

Chronic blockade of class I PI3-kinase attenuates Ang II-induced cardiac hypertrophy and autophagic alteration

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Abstract. – OBJECTIVE: Chronic Ang II stimulation is linked to cardiac remodeling characterized by fibrosis and cardiac hypertrophy. However, the underlying cellular mechanisms involved are not yet fully known. Here, we studied the molecular mechanisms underlying the chronic effect of Ang II on cardiac hypertrophy, fibrosis, and autophagy.

MATERIALS AND METHODS: The role of class I PI3-kinase in these actions of Ang II was studied using lentiviral vector-mediated expression of a dominant negative form of PI3-kinase subunit p85 α (Lv-DNp85) in the heart. Ang II was infused subcutaneously for 4 weeks on rats using osmotic pumps. Cardiac hypertrophy, fibrosis, reactive oxygen species (ROS), and autophagy were examined in four groups of rats (Ang II+Lv-DNp85, Ang II+Lv-GFP, Saline+Lv-DNp85, Saline+Lv-GFP).

RESULTS: Chronic infusion of Ang II induced severe cardiac hypertrophy and perivascular fibrosis in the heart. These effects were associated with a significant reduction in LC3 II and elevation in ROS levels, suggesting marked impairment of cardiac autophagy and increased generation of ROS. Cardiac transduction of Lv-DNp85 significantly attenuated Ang II-induced impairment of autophagy and elevation of ROS, as well as Ang II-induced cardiac hypertrophy and perivascular fibrosis. To study the cellular mechanisms underlying those actions of Ang II, phosphorylated Akt and mTOR were measured in hearts from these rats. Ang II increased phosphorylation of Akt and mTOR; and cardiac transduction of Lv-DNp85 significantly abolished Ang II-induced phosphorylation of Akt and mTOR, a signaling pathway inhibiting autophagy.

CONCLUSIONS: These results demonstrate that class I PI3-kinase, via activation of the Akt-mTOR pathway, is involved in Ang II-induced impairment of autophagy, elevation of ROS, cardiac hypertrophy, and fibrosis, suggesting a novel target for cardiac protection.

Key Words:

Angiotensin II, Cardiac hypertrophy, Fibrosis, Autophagy, PI3-kinase.

Introduction

Cardiac hypertrophy is initially an adaptive response to increased hemodynamic workload or to defects in the efficiency of the contractile machinery¹. However, in the long term it contributes to the development of heart failure and sudden cardiac death. Cardiac hypertrophy is often associated with diseases such as ischemic heart disease, hypertension, and heart failure, and is an important risk factor for subsequent cardiac morbidity and mortality². Key features of cardiac hypertrophy are increased cell size, enlarged organ size, changes in gene expression, and reorganization of the contractile machinery³. Various extracellular regulatory factors and intracellular signaling pathways are involved in the development of pathological cardiac hypertrophy². Elucidating the signaling pathways underlying cardiac hypertrophy is important for both our fundamental understanding of the process and development of potential therapeutic strategies for this condition.

PI3-kinase is an important intracellular kinase involved in controlling organ size^{4,6}. Three types of PI3-kinase have been discovered including class I, class II, and class III. Targeted overexpression of constitutively active class I PI3-kinase in the heart results in increased organ size, which is associated with a similar increase in cardiac myocyte size^{5,7}. Angiotensin II (Ang II) also plays an important role in cardiovascular physiolo-

ogy and pathology⁸. Circulating Ang II levels and the activity of the local cardiac renin-angiotensin system are increased in heart diseases, including cardiac hypertrophy⁹ and failure¹⁰. Chronic Ang II stimulation is linked to cardiac remodeling, which is characterized by fibrosis, myocyte hypertrophy, cardiac myocyte death, and metabolic alterations^{11,12}. However, the role of PI3-kinase in Ang II-induced cardiac hypertrophy has not been fully investigated. Much work has focused on the cardiomyocytes, especially neonatal cells¹³.

Recently, autophagy, a dynamic process involving the bulk degradation of cytoplasmic organelles and proteins, has been proven to be involved in the pathogenesis of cardiac hypertrophy and myocyte death¹⁴. Cardiac-specific loss of autophagy-related protein 5 (ATG5) leads to cardiac hypertrophy and dysfunction¹⁵. However, the effect of Ang II on cardiac autophagy and the underlying molecular mechanisms are still unknown. In the current study, we blocked class I PI3-kinase using lentiviral vector-mediated overexpression of dominant negative PI3-kinase P85 subunits in the rat heart in order to study the role of PI3-kinase in chronic Ang II infusion-induced cardiac hypertrophy and autophagy. Our results demonstrate that chronic blockade of PI3-kinase class I attenuated Ang II infusion-induced impairment of autophagy, elevation of ROS generation, cardiac hypertrophy, and fibrosis.

Materials and Methods

Animals

Experiments were performed on male Sprague-Dawley (SD) rats purchased from Charles River Laboratories (Wilmington, MA, USA). Rats were housed under controlled conditions with a 12-h light/dark cycle. Food and water were available to the animals *ad libitum*. All protocols were approved by the North Dakota State University Institutional Animal Care and Use Committee and by the Jilin University Institutional Animal Care and Use Committee.

Myocardial *in vivo* Gene Delivery

The lentiviral vector containing dominant negative class I PI3-kinase p85 α subunit (Lv-DNp85) and control Lv-GFP were constructed and titered as described in our previous publication¹⁶. The Lv-DNp85 and Lv-GFP were delivered into the heart using conventional intra-aortic root injection as described previously with minor

modifications¹⁷. Briefly, adult male SD rats were anesthetized with a mixture of oxygen (1 L/min) and isoflurane (3%), which was delivered through a nose cone¹⁸. Left anterior thoracotomy was performed in the left second intercostal space. Ligatures were looped around the ascending aorta and main pulmonary arteries for arterial occlusion. A PE-50 catheter was then inserted through the right carotid artery into the aortic root just above the aortic valve and below the occlusion ligature loop for measurement of pressure and for coronary arterial injections. Rats were then subjected to general hypothermia by external cooling with ice packs to below 26°C. Pulmonary arteries and aorta were occluded, followed by injection of cardioplegic solution (2 μ l/g body weight) containing (in mM): NaCl 110, KCl 20, CaCl₂ 1.2, MgCl₂ 16, and NaHCO₃ 10 via the arterial catheter. Lv-DNp85 or Lv-GFP (1 \times 10¹⁰ TU) was injected via the same catheter. Both occlusions were released after injection and the body temperature was heated back to normal with a heating pad. The chest was then closed and intrathoracic air was evacuated by suction.

Ang II Subcutaneous Infusion and Blood Pressure (BP) Recording

After cardiac injection of either Lv-DNp85 or Lv-GFP into the heart as described above, osmotic pumps were implanted for subcutaneous infusion of Ang II (200 ng/kg/min) using the method described in our previous publication^{19,20}. A four week period was allowed for recovery from surgery and gene expression in the heart. The rats were then anaesthetized with urethane (1 g/kg, i.p.), and BP was recorded using PE-10 catheters fused to PE-50 catheters. The vascular catheters were prefilled with heparinized saline (100 IU/ml), placed in the right femoral artery. Left ventricular pressure was recorded via right carotid artery catheterization. The vascular catheters were connected to a BP transducer and a bridge amplifier (AD instrument, Colorado Springs, CO, USA). The BP, heart rate (HR), and left ventricular dP/dt were collected and analyzed with PowerLab software (AD instrument). At the end of recording, hearts were collected and used for morphologic measurements and expression studies.

Assessment of Cardiac Hypertrophy and Fibrosis

The hearts were collected after BP and HR measurement and washed with saline solution. After heart weight and cardiac morphology were

determined, hearts were transversely sectioned. Some heart sections were quick frozen in liquid nitrogen for other assays and some sections were fixed in 10% formalin/PBS. Several sections of heart (4-5 μm thickness) were prepared and stained with hematoxylin and eosin (HE) and, then, visualized by light microscopy to evaluate morphology and cellular dimensions. The cross-sectional area of single myocytes was measured with Infinity Capture and Analyze Software. The outline of 100-200 cardiomyocytes was traced in each group. Sirius Red was used for collagen staining to assess perivascular fibrosis, which was expressed as the percentage of perivascular fibrotic area in the total area of the whole artery cross-section plus the perivascular fibrotic area.

Western Blots

The protein levels of PI3-kinase p85 α subunit, Akt, p-Akt, mTOR, p-mTOR, LC3 (including LC3-I and LC3-II) in the heart were assessed by Western blot analysis as described previously¹⁸. Primary antibodies (purchased from Santa Cruz Biotechnology Inc, Dallas, TX, USA or Cell Signaling Technology, Beverly, MA, USA) with dilution of 1:500 and a secondary antibody, anti-rabbit peroxidase-conjugated antibody (Bio-Rad, Hercules, CA, USA; dilution 1:15,000) were used. Immunoreactivity was detected by enhanced chemiluminescence autoradiography, and films were analyzed using Quantity One Software.

Immunofluorescence Staining

The immunofluorescence staining of heart sections was performed as we have described previously¹⁹. In brief, heart sections were fixed with formalin and were incubated with primary antibodies (rabbit anti-GFP antibody and mouse anti- α -actin antibody, dilution 1:500) overnight at 4°C. On the next day, the sections were washed and incubated with secondary antibody (Alexa Fluor 488 goat anti-rabbit IgG 1:1000 and Alexa Fluor 594 goat anti-mouse IgG 1:1000, Molecular Probe, Carlsbad, CA, USA) for 2 hours at room temperature. Heart sections were then washed with PBS/Tween, and mounted with anti-bleaching medium on a glass cover slip. The fluorescence staining was detected using an Olympus fluorescence microscope equipped with a color digital camera (Infinity 2), which was connected to a computer to capture and analyze images using Infinity Capture and Analyze Software.

PI3-Kinase and Reactive Oxygen Species (ROS) Assay

Rat heart tissue was rinsed with an ice-cold Hanks' solution and immediately lysed in NP-40 buffer at 4°C. The samples were immunoprecipitated using an anti-PI-3 kinase antibody (Cat# 06-195, Millipore Corp, Billerica, MA, USA) as described previously¹⁶, followed by class I PI-3 kinase assay using a competitive enzyme-linked immunosorbent assay kit (Cat# K-1000s, Echelon Biosciences, Salt Lake City, UT, USA) by exactly following the instruction provided by the manufacture. Each assay was repeated at least 3 times.

ROS generation was determined as in our previous study²¹ using a fluorogenic probe, dihydroethidium (DHE, excitation/emission wavelength: 485 nm/590 nm, purchased from Molecular Probes), which can be oxidized by superoxide generating fluorescence. Heart sections were embedded in a freezing medium and transverse sections (30 μm) of frozen tissue were obtained using a cryostat, collected on glass slides, and equilibrated for 10 min in Hanks' solution. Heart sections were loaded with DHE (2×10^{-6} M). Ethidium fluorescence of cardiomyocytes was detected by a fluorescent microscope (Nikon, Melville, NY, USA), and its intensity was analyzed using Quantity One Software (Bio-Rad, Hercules, CA, USA).

Statistical Analysis

All data are presented as means \pm SE. Statistical significance was evaluated by 1- or 2-way ANOVA, as appropriate, followed by either a Newman-Keuls or Bonferroni post hoc analysis, where appropriate. Differences were considered significant at $p < 0.05$ and individual probability values are noted in the figure legends.

Results

Effect of Lv-DNp85 Transduction on p85 Subunit Expression and PI3-Kinase Activity

Chronic Ang II stimulation is linked to cardiac remodeling that is characterized by interstitial fibrosis, cardiac hypertrophy, and apoptosis. To study the underlying intracellular signaling molecules involved, we examined the role of class I PI3-kinase in these actions of Ang II using lentiviral vector-mediated overexpression of a dominant negative PI3-kinase subunit p85 (Lv-DNp85). Four groups of SD rats ($n = 6$ rats in

each group) were implanted with subcutaneous osmotic minipumps for infusion of Ang II (200 ng/kg/min) or saline control, with cardiac transfer of Lv-DNp85 (2×10^{10} TU/ml, 200 μ l) or Lv-GFP (2×10^{10} TU/ml, 200 μ l). Cardiac injection of Lv-GFP significantly increased GFP expression in cardiac myocytes (Figure 1A-C). These results demonstrate that the transferred GFP expression using this viral vector is relatively specific for cardiac myocytes. Increased expression of DNp85 was also confirmed by Western blot in the hearts of rats that received injection of Lv-DNp85 (Figure 1D and E). Next, we examined the effect of Ang II and Lv-DNp85 on PI3-kinase class I by measurement of PI(3,4,5)P3 (PIP3) production. The results are presented in Figure

1F, demonstrating that subcutaneous Ang II infusion significantly increased PIP3 and that Ang II-induced increase in PI3-kinase activity were significantly attenuated by Lv-DNp85 transduction in the heart (Figure 1F).

Effect of Ang II Infusion and Cardiac Transfer of Lv-DNp85 on BP and HR

To determine the effect of class I PI3-kinase blockade on the action of Ang II in the heart, HR and BP were recorded via femoral catheterization in rats that received subcutaneous infusion of saline or Ang II with cardiac transfer of Lv-GFP or Lv-DNp85 for 4 weeks. The results are presented in Figure 2, demonstrating that cardiac transfer of neither Lv-GFP nor Lv-DNp85 al-

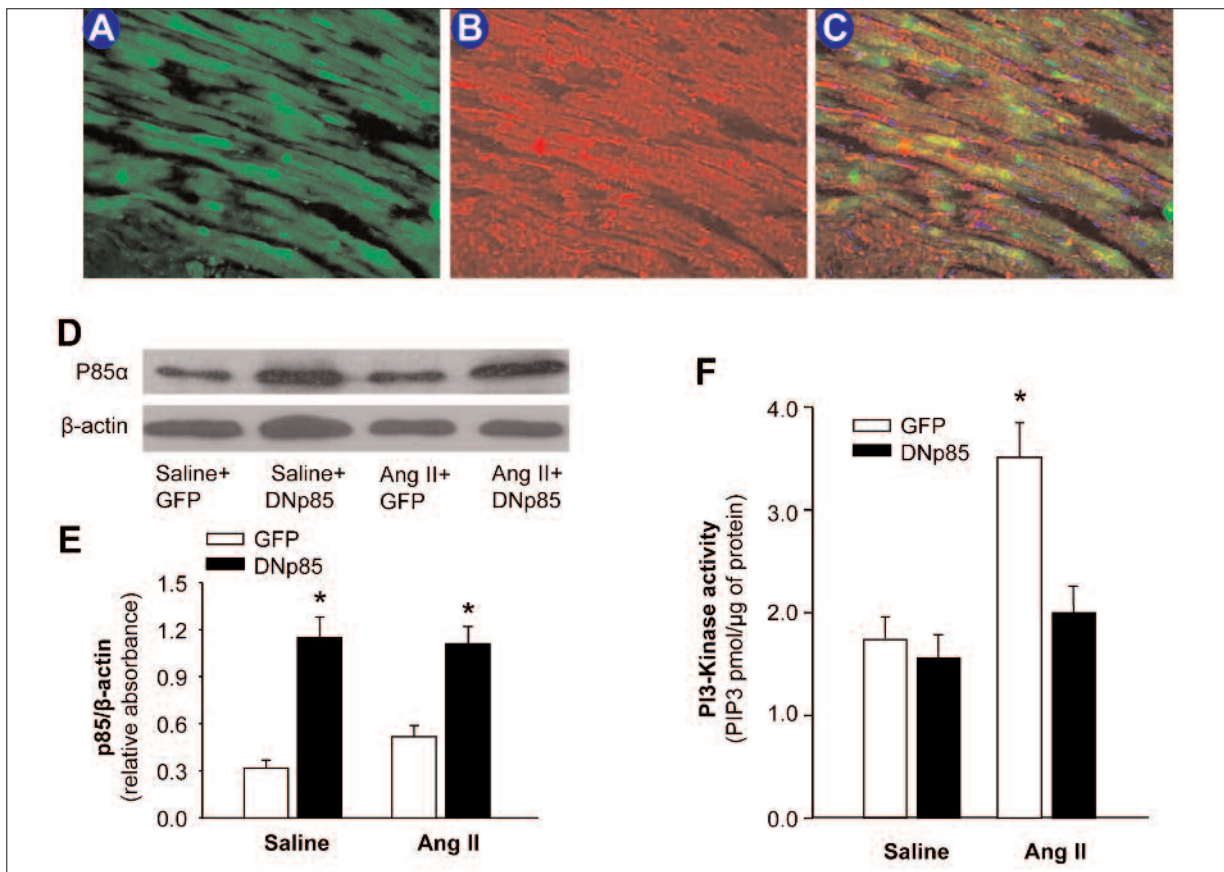


Figure 1. Effect of Ang II and cardiac transfer of Lv-DNp85 on class I PI3-kinase activity in the rat heart. **A-C**, Immunofluorescence images showing GFP expression in cardiac myocytes in rat hearts that received injection of Lv-GFP (40 \times magnification). **A**, Fluorescence micrograph demonstrating localization of GFP (green). **B**, Same field of cells as in A, immunostained with anti- α -actin (red), a cardiac myocyte marker. **C**, Overlap of A and B, indicating lentiviral vector-mediated GFP gene transduction in cardiac myocytes. **D**, Representative Western blot of heart lysate probed for PI3-kinase p85 subunit and β -actin in hearts from rats that received chronic subcutaneous infusion of Ang II or saline with cardiac transfer of Lv-DNp85 or Lv-GFP for four weeks. **E**, Bar graphs summarizing PI3-kinase p85 subunit protein levels in the heart of rats described in the above. Data are mean \pm SE (n=6 rats). * $p < 0.05$ as compared with rats that received cardiac transduction of Lv-GFP. **F**, Bar graphs showing class I PI3-kinase activity measured with and PIP3 ELISA kit in hearts from these rats describe in the above. Data are mean \pm SE (n=6 rats). * $p < 0.01$ as compared with rats that received saline and cardiac transduction of Lv-GFP.

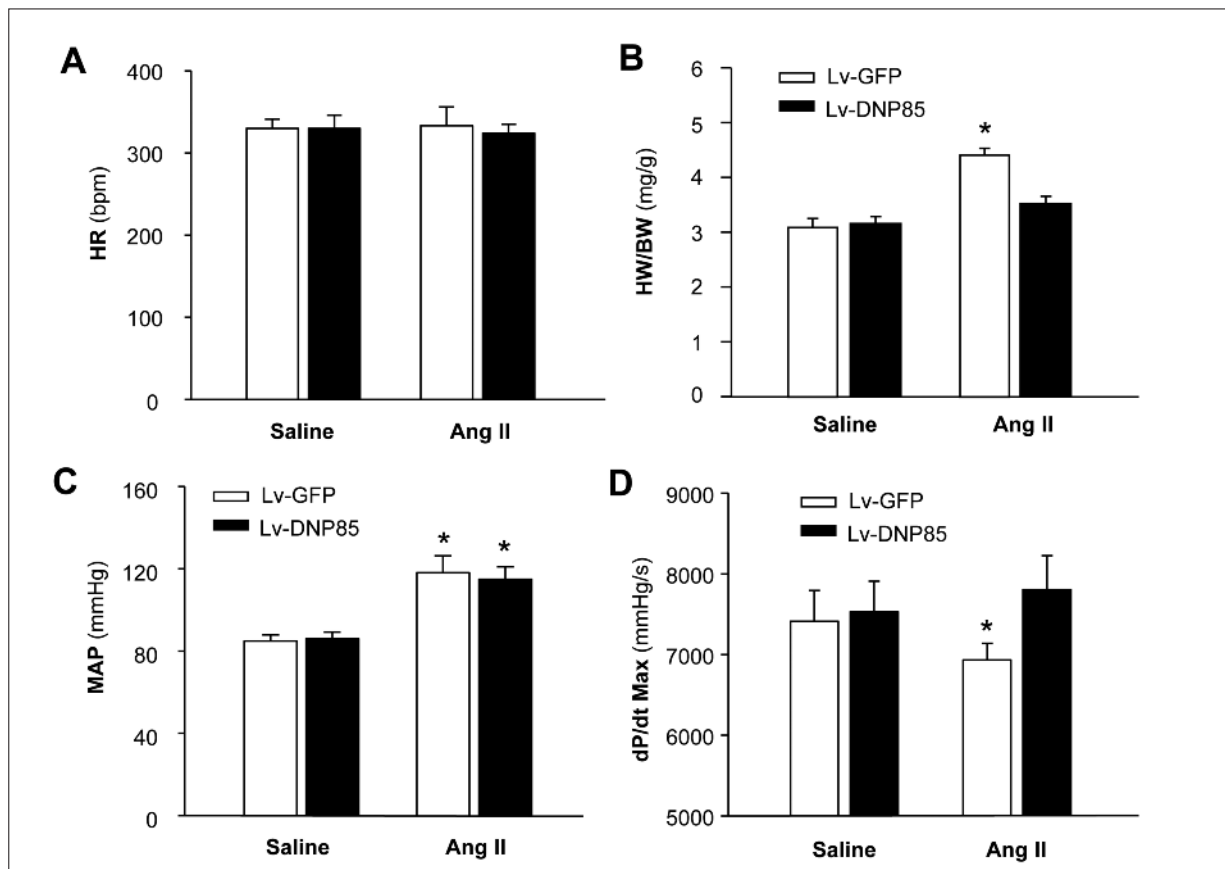


Figure 2. Effect of Ang II and Lv-DNp85 on heart rate, HW/BW ratio, blood pressure, and dP/dt Max. The heart rate (HR, in Panel **A**) and the mean arterial pressure (MAP) (in Panel **C**) were recorded via femoral arterial catheterization, and dP/dt Max (left ventricular contractility, in Panel **D**) was recorded via the left ventricular catheterization through the right carotid artery in rats that received chronic subcutaneous infusion of saline or Ang II with cardiac transduction of Lv-DNp85 or Lv-GFP control for 4 weeks. Heart weight versus body weight ratio (HW/BW, in Panel **B**) was measured after the rats were sacrificed at the end of each experiment. Data are presented as mean \pm SE from six rats in each group. * $p < 0.05$ as compared with rats that received saline and cardiac transduction of Lv-GFP.

tered mean arterial pressure (MAP) or HR in saline infused rats (Figure 2A and C). However, subcutaneous infusion of Ang II significantly increased MAP as compared with the saline control group (Figure 2C). Cardiac transfer of Lv-GFP or Lv-DNp85 did not alter Ang II-induced elevation in MAP (MAP increased by 46% and 45% in Lv-GFP group and in Lv-DNp85 group respectively, Figure 2C). In addition, Ang II subcutaneous infusion did not significantly alter HR in the rats that received cardiac injection of Lv-GFP or Lv-DNp85 as compared with saline-infused rats. These findings indicate that cardiac transfer of Lv-DNp85 did not alter either basal BP or the pressor response to Ang II administered subcutaneously, suggesting that genetic blockade of PI3 kinase in the heart has no noticeable effect on peripheral blood pressure.

Effect of Chronic Blockade of Class I PI3-Kinase and Ang II on Cardiac Hypertrophy

Cardiac hypertrophy was determined by measuring the ratio of heart weight to body weight (HW/BW) in rats infused subcutaneously with saline or Ang II together with cardiac transduction of Lv-GFP or Lv-DNp85. The body weight was not significantly altered by subcutaneous infusion of Ang II or cardiac transduction of Lv-DNp85 (BW: 343 ± 11 g in rats receiving saline and Lv-GFP; 347 ± 11 g in rats receiving saline and Lv-DNp85; 339 ± 13 g in rats receiving Ang II and Lv-GFP; 340 ± 8 g in rats receiving Ang II and Lv-DNp85; no significant difference between these groups, $p > 0.05$, $n = 6$). However, in Lv-GFP rats, subcutaneous infusion of Ang II (200 ng/kg/min for 4 weeks) significantly increased HW/BW as compared with saline infusion, sug-

gesting marked cardiac hypertrophy in the Ang II infusion group (Figure 2C). This Ang II-induced increase in HW/BW was significantly attenuated by cardiac transfer of Lv-DNp85 (Figure 2C).

Heart contractility was also measured using left ventricular catheterization in these four groups of rats. The results are presented in Figure 2D, demonstrating that chronic subcutaneous infusion of Ang II significantly reduced dP/dt max. This may be caused by cardiac fibrosis or decompensated hypertrophy. In the presence of Ang II, blockade of class I PI3-kinase significantly reversed Ang II-induced reduction in left ventricular dP/dt max (Figure 2D), suggesting that blockade of class I PI3-kinase markedly improve cardiac function.

Cardiac hypertrophy was also observed by examining cardiac morphology. Ang II infusion significantly increased cardiac myocyte cross sectional area as compared with saline infusion in rats that received cardiac injection of Lv-GFP, suggesting significant cardiac hypertrophy in Ang II-treated rats (Figure 3A, C, and E). The Ang II-induced cardiac hypertrophy was significantly attenuated by cardiac transfer of Lv-DNp85 (Figure 3B, D, and E). These results indicate that Ang II-induced cardiac hypertrophy is mediated by a class I PI3-kinase-dependent pathway.

Effect of Ang II and Class I PI3-Kinase Blockade on Cardiac Fibrosis

Cardiac remodeling is characterized by enhanced myocyte apoptosis, fibroblast proliferation, and fibrosis. Thus, we examined the effect of Ang II and PI3-kinase blockade on cardiac fibrosis in hearts from rats that received cardiac transfer of Lv-GFP or Lv-DNp85 along with subcutaneous infusion of saline or Ang II for 4 weeks. As shown in Figure 4, chronic subcutaneous infusion of Ang II significantly increased perivascular collagen deposition in the heart, and chronic blockade of class I PI3-kinase by transfer of Lv-DNp85 significantly attenuated Ang II-induced perivascular fibrosis. The results indicate that class I PI3-kinase may contribute to the fibrotic response to Ang II in the heart.

Effect of Ang II and Class I PI3-Kinase Blockade on Autophagy and Oxidant Stress

Altered autophagy has been shown to be involved in the pathogenesis of cardiac hypertrophy and myocyte death¹¹. Thus, we examined the chronic effect of Ang II and class I PI3-kinase blockade on cardiac autophagy by examining autophagosome marker microtubule-associated pro-

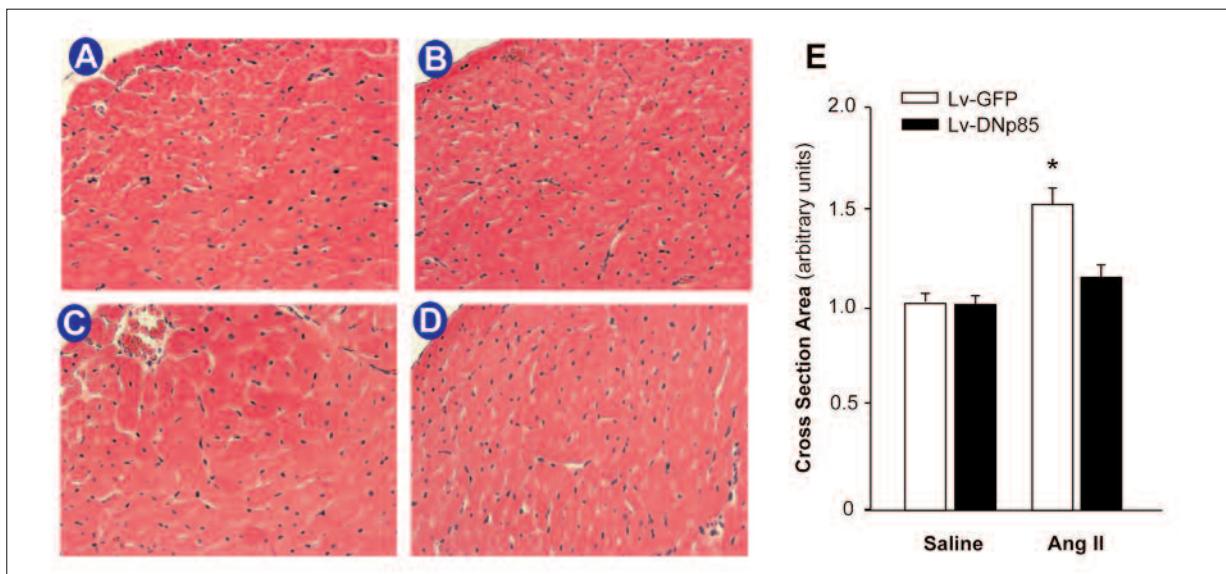


Figure 3. Chronic blockade of class I PI3-kinase attenuates Ang II infusion-induced cardiac hypertrophy. Cardiac morphological and histological alterations were examined 4 weeks after subcutaneous infusion of Ang II or saline with cardiac transduction of Lv-DNp85 or Lv-GFP, as described in Methods. **A**, Micrographs (40 × magnification) showing representative heart sections stained with hematoxylin/eosin in rats that received subcutaneous infusion of saline (**A** and **B**) or Ang II (**C** and **D**) with cardiac transduction of Lv-GFP (**A** and **C**) or Lv-DNp85 (**B** and **D**). **E**, Bar graphs summarizing cross-sectional areas of cardiac myocytes from transverse cardiac sections of each group of rats. Cross-sectional areas of 100 cells per rat were examined in random fields in 6 rats per group. * $p < 0.01$ as compared with rats that received saline plus Lv-GFP.

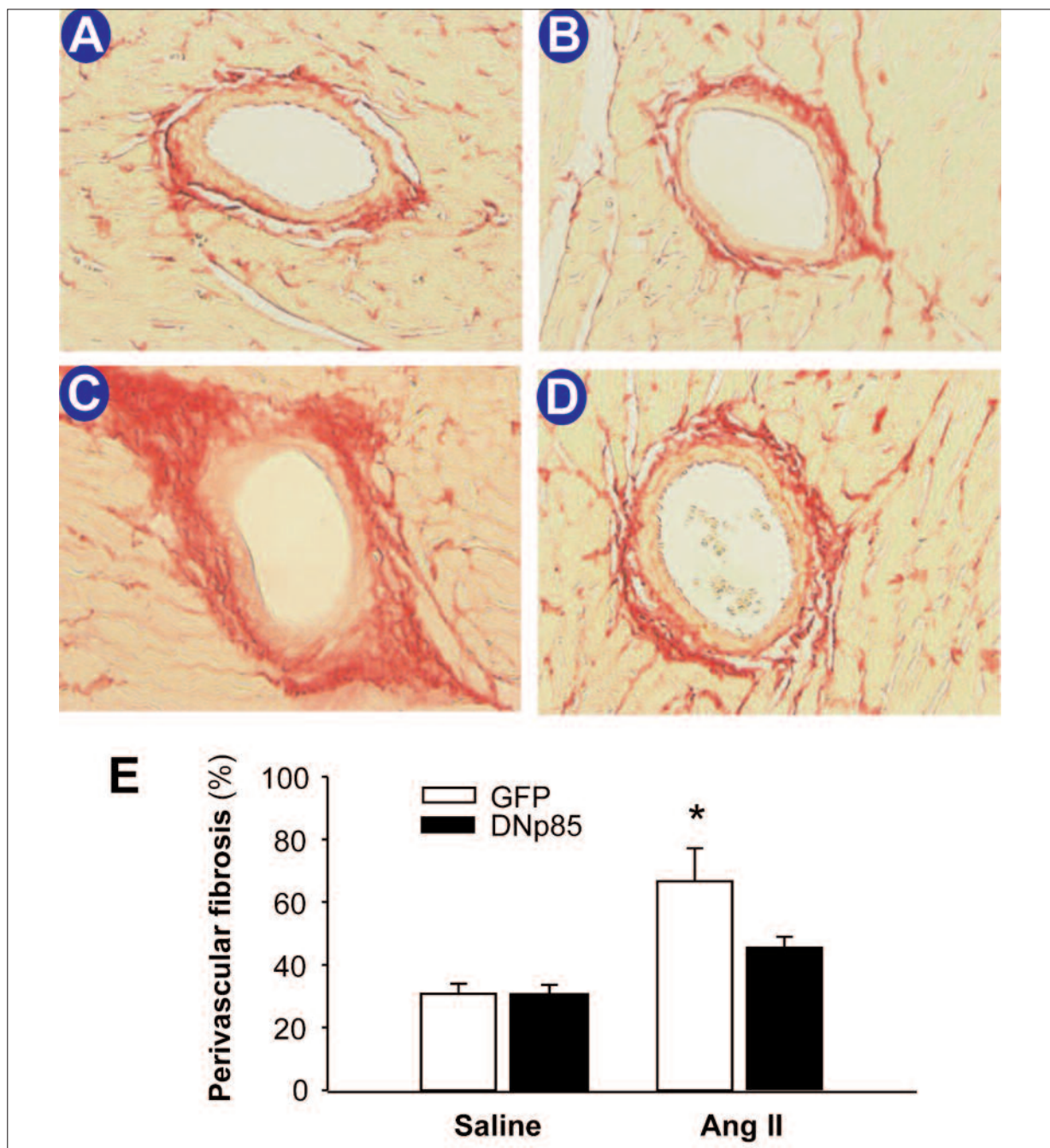


Figure 4. Effect of Ang II and blockade of PI3-kinase on cardiac fibrosis. A-D, Representative microscopic images showing myocardial perivascular collagen deposition (red staining) in rats 4 weeks after subcutaneous infusion of saline (**A** and **B**) or Ang II (**C** and **D**) with cardiac transduction of Lv-GFP (**A** and **C**) or Lv-DNp85 (**B** and **D**), as described in Methods. **E**, Bar graphs summarizing quantitative analysis of myocardial perivascular fibrosis, expressed as the ratio of perivascular collagen area to vessel cross-sectional area of the coronary arteries. Data are presented as mean \pm SE from 6 rats in each group, with 3 sections from each heart. * $p < 0.01$ as compared with rats that received saline plus Lv-GFP.

tein light chain 3 (LC3), with Western blot analysis in hearts from rats receiving cardiac transfer of Lv-GFP or Lv-DNp85 along with subcutaneous infusion of saline or Ang II for 4 weeks. LC3 protein usually has two forms, LC3-I and

LC3-II. During autophagy, the LC3-I is converted to LC3-II, which is recruited to the autophagosomes. Thus, the amount of LC3-II correlates with the number of autophagosomes, and is used a marker of autophagic activity.

Ang II infusion significantly decreased the protein levels of LC3 II (Figure 5A), indicating that cardiac autophagy was reduced by Ang II treatment. Moreover, blockade of class I PI3-kinase by transduction of Lv-DNp85 completely attenuated Ang II-induced reduction in LC3 II protein levels (Figure 5A), indicating that the class I PI3-kinase is involved in Ang II-induced impairment of autophagy in the heart.

It is well known that the function of autophagy as a protective cellular mechanism is involved in scavenging damaged mitochondria and other proteins, which could produce reactive oxygen species (ROS). Thus, we next examined ROS levels in the heart of rats receiving cardiac transfer of Lv-GFP or Lv-DNp85 along with subcutaneous infusion of saline or Ang II for 4 weeks. The ROS were measured with the fluorogenic probe, dihydroethidium (DHE), on heart sections. The results are expressed in Figure 5B, demonstrating that chronic Ang II infusion significantly increased ROS generation in the heart and that cardiac transduction of Lv-DNp85 atten-

uated Ang II- induced elevation in ROS levels by 76% (Figure 5B). In summary, Ang II infusion induced dramatic impairment of autophagy and elevation of ROS generation. Blockade of class I PI3-kinase attenuated Ang II-induced impairment of autophagy and elevation of ROS, an important factor known to be involved in the pathology of cardiac hypertrophy and fibrosis.

Effect of Ang II and Class I PI3-Kinase Blockade on Akt and mTOR Activity

To study the downstream signaling pathway of class I PI3-kinase in Ang II-induced impairment of autophagy, phosphorylation of Akt and mTOR activity was examined in hearts from rats receiving cardiac transfer of Lv-GFP or Lv-DNp85 along with subcutaneous infusion of saline or Ang II for 4 weeks. Chronic infusion of Ang II significantly increased phosphorylation of Akt (Figure 6A) and mTOR (Figure 6B). Ang II-induced increases in the phosphorylation of Akt and mTOR were significantly attenuated by cardiac transfer of Lv-DNp85 (Figure 6A and B).

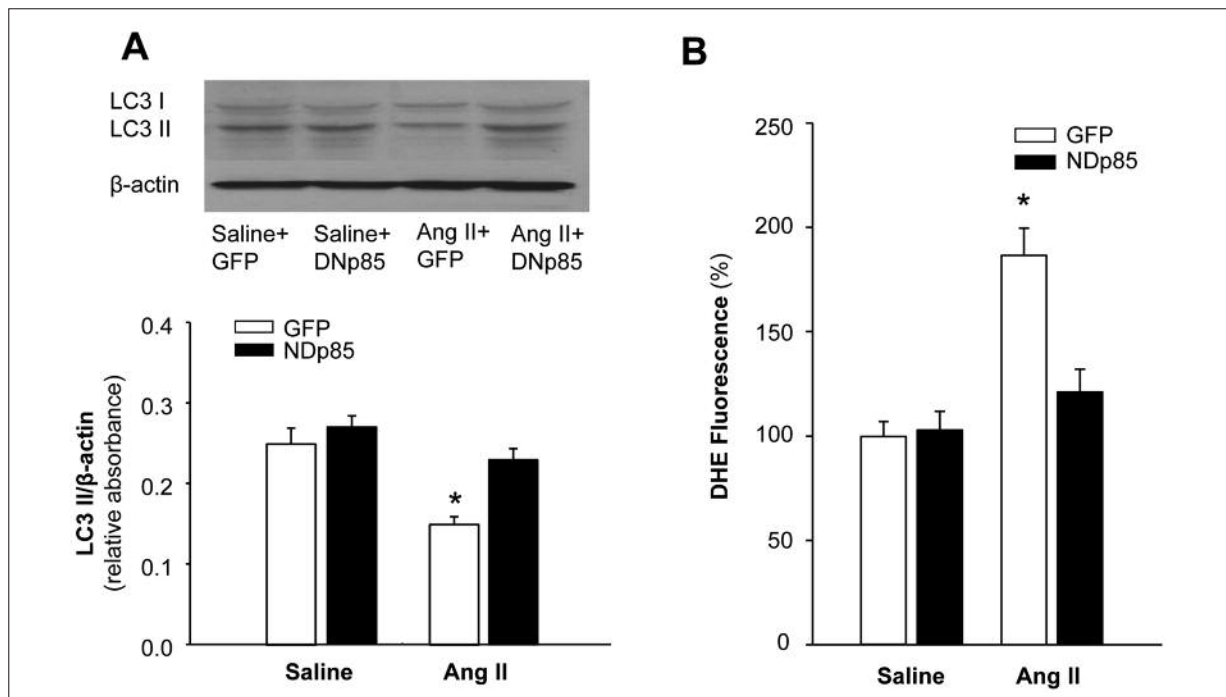


Figure 5. Effect of Ang II and blockade of class I PI3-kinase on cardiac autophagy and ROS production. **A**, Autophagy was examined by LC3 II protein levels (a marker of autophagy) in rats at 4 weeks after subcutaneous infusion of Ang II or saline with cardiac transduction of Lv-DNp85 or Lv-GFP. Data are presented as mean±SE of 3 independent experiments in 6 rats. **p* < 0.05 as compared with rats that received saline plus Lv-GFP. **B**, ROS levels were measured with a fluorogenic probe, DHE, staining in the heart sections of rats that received of chronic subcutaneous infusion of Ang II or saline with cardiac transfer of Lv-DNp85 or Lv-GFP for four weeks. Data represent the means ± SE of 5 heart sections/heart from total six rats after treatments as described above. **p* < 0.01, compared with rats that received saline and Lv-GFP.

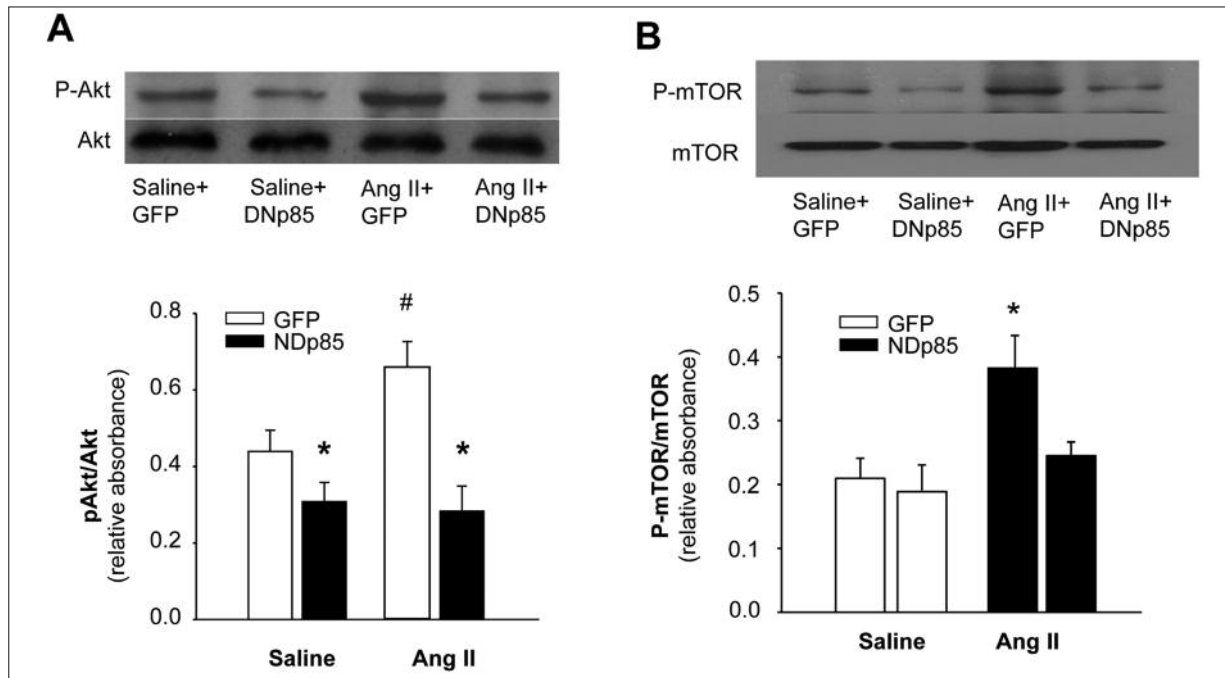


Figure 6. Effect of Ang II and blockade of class I PI3-kinase on phosphorylation of Akt and mTOR. **A**, Upper panel showing representative Western blots of heart lysate probed for phosphorylated Akt (P-Akt) and Akt. Lower panel: bar graphs summarizing phosphorylated Akt versus total Akt in hearts of rats that received chronic subcutaneous infusion of Ang II or saline with cardiac transfer of Lv-DNp85 or Lv-GFP for four weeks. Data are presented as mean \pm SE of 3 independent experiments in 6 rats. * $p < 0.05$ as compared with rats that received Lv-GFP in each group. # $p < 0.05$ compared with rats that received saline plus Lv-GFP. **B**, Representative Western blots performed on left ventricular lysates with antibodies against phosphorylated mTOR (P-mTOR) and mTOR in rats that received of chronic subcutaneous infusion of Ang II or saline with cardiac transfer of Lv-DNp85 or Lv-GFP for four weeks. Bar graph summarizing the blockade of PI3-kinase on phosphorylation of mTOR versus total mTOR in the heart of rats treated as described in the above. Data are mean \pm SE of 3 independent experiments in 6 rats. * $p < 0.01$, as compared with rats that received saline and Lv-GFP.

These results demonstrate that Akt-mTOR pathway activation may be involved in the downstream signaling of class I PI3-kinase in Ang II-induced impairment of autophagy, since mTOR has been reported to have negative regulatory effect on autophagy²².

Discussion

The current study provides the first *in vivo* evidence that chronic Ang II infusion induces cardiac hypertrophy and remodeling by impairment of autophagy and elevation of ROS through a class I PI3-kinase dependent mechanism. This conclusion is supported by the following observations: (1) Chronic subcutaneous infusion of Ang II significantly caused increased phosphorylation of Akt and mTOR, impaired autophagy, elevated ROS generation, myocardial hypertrophy, and fibrosis in the heart; (2) These actions of Ang

II were attenuated by blockade of class I PI3-kinase using cardiac transfer of dominant negative PI3-kinase p85 subunit (Lv-DNp85).

PI3-kinases are divided into three classes (class I, class II, and class III) according to their substrate specificity, distinctive function, and molecular structure. Class I PI3-kinase phosphorylates PtdIns, PtdIns(4)P, and PtdIns(4,5)P₂ to form PtdIns(3)P, PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ respectively. The major product of class I PI3-kinase *in vivo* is PtdIns(3,4,5)P₃ (also called PIP₃), which is usually used for measurement of class I PI3-kinase activity. The current study was focused on the class I PI3-kinase in the actions of Ang II on cardiac myocytes. Class I PI3-kinase is classified into two isoforms, class Ia and class Ib, based on their subunit composition. It has been reported that they may exert different functions in the heart. Class Ia PI3-kinase is activated by Tyr-kinases, such as insulin receptors, and promotes cardiac hypertrophy²³. Conversely, class Ib

PI3-kinase is activated by G-protein-coupled receptors, such as Ang II receptors, and regulates cardiac contractility²⁴. However, the current study demonstrates that blockade of p85 α subunit, an adapter protein of class Ia PI3-kinase, also attenuated Ang II-induced cardiac hypertrophy and remodeling, suggesting that class Ia may be also involved in G-protein-coupled receptor actions. This result has also been observed *in vitro* using cultured cardiomyocytes, showing that p110 and p110 α , catalytic subunits of class Ia, contribute to the actions of several G-protein-coupled receptors^{13,24}. However, the coupling mechanism between class Ia PI3-kinase and receptor remains to be determined.

Elevated plasma Ang II levels and overproduction of Ang II within the heart occur in patients and animal models with cardiac hypertrophy and heart failure^{8,10}; thus, increased Ang II levels may contribute to the pathogenesis of cardiac hypertrophy and fibrosis. This notion is also supported by our current observation that chronic infusion of Ang II induces severe cardiac hypertrophy and fibrosis in rats. However, the underlying cellular mechanisms are still not fully clear. A recent study demonstrated that cardiac specific knockout of autophagy related protein leads to cardiac hypertrophy and dysfunction¹⁵. To this end, we examined the effect of Ang II on cardiac autophagy. Our results indicate that chronic subcutaneous infusion of Ang II caused severe cardiac hypertrophy, which was associated with impaired cardiac autophagy, as evidenced by reduced LC3-II levels in the heart. The results suggest that reduced cardiac autophagy may contribute, at least partially, to Ang II-induced cardiac hypertrophy and remodeling. The results of the current study differ from previous publications showing that Ang II increases cardiac autophagy in cultured neonatal cardiomyocytes^{25,26}. The reason for this discrepancy is not clear, but may be due to several possibilities including the differences between adult heart vs neonatal heart, *in vivo* heart vs *in vitro* cell cultures, acute vs chronic studies, or different isoform of PI3-kinase involved. For example, our study demonstrated that the inhibitory effect of Ang II on autophagy is mediated by class I PI3-kinase. Pan et al²⁵ reported that Ang II-induced increase in autophagy is mediated by class III PI3-kinase. More interestingly, a recent study demonstrated that the autophagic response to Ang II is both time- and dose-dependents²⁷. The early exposure or low

dose of Ang II causes a stimulatory response in autophagy, whereas long-time exposure or high doses of Ang II leads to an impaired response. Enhanced autophagy has also been observed in other cardiac conditions, such as ischemia and pressure overload²⁸. The autophagy-impairing effect of Ang II could exaggerate these cardiac conditions, thereby leading to worsening of the damage to cardiac myocytes, followed by myocardial decompensation. Thus, targeting cellular pathways underlying Ang II-induced impairment of cardiac autophagy could protect the cardiac myocyte from stress-induced damage.

Autophagy is a self-degradative process that is involved in removing and recycling damaged proteins and cellular organelles, maintaining intracellular homeostasis, and cell survival²⁸. Cardiac myocytes are extremely long-lived, as their capacity to regenerate in an adult heart is limited, and the contractile action of the heart is a life-long activity. Thus, a continual process of cellular repair involving removal and replacement of damaged structures must be in place. Impaired cardiac autophagy could result in intracellular accumulation of damaged organelles and proteins, which may trigger production of ROS. For example, autophagy is the primary avenue for disposing of mitochondria (this process is also noted as mitophagy). If not removed due to impaired autophagy, damaged mitochondria may continue to generate high levels of ROS that could damage cellular protein, lipids, and DNA. In addition, mitochondria DNA (mtDNA) contains unmethylated CpG motifs, which are pro-inflammatory²⁹. Accumulation of mtDNA could cause a toll-like receptor inflammatory response that contributes to cardiac damage and heart failure. This mechanism is confirmed in the current study, which indicates that chronic infusion of Ang II significantly increases ROS generation in cardiac myocytes. Enhanced ROS production and inflammatory response are well recognized in Ang II-induced cardiac hypertrophy, fibrosis, and failure³⁰.

The intracellular signaling pathways underlying Ang II-induced impairment of cardiac autophagy and cardiac hypertrophy are not yet clear, but likely involve a role for PI3-kinase. A previous study suggests the involvement of PI3-kinase in Ang II-induced hypertrophy and ROS formation in cultured cardiomyocytes, based on the inhibitory effects of the PI3-kinase inhibitors Ly294002 and wortmannin¹³. However, these in-

hibitors block all three classes of PI3-kinases, and can be used neither for heart-specific *in vivo* studies nor for long-term observation. Thus, we used lentiviral vector-mediated overexpression of the dominant negative p85 subunit to inhibit class I PI3-kinase specifically in the heart. Our results demonstrate that chronic blockade of class I PI3-kinase significantly attenuates Ang II-induced phosphorylation of Akt and mTOR, as well as Ang II-induced hypertrophy and impairment in cardiac autophagy, suggesting that PI3-kinase dependent phosphorylation of Akt and mTOR could be involved in the Ang II-induced impairment of cardiac autophagy. Given that activated Akt enhances the expression of mTOR protein, a suppressor of autophagy initiation³¹, we may speculate that Ang II may depress cardiac autophagy by stimulation of a class I PI3-kinase/Akt/mTOR pathway, leading to cardiac damage and decompensation. Therefore, drug targeting of this signaling pathway could protect the myocardium from damage. Indeed, pre-treatment with rapamycin, an mTOR inhibitor, reduces infarct size in the heart³².

Conclusions

We provide the first evidence that chronic treatment of Ang II induces cardiac hypertrophy, perivascular fibrosis, and impaired cardiac autophagy via stimulation of the class I PI3-kinase pathway. These results are not only highly significant from a pathophysiological perspective, but also have important pharmacological implications in the control of myocardial hypertrophy to prevent decompensation and failure in cardiac function.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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