrophy is induced by various factors, including pressure (47) and volume overload (48) and pharmaceutical induction, such as induction using angiotensin II (49) and ISO (50). ISO is a β-receptor agonist that causes acute

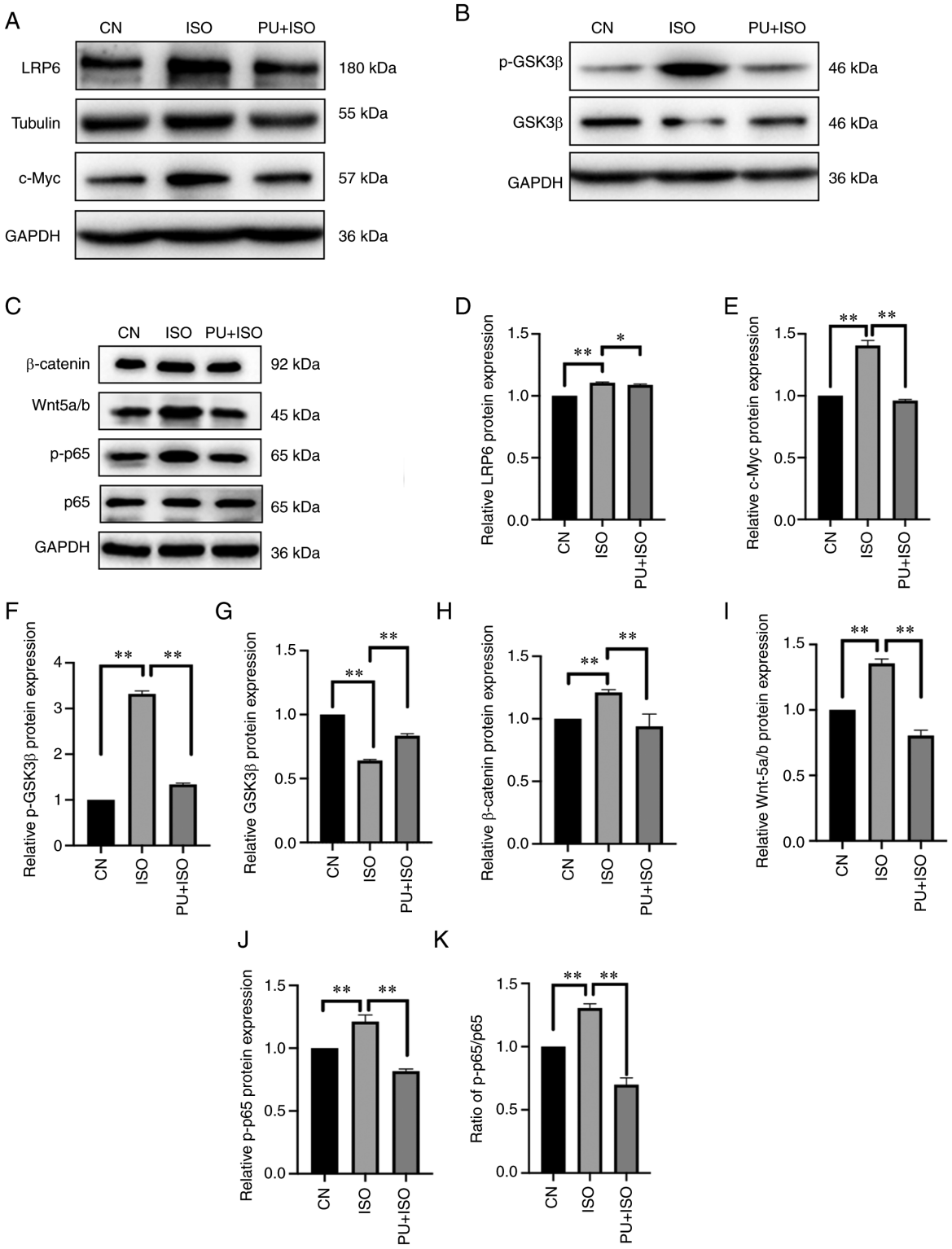

Figure 5. Effect of PU on expression of components in the Wnt/β-catenin and NF-κB signaling pathways. (A) Representative western blotting images of LRP6, c-Myc, p-GSK3β and GSK3β. (B) Representative western blotting images of p-GSK3β and GSK3β. (C) Representative western blotting images of β-catenin, Wnt5a/b, p-p65 and p65. (D) LRP6, (E) c-Myc, (F) p-GSK3β, (G) GSK3β, (H) β-catenin, (I) Wnt5a/b and (J) p-p65 protein expression levels were semi-quantified. (K) Ratio of p-p65/p-65. The blots were analyzed using ImageJ. *P<0.05 and **P<0.01. CN, control; ISO, isoproterenol; PU, puerarin; LRP6, low-density lipoprotein receptor-related protein 6; p-, phosphorylated; GSK3β, glycogen synthase kinase 3β.

myocardial contraction, ischemia, hypoxia and cause damage to myocardial cells due to production of a large number of peroxynitrite and oxygen free radicals in rats (51). Prabhu *et al* (52) reported that activities of myocardial mitochondrial cathepsin-D and β-glucuronidase are significantly decreased by high-dose ISO and that ISO decreases stability of the myocardial mitochondrial membrane. ISO is widely used to induce MH (50,53). There is evidence demonstrating that ISO stimulation leads to MH (53). ISO activates MAPK in cardiomyocytes and phosphorylates Raf/MEK/ERK signaling

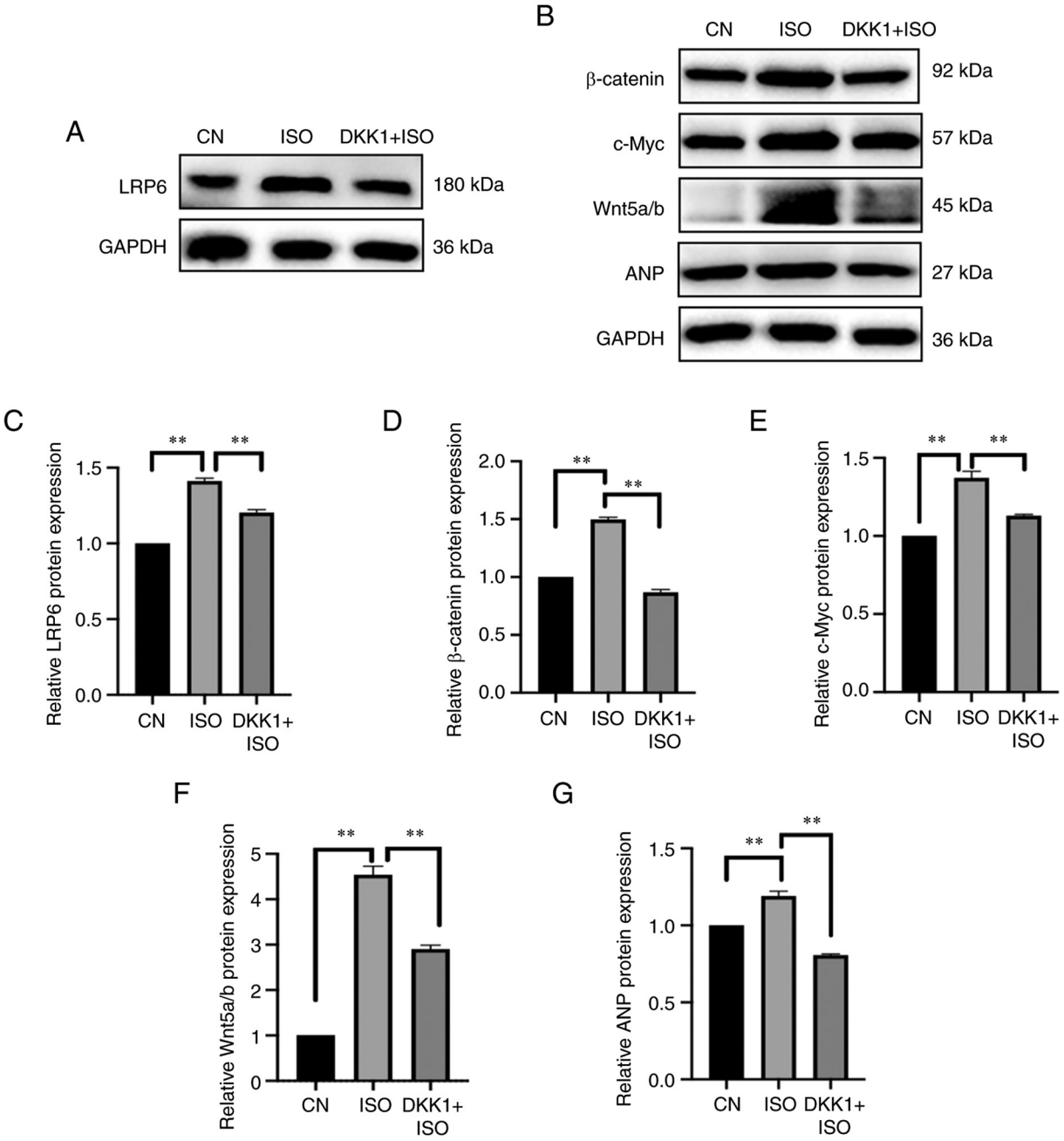

Figure 6. Effect of DKK1 on expression of protein in the signaling Wnt pathway. (A) Representative western blotting images of LRP6. (B) Representative western blotting images of β-catenin, Wnt5a/b, c-Myc and ANP. (C) LRP6, (D) β-catenin, (E) c-Myc, (F) Wnt5a/b and (G) ANP protein expression levels were semi-quantified. The blots were analyzed using ImageJ. **P<0.01. DKK1, Dickkopf-1; CN, control; ISO, isoproterenol; LRP6, low-density lipoprotein receptor-related protein 6; ANP, atrial natriuretic peptide.

pathway components (54). The increase of p-ERK1/2 protein expression levels is a downstream effect of ISO-induced protein kinase C ε activation, which leads to MH (55). A previous study reported that chronic infusion of ISO results in compensatory cardiac hypertrophy associated with protein kinase A activity and N-terminal cleaved histone deacetylase production (56). The mechanism of ISO-induced myocardial injury may be associated with oxidative stress, calcium overload, inflammation and lipid peroxidation (57). It has been confirmed that in ISO-induced MH, ISO treatment markedly increases reactive oxygen species levels in cardiomyocytes (58), activates the NF-κB signaling pathway in AC16 cells and induces an inflammatory response (59). Further studies are required to determine the mechanism underlying ISO-induced MH. Based on a previous study (60), 10 μM ISO was chosen as the induction concentration for the present study.

In failing human hearts, there is a significant increase in both mRNA and protein expression of ANP, BNP and β-MHC (61); whereas expression of these three genes is normally stable in normal cardiomyocytes (62,63). A previous study reported that PU decreases mRNA expression levels of MH markers ANP and BNP in a dose-dependent manner, with 40 μM being the optimal concentration (64). Combined with the results from the present CCK-8 assay, 40 μM was selected as the concentration to induce MH for subsequent experimentation in the present study. It has been reported that the early

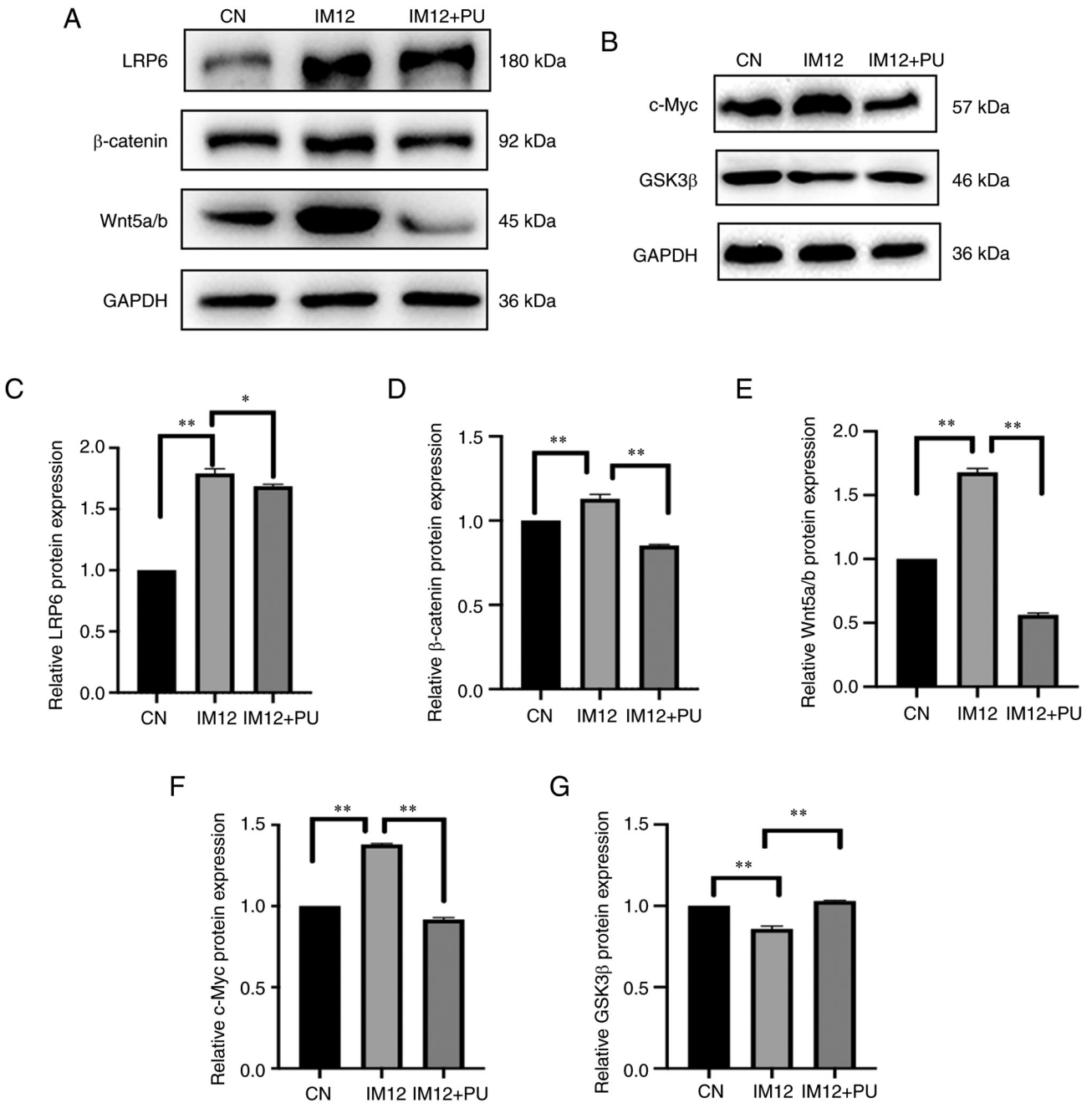

Figure 7. Effect of IM-12 and PU on expression of proteins in the Wnt signaling pathway. (A) Representative western blotting images of LRP6, β-catenin and Wnt5a/b expression. (B) Representative western blotting images of c-Myc and GSK3β expression. (C) LRP6, (D) β-catenin, (E) Wnt5a/b, (F) c-Myc and (G) GSK3β protein expression levels were semi-quantified. Blots were analyzed using ImageJ. *P<0.05 and **P<0.01. CN, control; PU, puerarin; LRP6, low-density lipoprotein receptor-related protein 6; GSK3β, glycogen synthase kinase 3β.

stage of MH is characterized by the increase of the surface area of cardiomyocytes (65). Changes in cell surface area (quantified and analyzed using immunofluorescence) directly reflect progression of cardiac hypertrophy; this method is used to assess MH. In a previous study, H9c2 cells were stained with crystal violet and surface area was observed as a measure of MH to confirm that the surface area of CD38 knockdown cells was not enlarged (66). Another study used wheat germ agglutinin staining to evaluate the size of cardiomyocytes from heart sections (67). A previous study used surface area of cells stained with gentian violet to assess MH (68). Studies have also aimed to use cell surface area to assess the role of miR-339-5p in cardiomyocyte hypertrophy (69) and determine the therapeutic potential of pyrroloquinoline quinone for ISO-induced cardiac hypertrophy (59). In the present study, pretreatment with PU was demonstrated to exert protective effects against ISO-induced MH, demonstrated by decreased area of cardiomyocytes and expression of markers of cardiac hypertrophy. Therefore, PU may be a promising clinical agent for the prevention and treatment of MH, although its mechanism remains to be fully elucidated.

The signal transduction mechanism underlying MH is complex. Inflammation (70) and oxidative stress (71) have both been previously demonstrated to cause alterations leading to

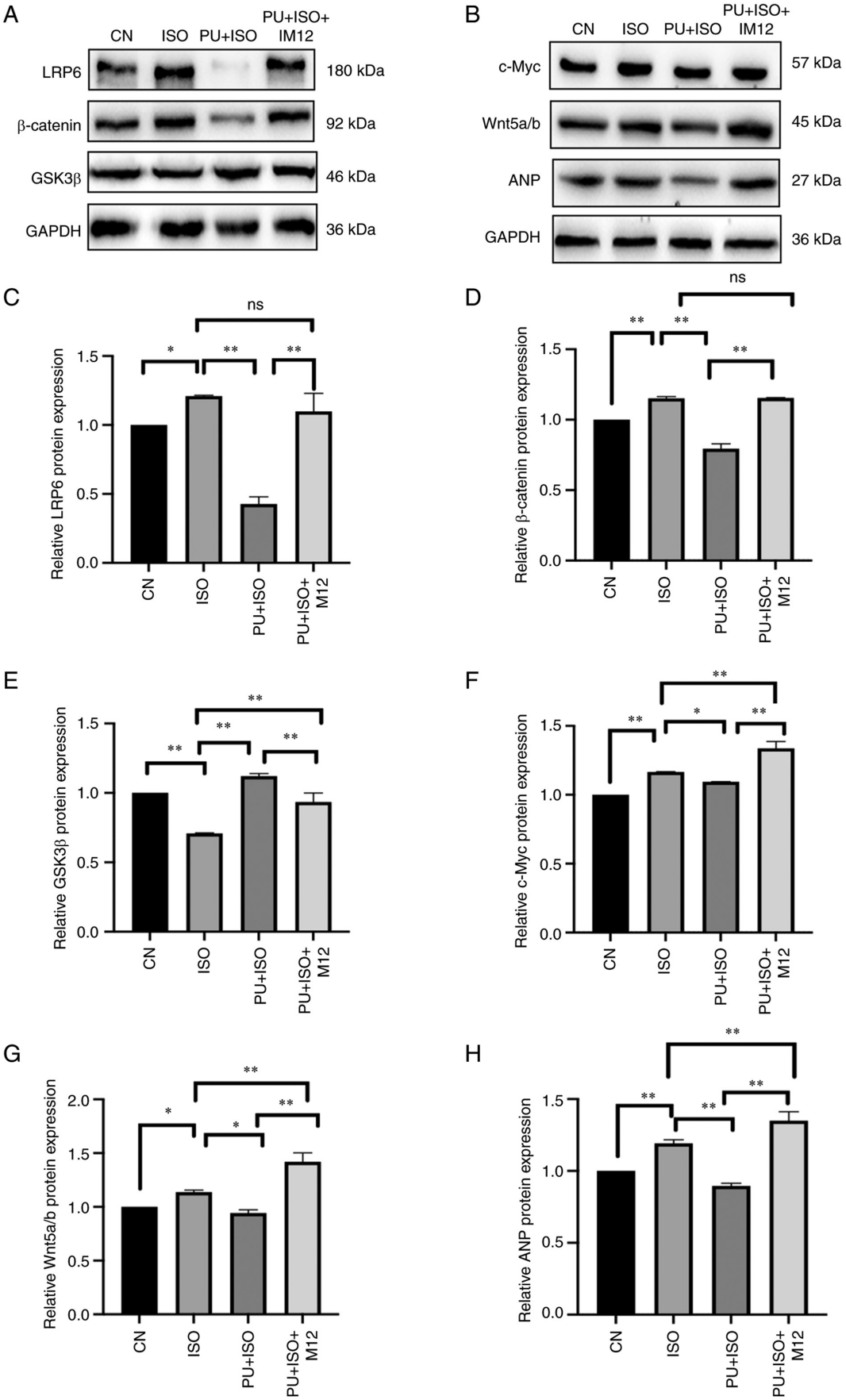

Figure 8. Effect of ISO, PU and IM-12 on expression of proteins in the Wnt signaling pathway. (A) Representative western blotting images of LRP6, GSK3β and β-catenin expression. (B) Representative western blotting images of c-Myc, Wnt5a/b and ANP expression. (C) LRP6, (D) β-catenin, (E) GSK3β, (F) c-Myc, (G) Wnt5a/b and (H) ANP protein expression levels were semi-quantified. The blots were analyzed using ImageJ. *P<0.05 and **P<0.01. CN, control; ISO, isoproterenol; PU, puerarin; LRP6, low-density lipoprotein receptor-related protein 6; GSK3β, glycogen synthase kinase 3β; ANP, atrial natriuretic peptide; ns, not significant.

MH and heart failure. A previous systematic study of Wnt on a genome-wide level reported an association between MH and the Wnt expression profile (72). The Wnt signaling pathway has also been reported to be associated with cardiac disease; excessive stimulation of Wnt signaling is detrimental to cardiovascular pathology (42). PU has been reported to demonstrate anti-inflammatory and antioxidant activities (73). However, the potential effects of PU on MH and the Wnt signaling pathway in cardiomyocytes remain poorly understood. Therefore, the present study aimed to explore if PU demonstrate effects against MH in an *in vitro* model, with specific focus on the Wnt/β-catenin signaling pathway.

The Wnt signal transduction pathway is divided into classical and non-classical branches (74). The present study verified the expression of associated proteins and demonstrated that PU inhibited ISO-induced MH by blocking the classical branch of the Wnt/β-catenin signaling pathway. Wnt signaling is typically activated by the membrane receptor complex consisting of frizzled (Fzd), LRP5/6 and disheveled (Dvl) (75). Following activation of the Wnt receptor, Fzd binds to Wnt protein and LRP5/6 to form a receptor complex (76). Dvl is recruited by the receptor complex and activated via phosphorylation before binding to the β-catenin degradation complex, which separates β-catenin from the degradation complex to promote stabilization and aggregation before β-catenin translocates into the nucleus (77). β-catenin is a core component of the Wnt/β-catenin signaling pathway and is reported to be upregulated in cardiomyocytes from patients with acute myocardial infarction and heart tissue of spontaneously hypertensive rats (78). However, GSK3β mediates both β-catenin-dependent and -independent cascades (79). A previous study reported that GSK3β exerts negative regulatory effects on the Wnt signaling pathway (80). In the nucleus, β-catenin binds to T cell factor/lymphoid enhancer factor (Tcf/Lef) to activate transcription of Wnt target genes (81). c-Myc and Tcf/Lef are downstream targets of the Wnt signaling pathway; a previous study reported that activity of these targets is inhibited by low expression of β-catenin (82).

In the present study, western blotting was used to assess expression of core proteins in the Wnt signaling pathway; expression levels of these proteins in the ISO group was significantly increased but significantly reversed by PU pretreatment. However, GSK3β protein expression levels exhibited an opposite trend, which is likely due to its negative regulatory mechanism of Wnt; following activation of Wnt signaling, activity of GSK3β is hampered (83). A previous study reported that decreased expression of GSK3β in cardiac fibroblasts that led to adverse ventricular remodeling (84). Therefore, inhibition of GSK3β may increase the activity of Wnt signaling, thereby leading to cardiac hypertrophy. PU induces expression of GSK3β, as demonstrated in the present study, and serves a protective role. Furthermore, numerous studies have demonstrated that the Wnt/β-catenin and NF-κB signaling pathways exhibit crosstalk and this interaction is enhanced by the inflammatory response (85,86). In the present study, it was demonstrated that, in the ISO group, the protein expression of Wnt5a/b and β-catenin and the phosphorylation levels of p65 were significantly increased compared with the control but significantly decreased by pretreatment with PU. These observations are in accordance with those in previous studies which suggested that there may be synergy between the two signaling pathways during the development of MH (87,88). However, more evidence is needed to support the synergistic effect.

To verify whether inhibition of the Wnt signaling pathway ameliorated ISO-induced MH, DKK1, an inhibitor of the Wnt signaling pathway, was used. DKK1 is a member of the DKK family that is reported to antagonize the Wnt/β-catenin signaling pathway by decreasing β-catenin and increasing OCT4 expression (89). The results of the present study demonstrated that the DKK1 group exhibited trends in the expression of protein in the Wnt signaling pathway that were comparable to those follow PU pretreatment, in that PU exerted inhibitory effects on Wnt signaling comparable with those mediated by the known Wnt inhibitor. IM-12, an activator of the Wnt signaling pathway, has been reported to increase expression of β-catenin and downstream proteins while suppressing expression of GSK-3β (46). The present study assessed whether IM-12 reversed the inhibitory effects of PU on ISO-induced MH, thus confirming whether PU exerted its effects via inhibition of the Wnt signaling pathway. The present study demonstrated that PU inhibited expression of Wnt signaling pathway proteins induced by IM-12 and increased expression of GSK-3β. Furthermore, IM-12, the specific activator of the Wnt signaling pathway, was demonstrated to eliminate the protective effects of PU on MH. In conclusion, PU served a cardioprotective role partially via inhibition of the Wnt signaling pathway.

However, in the present study only core proteins in the Wnt signaling pathway were assessed; therefore the detailed underlying molecular mechanism in the upstream regulation of Wnt/β-catenin signaling during MH was not fully elucidated. Since Wnt signaling induces nuclear localization of β-catenin (90), it is important to determine the localization and expression of β-catenin. These limitations of the present study require addressing in future. In future studies, the inhibitor-like mechanism of PU and its effects on other disease caused by aberrant activation of the Wnt signaling pathway, including colon cancer, hepatocellular carcinoma and pancreatic, lung and ovarian cancer (91), should be explored.

In summary, the present study demonstrated that pretreatment of AC16 cells with PU attenuated ISO-induced MH. The underlying mechanism was associated with inhibition of protein expression in the Wnt/β-catenin signaling pathway and NF-κB activity. These results suggested that PU may be a potential agent for treating MH. Based on the importance of the Wnt signaling pathway for initiation, maintenance, progression and relapse of MH, PU may serve an inhibitor-like role for the treatment of cardiovascular and associated disease caused by hyperactivation of the Wnt signaling pathway. Furthermore, the present study may provide a novel direction for development and use of agents for MH treatment.

Acknowledgements

Not applicable.

Funding

The present study was supported by Shanghai Special Project of Biomedical Science and Technology (grant no. 21S11901700).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XW performed experiments, analyzed data and wrote the manuscript. KH performed experiments and data analysis. LM designed the study, analyzed data and revised the manuscript. LW performed data analysis. YY participated in discussions and analyzed data. YL designed the study and obtained funding. XW, KH, LM and YL confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for participation

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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