3,3'-Diindolylmethane attenuates cardiac H9c2 cell hypertrophy through 5'-adenosine monophosphate-activated protein kinase-α

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Abstract. 3,3'-Diindolylmethane (DIM) is the major product of the acid-catalyzed condensation of indole-3-carbinol (I3C), a component of extracts of Brassica food plants. Numerous studies have suggested that DIM has several beneficial biological activities, including elimination of free radicals, antioxidant and anti-angiogenic effects and activation of apoptosis of various tumor cells. In the present study, an *in vitro* model was established, using 1 μM angiotensin II (Ang II) in cultured rat cardiac H9c2 cells, to observe the effects of DIM on cardiac hypertrophy. Following 24 h stimulation with DIM (1, 5, and 10 μM) with or without Ang II, cells were characterized by immunofluorescence to analyze cardiac α-actinin expression. Cardiomyocyte hypertrophy and molecular markers of cardiac hypertrophy were assessed by quantitative polymerase chain reaction. Atrial natriuretic peptide, brain natriuretic peptide and myosin heavy chain β mRNA expression were induced by Ang II in H9c2 cells treated with the optimal concentration of DIM for 6, 12, and 24 h. The levels of phosphorylated and total proteins of the 5' AMP-activated protein kinase α (AMPKα)/mitogen-activated protein kinase (MAPK)/mechanistic target of rapamycin (mTOR) signaling pathways in H9c2 cells treated with DIM for 0, 15, 30, and 60 min induced by Ang II were determined by western blot analysis. The results showed that DIM attenuated cellular hypertrophy *in vitro*, enhanced the phosphorylation of AMPKα and inhibited the MAPK-mTOR signaling pathway in response to hypertrophic stimuli.

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Introduction

Cardiac hypertrophy is an important compensatory mechanism for pathophysiological states, involving an increase in size and/or thickness of the ventricles of the heart. Cardiac hypertrophy is an adaptive response to increased workload or to defects in the efficiency of the contractile machinery of the heart. Sustained hypertrophic stimulation, however, is a maladaptive response causing adverse effects, including cardiomyocyte fetal gene re-expression and cardiac interstitial fibrosis (1,2). Cardiac hypertrophy is a well-established risk factor of cardiovascular mortality, and can significantly increase the incidence of cardiovascular events, including sudden death, ventricular arrhythmia, myocardial ischemia and heart failure, and can thus contribute to an increase in the rate of mortality (3). The mechanistic process of cardiac hypertrophy remains to be fully elucidated. Current therapies for cardiac hypertrophy have predominantly focused on the regulation of hemodynamics (4); however, an effective method to prevent and treat cardiac hypertrophy remains to be developed. Pharmacological interventions targeting the molecular changes involved in cardiac hypertrophy may be a promising approach for the prevention of cardiac hypertrophy and progression to heart failure.

3,3'-Diindolylmethane (DIM) is a Traditional Chinese Medicine that is extracted from Brassica plants. It is the major *in vivo* product derived from the acid-catalyzed condensation of indole-3-carbinol (I3C). Numerous studies have suggested that DIM has various properties, including eliminating free radicals, antioxidant and anti-angiogenic effects and promoting apoptosis in tumor cells (5-7). DIM can affect mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K)/Akt and the nuclear factor-κB (NF-κB) signaling pathways (6,8,9), and functions of anti-cancer agents (8), anti-angiogenic (6) and anti-inflammatory (9) processes. In a previous study by our group (10), it was shown that DIM prevented the development of cardiac hypertrophy and could revert established cardiac hypertrophy through the promotion of AMPKα and inhibition of mechanistic target of rapamycin (mTOR) signaling *in vivo*. Furthermore, the cardioprotective effects of DIM were ameliorated in mice lacking functional 5' AMP-activated protein kinase α (AMPKα)2 (10).

Angiotensin II (Ang II) has a central function in the regulation of the cardiovascular system (11). Activation of the

renin-angiotensin system (RAS), and the subsequent generation of Ang II, are important mediators of myocardial fibrosis, pathological hypertrophy and heart failure (12,13). The purpose of the present study was to use an *in vitro* model of hypertrophy, induced by 1 μM Ang II in cultured rat cardiac H9c2 cells, to evaluate the effects of DIM on the cellular response.

Materials and methods

Materials. The primary antibodies included phosphorylated (p)-AMPKα (sc101631; Santa Cruz, Santa Cruz, CA, USA), p-c-Jun, total (T-)c-Jun, T-AMPKα (BS4046, BS1061, BS6271, respectively; Bioworld Technology Inc., St. Louis Park, MN, USA) and p-mTOR (2971), T-mTOR (2983; Cell Signalling Technology, Inc.), p-p70S6K (9234), T-p70S6K (2708), p-S6 (2215), T-S6 (2217), p-eIF4E (9741), T-eIF4E (2067P), p-4E-BP1 (2855), T-4E-BP1 (9644), p-mitogen-activated protein kinase kinase (MEK)1/2 (9154), T-MEK1/2 (9122), p-extracellular signal-regulated kinase (ERK)1/2 (4370), T-ERK1/2 (4695), p-p38 (511), T-p38 (9212), p-c-Jun N-terminal kinase (JNK)1/2 (4668P), T-JNK1/2 (9258) and GAPDH (2118) (all purchased from Cell Signalling Technology, Inc., Danvers, MA, USA). DIM was purchased from Sigma-Aldrich (St. Louis, MO, USA; D9568-5G) and dissolved in dimethyl sulfoxide (RNBC0311; Sigma-Aldrich) for the *in vitro* bioassay.

Cell culture. Rat cardiac H9c2 cells (Cell Bank of the Chinese Academy of Sciences, Shanghai, China) were cultured in Dulbecco's modified Eagle's medium (DMEM, C11995; Gibco-BRL, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL, 10099-133), 100 U/ml penicillin/100 mg/ml streptomycin (Gibco-BRL, 15140) and 5% CO_2 at 37˚C. The media was exchanged every 1-2 days and subcultured to 70-80% confluency. Cells were plated at an appropriate density according to each experimental design. H9c2 cells were seeded in six-well plates at a density of $0.25x10^6$ cells/well. Following 24 h adherence, the culture medium was changed to serum-free DMEM 12 h prior to the experiment. Cells were incubated with DIM (1, 5, and 10 μM) in serum-free DMEM at 37˚C with or without 1 μM AngII (A9525; Sigma-Aldrich) for 24 or 48 h.

Immunofluorescence. Cells were analyzed for cardiac α-actinin expression by immunofluorescence, in order to identify rat cardiac H9c2 cells and assess cardiomyocyte hypertrophy. The cells were washed with phosphate-buffered saline (PBS), fixed with RCL2® (RCL2-CS24L; ALPHELYS, Plaisir, France) and permeabilized in 0.1% Triton™ X-100 in PBS. The cells were then incubated with anti-α-actinin (05-384; Millipore, Billerica, MA, USA) at a dilution of 1:100 in 1% goat serum. The cells were then incubated with Alexa FluorH 488 goat anti-mouse immunoglobulin (Ig)G (A11001; Invitrogen Life Technologies, Carlsbad, CA, USA) secondary antibody. The cells on coverslips were mounted onto glass slides with Slow Fade Gold antifade reagent with DAPI (S36939; Invitrogen Life Technologies). The outline of 40 cells from each group were visualized by light microscopy (BX51TRF; Olympus Corporation, Tokyo, Japan). A single cell was measured using a quantitative digital image analysis system (Image-Pro Plus version 6.0; Media Cybernetics, Rockville, MD, USA).

Quantitative polymerase chain reaction (qPCR). qPCR was used to detect RNA expression levels of hypertrophic and fibrotic markers. Total RNA was extracted from frozen, pulverized mouse cardiac tissue using TRIzol™ (15596-026; Roche Diagnostics, Mannheim, Germany). RNA yield and purity were evaluated using a SmartSpec Plus Spectrophotometer (Bio-Rad, Hercules, CA, USA), comparing the A260/A280 and A230/260 ratios. The RNA (2 μg of each sample) was reverse-transcribed into cDNA using oligo(DT) primers and the Transcriptor First Strand cDNA Synthesis kit (04896866001; Roche Diagnostics). The PCR products were quantified using a LightCycler 480 SYBR® Green 1 Master Mix (04707516001; Roche Diagnostics). Following an initial 5 min denaturation step at 95˚C, a total of 42 primer-extension cycles were carried out. Each cycle consisted of a 10 sec denaturation step at 95˚C, a 20 sec annealing step at 60˚C, and a 20 sec incubation at 72˚C for extension. Then a final extension step was performed at 72 ˚C for 10 min. The double standard curve was used to quantify the PCR results. Calibrator Normalized Ratio = (concentration of sample target / concentrations of sample reference) / (concentration of calibrator target / concentration of calibrator reference).The results were normalized against GAPDH gene expression. The sequences of the oligonucleotide primers (Sangon Biotech, Shanaghai, China) were as follows: : Atrial natriuretic peptide (ANP) forward, 5'-AAAGCAAACTGAGGGCTCTGCTCG-3', reverse, 5'-TTCGGTACCGGAAGCTGTTGCA-3'; brain natriuretic peptide (BNP) forward, 5'-CAGCAGCTTCTGCATC GTGGAT-3', reverse, 5'-TTCCTTAATCTGTCGCCGCTGG-3'; myosin heavy chain β (β-MHC) forward, 5'-TCTGGACAGCTC CCCATTCT-3', reverse, 5'-CAAGGCTAACCTGGAGAA GATG-3'; and GAPDH forward, 5'-GACATGCCGCCTGGAGA AAC-3', and reverse, 5'-AGCCCAGGATGCCCTTTAGT-3'.

Western blot analysis. Cultured cardiac H9c2 cells were lysed in radioimmunoprecipitation (RIPA) lysis buffer (720 μl RIPA, 20 μl PMSF (1 mM), 100 μl complete, 100 μl cOmplete (Roche, Indianapolis, IN, USA, 04693124001), 100 μl phostop (Roche, 04906837001), 50 μl NaF (1 mM), 10 μL Na_3VO_4, per ml) and 30 μg cell lysate was used for protein separation by 10% SDS-PAGE. The proteins were then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). Specific protein expression levels were normalized to the GAPDH protein levels of the total cell lysate and cytosolic proteins on the same PVDF membranes. The following primary antibodies were used: p-AMPKα, p-c-Jun, T-c-Jun, T-AMPKα, p-mTOR, T-mTOR, p-p70S6K, T-p70S6K, p-S6, T-S6, p-eIF4E, T-eIF4E, p-4E-BP1, T-4E-BP1, p-mitogen-activated protein kinase kinase (MEK)1/2, T-MEK1/2, p-extracellular signal-regulated kinase (ERK)1/2, T-ERK1/2, p-p38, T-p38, p-c-Jun N-terminal kinase (JNK)1/2, T-JNK1/2 and GAPDH. The primary antibodies were diluted at 1:1,000, with the exception of p-AMPKα, which was diluted 1:200. Antibody incubation was performed overnight with gentle shaking at 4˚C. Quantification of the western blots was performed using an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA). The secondary antibodies, goat anti-rabbit IRdye® 800 CW (LI-COR, 926-32211) IgG and goat anti-mouse IRdye® 800 CW (LI-COR, 926-32210), were used at a 1:10,000 dilution at 37˚C in Odyssey blocking for 1 h. The blots were scanned using an infrared Li-Cor scanner, allowing

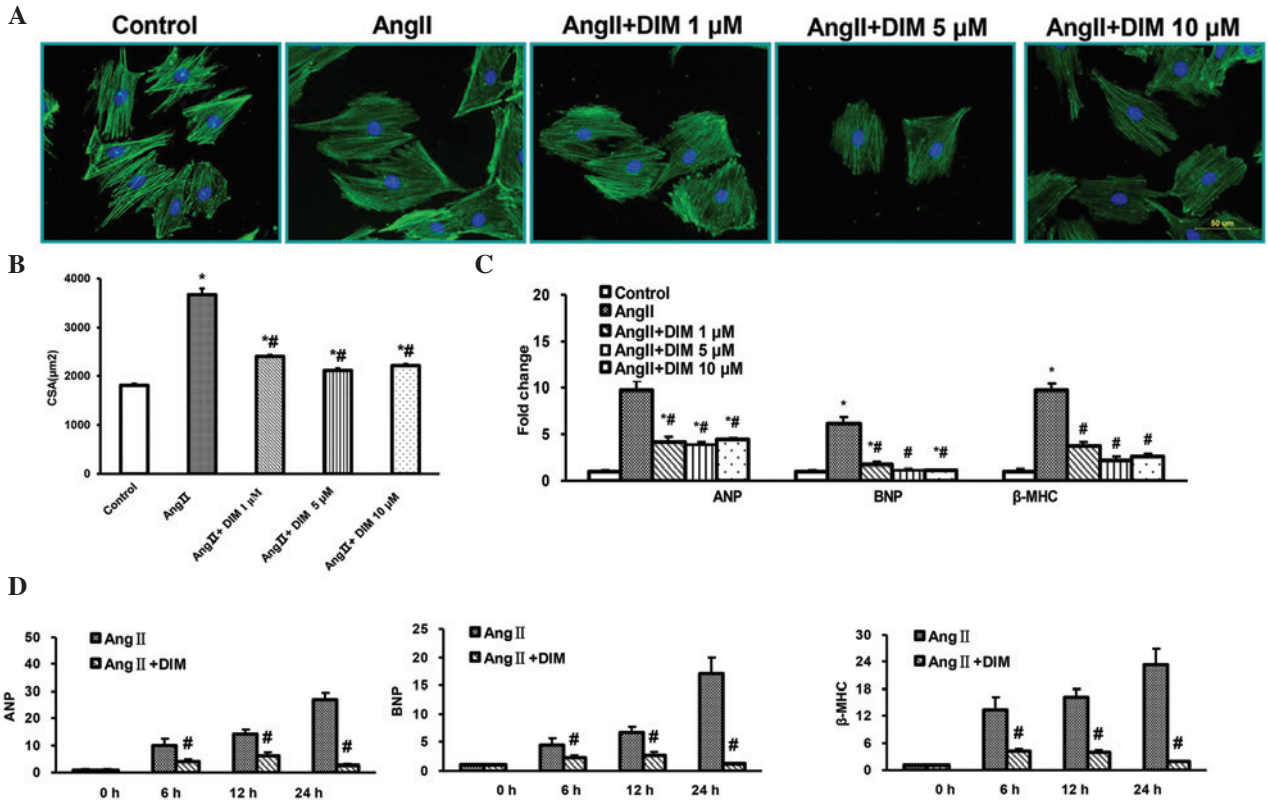

Figure 1. DIM attenuates cardiac H9c2 cell hypertrophy *in vitro*. (A and B) Effects of DIM on the reduction in size of H9c2 cells induced by Ang II (1 μM) for 24 h. (A) Representative immunofluorescent staining. Scale bar, 10 μm. (B) Quantitative analysis of immunofluorescence. *P<0.05 vs control group. #P<0.05 vs Ang II group. (C) qPCR analysis of the mRNA levels of ANP, BNP and β-MHC induced by DIM (1, 5, 10 μM) with Ang II (1 μM) for 24 h. *P<0.05 vs control group. #P<0.05 vs Ang II group. (D) qPCR analysis of the mRNA levels of ANP, BNP and β-MHC induced by DIM (5 μM) with Ang II (1 μM) at the time-points indicated. #P<0.05 vs Ang II group at the same time-point. ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; Ang II, angiotensin II; DIM, 3,3'-diindolylmehane; β-MHC, myosin heavy chain β; CSA, cell surface area; qPCR, quantitative polymerase chain reaction analysis.

for simultaneous detection of two targets (phosphorylated and total protein) within the same experiment.

Statistical analysis. Data are expressed as the mean ± standard error of the mean. Differences among groups were determined by two-way analysis of variance followed by Tukey's post-hoc test. Comparisons between two groups were performed using an unpaired Student's t-test. Statistical analyses were conducted using SPSS version 13.0 (SPSS Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

DIM attenuates cardiac H9c2 cell hypertrophy. An *in vitro* model using Ang II (1 μM) in cultured rat cardiac H9c2 cells was established in order to confirm the effects of DIM on cardiac hypertrophy. Following stimulation with DIM (1, 5, and 10 μM) with or without Ang II (1 μM) for 24 h, cells were characterized for cardiac α-actinin expression by immunofluorescence, to assess cardiomyocyte hypertrophy. H9c2 cells showed an enlarged cell surface area as compared with those induced by DIM (1, 5, and 10 μM) with Ang II for 24 h (Fig. 1A and B). qPCR demonstrated that DIM (1, 5, and 10 μM) markedly decreased the induction of ANP, BNP and β-MHC mRNA expression induced by Ang II (1 μM), most significantly in cells treated with 5 μM DIM (Fig. 1C). There were no significant differences between the DIM 5 μM and DIM 10 μM groups. Additionally, qPCR demonstrated that cells treated with 5 μM DIM for 6, 12, and 24 h exhibited a markedly decreased induction of ANP, BNP and β-MHC mRNA expression by Ang II (Fig. 1D). These findings indicated that DIM attenuated cell hypertrophy *in vitro*.

DIM promotes p-AMPKα activity and inhibits mTOR signaling in response to Ang II in vitro. In a previous study by our group, it was identified by western blotting that DIM could enhance the phosphorylation of AMPKα and block mTOR signaling induced by pressure overload *in vivo* (7). In the present study, the activation of AMPKα and mTOR signaling by Ang II (1 μM) in H9c2 cells treated with DIM (5 μM) or PBS for 0, 15, 30 and 60 min was evaluated for the first time, to the best of our knowledge. The activation of AMPKα was increased in H9c2 cells treated with 5 μM DIM, as compared with that in response to 1 μM AngII alone. The levels of phosphorylated mTOR, p70S6K, S6, eIF4E and 4E-BP1 were significantly increased in H9c2 cells in response to 1 μM Ang II. However, the increased levels of mTOR, p70S6K, S6, eIF4E and 4E-BP1 were reduced in cells treated with 5 μM DIM (Fig. 2A and B). These findings indicated that DIM attenuated cell hypertrophy, predominantly through stimulating p-AMPKα activity and inhibiting mTOR signaling *in vitro*.

DIM inhibits MAPK signaling in response to hypertrophic stimuli in vitro. MAPK signaling has a central function in cardiac hypertrophy (14). The state of activation of MAPK

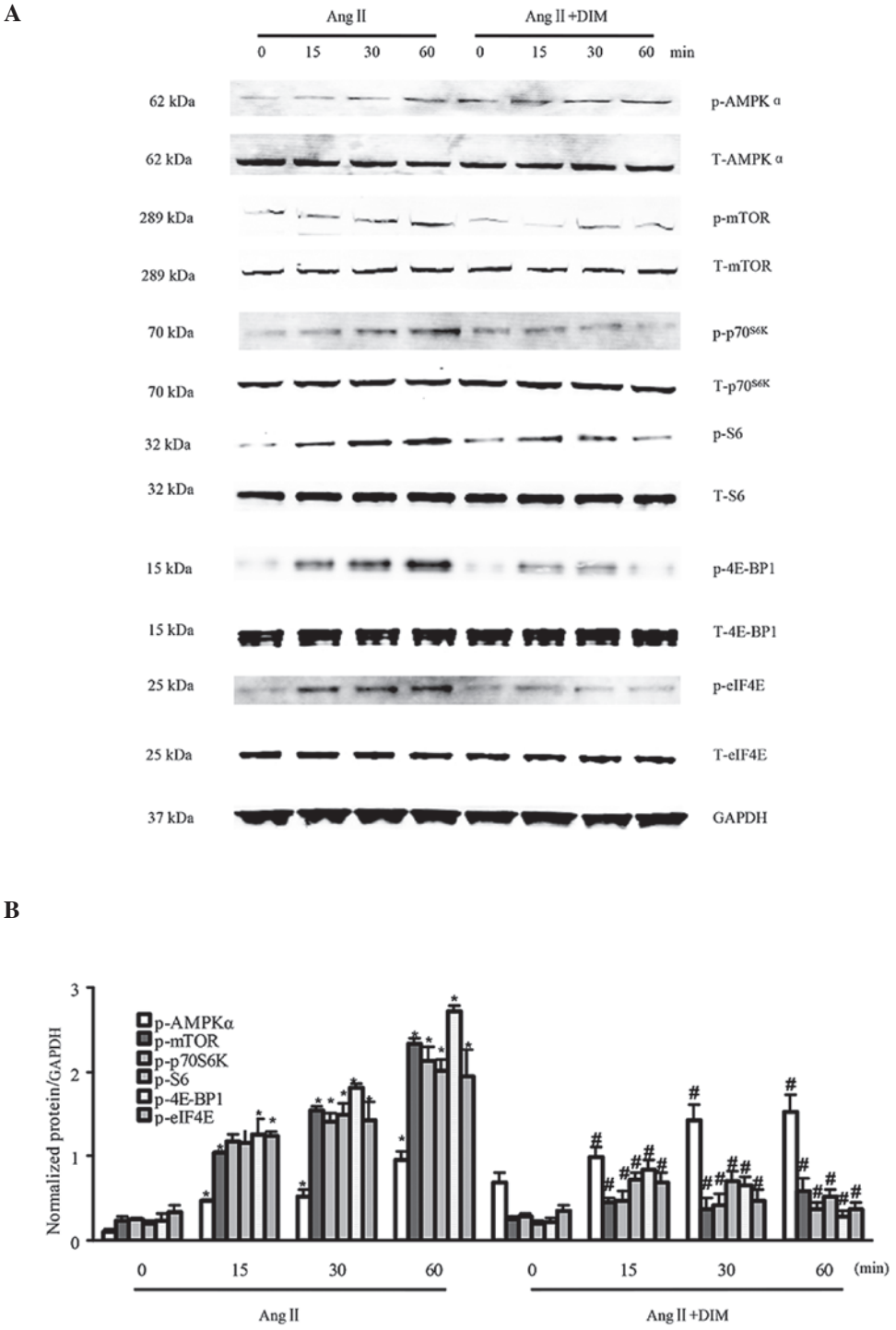

Figure 2. DIM enhances p-AMPKα activity and inhibits mTOR signaling in response to hypertrophic stimuli *in vitro*. (A and B) The levels of total (T-) and phosphorylated (p-)AMPKα, mTOR, p70S6K, S6, eIF4E, and 4E-BP1 expression in H9c2 cells treated with DIM (5 μM) with Ang II (1 μM) at the time-points indicated. (A) Representative blots. (B) Quantitative results. *P<0.05 vs Ang II group at time-point 0. $^{\#}$P<0.05 vs Ang II group at the same time-point. Ang II, angiotensin II; DIM, 3,3'-diindolylmehane; p-AMPKα, phosphorylated 5' AMP-activated protein kinase α; mTOR, mammalian target of rapamycin.

signaling in H9c2 cells treated with 5 μM DIM or PBS for 0, 15, 30, and 60 min by 1 μM Ang II, was therefore evaluated. It was identified that the phosphorylated levels of MEK1/2, ERK1/2, p38, JNK1/2 and c-Jun were significantly increased in H9c2 cells in response to Ang II (1 μM). However, the increased levels of MEK1/2, ERK1/2, JNK1/2 and c-Jun were attenuated in cells treated with 5 μM DIM, whereas p38 was similarly activated in DIM- and PBS-treated groups (Fig. 3A and B). The results indicated that DIM significantly ameliorated cardiac hypertrophy through direct inhibition of the MAPK signaling pathway *in vitro*.

Discussion

In the present study, the role of DIM in cultured rat cardiac H9c2 cells with induced hypertrophy by Ang II *in vitro* was examined. The results demonstrated that DIM significantly ameliorated cardiac cell hypertrophy by stimulating p-AMPKα

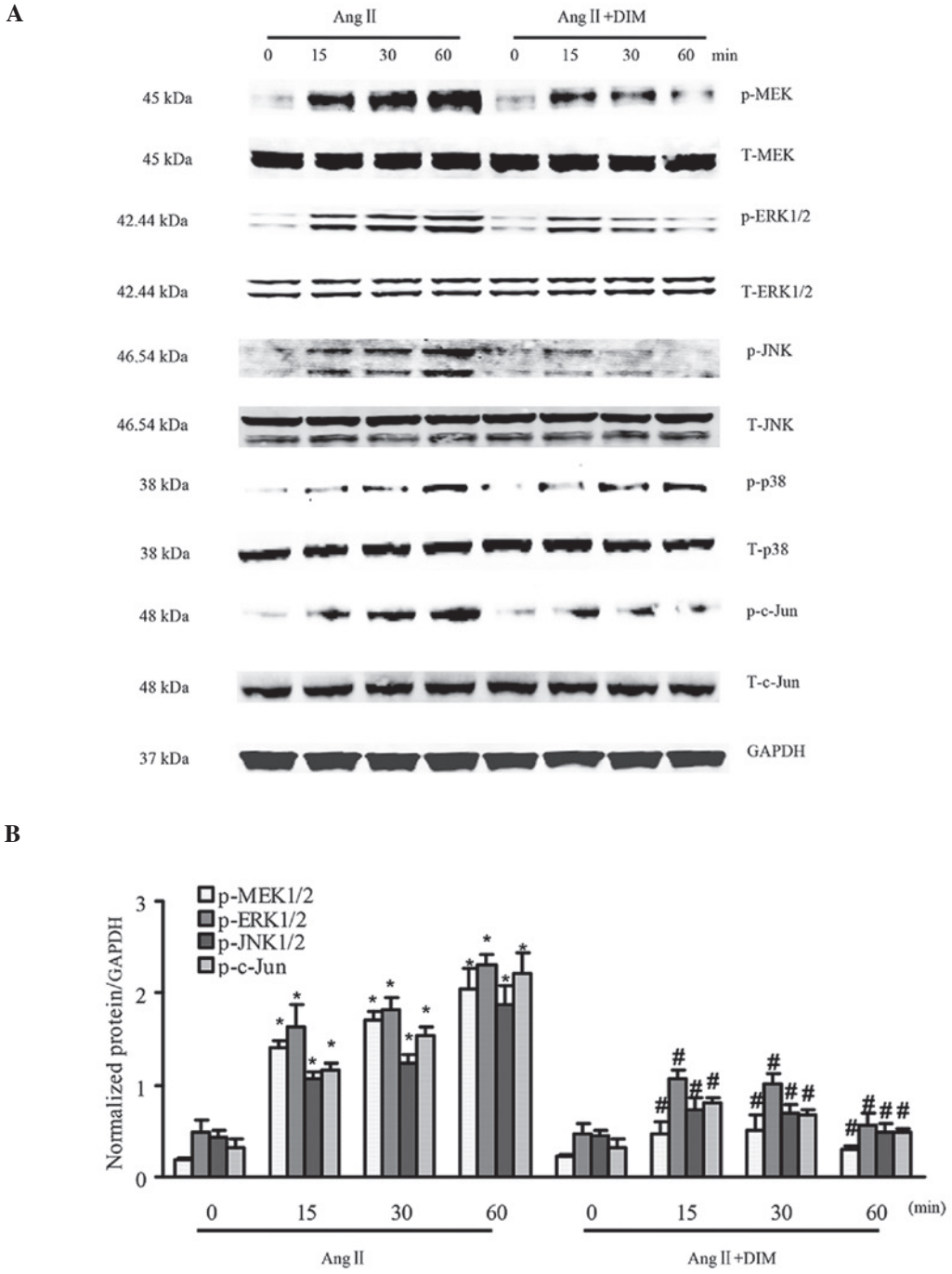

Figure 3. DIM inhibits MAPK signaling induced by Ang II. (A and B) The levels of total (T-) and phosphorylated (p-)MEK1/2, ERK1/2, p38, JNK1/2 and c-Jun expression in H9c2 cells treated with DIM (5 μM) with Ang II (1 μM) at the indicated time-points. (A) Representative blots. (B) Quantitative results. *P<0.05 vs Ang II group at time-point 0. $^{\#}$P<0.05 vs Ang II group at the same time-point. Ang II, angiotensin II; DIM, 3,3'-diindolylmehane; MAPK, mitogen-activated protein kinase; p-MEK, phosphorylated mitogen-activated protein kinase kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase.

activity and disrupting mTOR and MAPK signaling in response to Ang II stimuli *in vitro*. These findings suggested that DIM has a protective role in the regulation of rat cardiac H9c2 cell hypertrophy induced by Ang II *in vitro*.

H9c2 cells have been used as an alternative source for non-proliferating adult cardiomyocytes, originating from the ventricular muscle of the embryonic rat heart (15,16). H9c2 cells display similarities to adult cardiomyocytes in their morphology, protein expression, signaling and hypertrophy-associated characteristics (15). Previous studies have cultured primary rat neonatal cardiomyocytes (PNCM) and H9c2 cells and treated the cells with Ang II and endothelin-1 (ET-1) to promote a hypertrophic response (17). Ang II and ET-1 are closely associated hypertrophic stimuli and have been observed to cause a similar increase in cell size, rearrangement of contractile proteins and upregulation of hypertrophic genes in both PNCM and H9C2 cells (15). These findings validate the importance of H9C2 cells as a model for *in vitro* studies of cardiac hypertrophy (15). The present study used an *in vitro* model of hypertrophy with 1 μM Ang II in cultured rat cardiac H9c2 cells, to demonstrate the effects of DIM on the cellular phenotype. It was shown that DIM (1, 5, and 10 μM) inhibited the enlargement of the cell

surface area and decreased the induction of ANP, BNP and β-MHC mRNA expression induced by Ang II. The effect was most significant in the 5 μM DIM-treated group. In addition, 5 μM DIM markedly decreased the induction of ANP, BNP and β-MHC mRNA expression induced by Ang II for 6, 12, and 24 h. There were no significant differences between the 5 and 10 μM DIM-treated groups. In a previous study by our group (10), it was demonstrated that DIM attenuated cardiac hypertrophy with improvements to the cardiomyocyte cross-sectional area in both the forward and reverse experiments, and blunted the expression of hypertrophic markers (10).

Diverse intracellular signaling pathways affecting nuclear factors and the regulation of gene expression, are implicated in the pathological processes involved in cardiac hypertrophy, including AMPK/mTOR (18), MAPKs (19), PI3K/AKT (20) and NF-κB (21). AMPK is highly expressed in cardiac myocytes and functions as a trimer of the α(1-2) catalytic subunit with the β(1-2) and γ(1-3) regulatory subunits (22). A previous study (23) has demonstrated that 5-aminoimidazole-4-carboxamide riboside, an AMPK activator, attenuated cardiac hypertrophy induced by pressure overload *in vivo*. Another study (18) showed that mice deficient of the major α catalytic subunit in cardiac muscle, AMPKα2, were protected from pressure-overload-induced left ventricular remodeling, which was associated with regulating mTOR/p70S6K signaling (18). In our previous study (10), it was found that DIM not only prevented the development of cardiac hypertrophy, but also reversed the established cardiac hypertrophy by regulating AMPKα and mTOR signaling *in vivo*. The cardioprotective effects of DIM were ameliorated in mice lacking functional AMPKα2 (10).

The MAPK signaling pathway consists of a sequence of successively functioning kinases that ultimately result in the dual phosphorylation and activation of p38, JNKs and ERKs (24). MEK-ERK1/2 signaling is sufficient to promote cardiac hypertrophy *in vivo* (25). However, pharmacological inhibition of MEK1-ERK1/2 may diminish the beneficial effects that are associated with compensated hypertrophy, whereas the inhibition of p38 and/or JNK may lead to a maladaptive hypertrophic response over a longer period (20). The status of MAPK signaling was evaluated in the presented hypertrophic model. This study showed that MEK, ERK1/2, JNK1/2 and c-Jun activation were inhibited in DIM-treated hearts and H9c2 cells (5 μM) as compared with the control groups stimulated with Ang II (1 μM). The findings indicated that the inhibitory effects of DIM on cardiac hypertrophy are partly mediated through MAPK signaling.

In conclusion, the present study indicated that DIM protected against rat cardiac H9c2 cell hypertrophy in response to Ang II. These observations have revealed new insights into the pathogenesis of cardiac remodeling and may have considerable implications for the development of strategies for the treatment of cardiac hypertrophy and heart failure through the application of DIM. Additional studies are required to explore the potential clinical uses of DIM.

Acknowledgements

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