The Anti-Ischemic Effects of PIK3IP1 in Reducing the Likelihood of Cardiac Cell Death are Mediated Through its Interactions with the ETA-PI3Ky-AKT Axis

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Abstract

Oxidative stress, caused by the accumulation of reactive oxygen species (ROS) during acute myocardial infarction (AMI), is one of the main factors leading to myocardial cell damage and programmed cell death. Phosphatidylinositol-3-kinase -AKT (PI3K-AKT) signaling is essential for regulating cell proliferation, differentiation, and apoptosis. Phosphoinositide-3-kinase (PI3K)-interacting protein 1 (PIK3IP1) is an intrinsic inhibitor of PI3K in various tissues, but its functional role during AMI remains unknown. In this study, the anti-ischemic role of PIK3IP1 in an in vitro AMI setting was evaluated using H9c2 cells. The MTT assay demonstrated that cell viability decreased significantly with treatment of H₂O₂ (200 -500 µM). The TUNEL assay results revealed substantial cellular apoptosis following treatment with 200 µM H₂O₂. Under the same conditions, the expression levels of hypoxia-inducible factor (HIF-1_α), endothelin-1 (ET-1), bcl-2-like protein 4 (BAX), and cleaved caspase-3, were elevated, whereas those of PIK3IP1 and Bcl-2 decreased significantly. PIK3IP1 overexpression inhibited H₂O₂-induced, and PI3K-mediated, apoptosis; however, PIK3IP1 knockdown reversed this effect, suggesting that PIK3IP1 functions as an anti-apoptotic molecule. To identify both the upstream and downstream molecules associated with PIK3IP1, ET-1 receptor type-specific antagonists (BQ-123 and BQ-788) and PI3K subtype-specific antagonists (LY294002 and IPI-549) were used to determine the participating isoforms. Co-immunoprecipitation was performed to identify the binding partners of PIK3IP1. Our results demonstrated that ROS-induced cardiac cell death may occur through the ETA-PI3K_γ-AKT axis, and that PIK3IP1 inhibits binding with both ETA and PI3K_γ. Taken together, these findings reveal that PIK3IP1 plays an anti-ischemic role by reducing the likelihood of programmed cell death via interacting with the ETA-PI3K_γ-AKT axis.

Introduction

Cardiovascular diseases, such as acute myocardial infarction (AMI), dilated cardiomyopathy, and congestive heart failure are the leading causes of death worldwide¹. In AMI, oxidative stress induces the production of large amounts of reactive oxygen species (ROS), impairs functioning of the vascular tissues, and leads to cardiac cell death. However, the major pathways involved in AMI pathogenesis remain unknown. Thus, in the present study, we aimed to investigate the anti-apoptotic pathways involved in AMI.

Phosphoinositide-3-kinase (PI3K) is a crucial hub protein involved in cell survival, growth, apoptosis, proliferation, and immune responses in adaptive and maladaptive cardiac hypertrophy². PI3K converts phosphatidylinositol-4,5-bisphosphate (PIP₂) to phosphatidylinositol (3,4,5)-tris-phosphate (PIP₃)³. After phosphorylation, PIP₃ recruits and promotes activation of downstream effectors such as PDK1 (phosphoinositide-dependent kinase-1) and protein kinase B (AKT)⁴. PI3K is a holoenzyme with four distinct subtypes, which include the class 1A subtypes PI3K_α, PI3K_β and PI3K_δ, and the class 1B subtype PI3K_γ⁵. Upon PI3K_α activation in response to stimulation of receptor tyrosine kinase by agonists such as insulin-like growth factor 1 (IGF-1), AKT induces compensatory physiological hypertrophy⁶. In contrast,

PI3K γ activation negatively regulates cardiac contractility during pressure overload⁷. Moreover, p110 γ is activated as a result of G-protein-coupled receptor (GPCR) stimulation by agonists such as endothelin-1 (ET-1)⁸.

PI3K-interacting protein 1 (PIK3IP1) is a transmembrane protein that negatively regulates PI3K activity, thereby inhibiting AKT phosphorylation^{9,10}. Our previous study investigated the functional role of PIK3IP1 in IGF1-mediated physiological hypertrophy, in which the class 1A PI3K α isoform is involved¹¹. However, the functional role of PIK3IP1 in GPCR-mediated pathological hypertrophy, in which the class 1B isoform PI3K γ is involved, remains unknown.

ET-1 activates several signal transduction pathways linked to cellular hypertrophy, growth, migration, and proliferation in several cell types, including cardiac tissues¹³. In cardiac cells, ET-1 may play an important role in the pathophysiology of chronic heart failure and ischemic heart diseases¹². Using a rat model of myocardial infarction (MI), such as myocardial stunning, Klainguti *et al.* (2000) reported that a surge in myocardial ET-1 level after 20 min of ischemia led to apoptosis in the post-ischemic condition¹³. Additionally, during hypoxia, the expression of ET-1 is elevated by hypoxia-inducible factor 1 α (HIF-1 α)¹⁴. ET-1 exerts its biological action through the activation of two receptor subtypes— endothelin A receptor (ET_A) and endothelin B receptor (ET_B), both of which belong to a large family of GPCRs. Activation of each receptor subtype, however, exerts opposing pathophysiological effects. For instance, the ET_A receptor leads to vasoconstriction and exerts pro-oxidative actions, whilst stimulation of ET_B may elicit vasodilatation in healthy subjects due to stimulation of nitric oxide production¹⁵.

The present study used H9c2 cells and H₂O₂ treatment to examine the pathways underlying ROS-induced cardiac programmed cell death, focusing on the anti-ischemic effects of PIK3IP1. Our results suggest that PIK3IP1 plays an essential protective role in ischemic hypoxia-induced cardiac cell apoptosis via interacting with ET_A-PI3K γ /AKT-mediated signaling.

Results

H₂O₂-induced apoptosis of H9c2 cardiomyocytes is accompanied by decreased PIK3IP1 and Bcl-2 expression, but increased HIF-1 α , ET-1, BAX, and cleaved caspase-3 expression

H9c2 cardiomyocytes were treated with the indicated concentrations (10-500 μ M) of H₂O₂ for 24 h and the cell viability was examined using the MTT assay to determine the optimal concentration of H₂O₂ for the subsequent experiments (Fig. 1A). Cell viability decreased in a dose-dependent manner, with 200 and 500 μ M H₂O₂ substantially reducing cell viability. Therefore, 200 μ M H₂O₂ was selected for use in the TUNEL assay to measure apoptosis-induced DNA fragmentation as an inducer of apoptosis. The results demonstrated that the number of TUNEL-positive cells increased substantially after 24 h treatment with 200 μ M H₂O₂ (Fig. 1B). Immunoblotting was performed to investigate the effects of 200 μ M H₂O₂ treatment on the expression of proteins involved in the ROS-mediated apoptosis pathways in

cardiac cells (Fig. 1C). The transcription factor HIF-1 α plays an important role in cellular responses to systemic oxygen levels in mammals¹⁶. Our results showed that HIF-1 α expression increased within 1 h in response to 200 μ M H₂O₂ treatment, however this effect was partially reversed with prolonged incubation (3-24 h). Interestingly, the expression of ET-1 gradually increased in the presence of 200 μ M H₂O₂, but that of PIK3IP1 decreased within the same time range. The levels of proapoptotic proteins, BAX and cleaved caspase-3 (c-Casp3), increased similar to that of ET-1; consistently, the levels of antiapoptotic protein Bcl-2 decreased. These results suggest that ET-1 acts as a pro-apoptotic protein, whereas PIK3IP1 acts as an anti-apoptotic protein during ROS-rich ischemic condition.

PIK3IP1 overexpression inhibits H₂O₂-induced cell death via downregulating p110 α , p110 γ , p-AKT, and cleaved caspase-3 expression

To confirm the anti-apoptotic role of PIK3IP1 in ROS-generating conditions, PIK3IP1 was overexpressed as described in **Materials and Methods**. A recombinant open reading frame cDNA clone system, expressing DYKDDDDK-tagged PIK3IP1 (GenScript), was used for overexpression experiments. Western blot analysis revealed a specific band (DYKDDD) for PIK3IP1 in pcDNA3.1-PIK3IP1-transfected (PIK) cells, but not in pcDNA3.1-transfected control (Vec) cells (Fig. 2A). The total expression of PIK3IP1 in PIK cells was approximately two-fold higher than that of Vec cells. H₂O₂ treatment decreased the expression of PIK3IP1 in both the PIK and Vec cells. Next, we examined the effects of PIK3IP1 overexpression on the expression of proteins involved in the PI3K-AKT signaling pathway. Western blot analysis revealed that H₂O₂-induced upregulation of p110 α , p110 γ , and p-AKT expression was significantly decreased in PIK3IP1-overexpressing cells (Fig. 2B). The level of cleaved caspase-3 protein, a cell death marker, was also significantly decreased in PIK3IP1-overexpressing cells, suggesting that PIK3IP1 overexpression could inhibit cardiomyocyte cell death. TUNEL assays were performed to quantitate apoptotic cells in the experimental groups. The number of TUNEL-positive cells was significantly increased in the H₂O₂-treated cells, but decreased significantly in the PIK3IP1-overexpressing cells (Fig. 2C). These results suggest that PIK3IP1 plays an anti-apoptotic role by inhibiting PI3K-AKT signaling during H₂O₂ exposure.

PIK3IP1 knockdown increased H₂O₂-induced cell death via upregulating p110 α , p110 γ , p-AKT, and cleaved caspase-3 expression

To determine whether downregulation of PIK3IP1 level would exacerbate cardiomyocyte cell death via inhibiting PI3K, PIK3IP1 knockdown (KD) was performed using siRNA, as described in **Materials and Methods**. H9c2 cells were transfected with negative control (siNC) and PIK3IP1 siRNAs (siPIK, all 25 nM), and subsequently treated with H₂O₂. The efficiency of PIK3IP1 KD was confirmed using western blotting. Expression of PIK3IP1 was significantly decreased in siPIK-transfected cells compared to siNC-transfected cells (by approximately 50%) (Fig. 3A). The expression of p110 α , p110 γ , p-AKT, and cleaved caspase-3 proteins increased significantly in siPIK-transfected cells. Addition of 200 μ M H₂O₂ showed additive effects on the protein levels (Fig. 3B), suggesting that H₂O₂ increases cardiomyocyte cell death in PIK3IP1-deficient cells by directly activating the PI3K-AKT pathway. The number of TUNEL-positive

cells significantly increased in the H₂O₂-treated group, which was amplified in the PIK3IP1 KD group (Fig. 3C). This indicated that inhibition of PIK3IP1 induces apoptotic signaling and aggravates H₂O₂-induced apoptosis.

H₂O₂ induced cardiomyocyte cell death via activating ET_A-PIK3IP1 binding

Increasing evidence suggests that ET-1 is involved in the processes of myocardial ischemia and infarction. For instance, a 2-10 fold increase in ET-1 concentration has been reported during the early onset of myocardial infarction¹⁷⁻²⁰.

Under hypoxic conditions, HIF-1 α expression activates ET-1 gene expression, thereby increasing ET-1 levels; however, the effects of ET-1 on apoptosis in cultured cardiomyocytes remains controversial²¹. In the present study, H9c2 cells were treated with H₂O₂ for 24 h after pretreatment with either an ET_A inhibitor, BQ-123 (10 μ M), or an ET_B inhibitor, BQ-788 (10 μ M), and a TUNEL assay was conducted. The number of TUNEL-positive cells significantly increased in the H₂O₂-treated group; this effect was inhibited in the BQ-123-treated group, but not in BQ-788-treated cells (Fig. 4A). These results imply that ET_A receptors, but not ET_B receptors, mediate hypoxia-induced injury through endogenous ET-1. To investigate the relationship between ET_A and PIK3IP1, co-immunoprecipitation experiments were conducted. The H9c2 cells were lysed in NP-40 buffer, and anti-PIK3IP1 antibodies were incubated with protein G conjugated magnetic beads (Dynabeads, Invitrogen 10003D) to facilitate the formation of antibody-bead complex. The bound proteins were eluted and subjected to western blotting. ET_A, but not ET_B co-immunoprecipitated with PIK3IP1 (Fig. 4B; Supplementary Fig. 1). Furthermore, following PIK3IP1 KD (using siPIK), 3 h incubation with BQ-123 rescued the protein expression of PIK3IP1 (Fig. 4B), suggesting that ET_A inhibitor decreased the efficiency of siRNA interference. Co-immunoprecipitation analysis indicated that PIK3IP1 was preferentially bound to ET_A, but not to ET_B (Supplementary Fig. 1).

H₂O₂ induces H9c2 cell death through the ET_A-PIK3IP1-PI3K γ axis

To verify the preferential role played by ET_A in H₂O₂-induced cardiac cell apoptosis, the downstream PI3K-AKT signaling molecules were examined in the presence, or absence, of the ET_A- and ET_B-specific inhibitors (BQ-123 and BQ-788, respectively) by western blotting. Following pretreatment with either BQ-123 or BQ-788, H9c2 cells were treated with H₂O₂ for 3 h. The expression of p110 α and p110 γ isoforms was upregulated following H₂O₂ treatment, however their expression levels diminished in the BQ-123-pretreated group. BQ-788 pretreatment decreased p110 α expression, but not p110 γ (Fig. 5A). These data suggest that p110 γ is sensitive only to ET_A, but not to ET_B. Further experiments were conducted using LY294002, a p110 α -specific inhibitor, and IPI-549, a p110 γ -specific inhibitor. In H9c2 cells treated with H₂O₂ and LY294002, p110 α expression was downregulated, but that of p110 γ was not. In the group treated with H₂O₂ and IPI-549, p110 γ expression was downregulated, but, that of p110 α was not. Additionally, the downstream p-AKT and cleaved caspase-3 levels decreased in the LY294002 and IPI-549 treated groups. However, the degree of inhibition was greater in the IPI-549-treated p110 γ group than in

the LY294002-treated p110 α group (Fig. 5B). Figure 5 and Supplementary Fig. 2. show that both p110 γ and p110 α were co-immunoprecipitated with PIK3IP1, suggesting that both isoforms have a tendency to bind PIK3IP1. These findings together imply that the PIK3IP1-PI3K γ -AKT axis plays an essential role in H₂O₂-induced cardiomyocyte cell death.

Discussion

AMI, also commonly known as 'heart attack', is a serious health problem worldwide with a high mortality rate. In AMI, the myocardial ischemia produces a large amount of ROS due to oxidative stress, causing serious damage to the myocardial cell membrane and apoptosis of cardiomyocytes²². In a study of ischemia–reperfusion injury, overexpression of Bcl-2 protein was found to decrease the infarct size, apoptosis, and functional injuries of cardiomyocytes²³, suggesting that apoptosis plays a major role in the injuries caused by myocardial infarction. Therefore, the suppression of apoptosis is important for reducing myocardial infarct size. However, the essential signaling pathways associated with AMI-induced apoptosis remain to be studied. The present study focused on identifying the molecular players, and their interaction cascades, involved in the ROS-induced apoptosis pathways using H9c2 cell line.

ROS-activated mitochondria-dependent apoptotic pathways have been studied using both in vivo and in vitro systems²⁴. However, most studies concerning apoptosis have come from cultured cells, such as primary neonatal rat cardiomyocyte, atrial cardiac muscle derived HL-1 cell line, and the cardiomyocyte-like myoblast cell line H9c2²⁵. Since H9c2 cells are known to be more sensitive to hypoxia than HL-1 cells²⁶, the present study utilized a well-characterized H9c2 cell line derived from rat cardiac myoblasts. The results displayed in Fig. 1 indicate that H9c2 cell viability substantially decreased following treatment with 100–500 μ M H₂O₂, which was associated with elevated programmed cell death. Western blot analysis showed that HIF-1 α expression was upregulated within 1 h of treatment, followed by upregulation of ET-1 and mitochondrial apoptosis markers such as BAX and cleaved Caspase-3. In contrast, the expression of anti-apoptotic PIK3IP1 and Bcl-2 proteins was downregulated, suggesting that the in vitro experimental system with H9c2 cells was well established for the present study.

PI3K signaling plays an important role in the pathogenesis of cardiovascular diseases^{27,28}. However, different types of PI3K isoforms may perform distinct functions. For example, PI3K α may play an important role in the induction of physiological cardiac hypertrophy upon exercise^{29,30}, whereas PI3K γ may play a pathological role in the induction of congestive heart failure, considering PI3K γ -mediated inhibition of cardiac contractility in failing hearts^{31–33}. Previous studies demonstrated that PI3K $\gamma^{-/-}$ mice display increased ischemia-reperfusion injury, and PI3K γ kinase-dead knock-in (PI3K $\gamma^{KD/KD}$) mice exhibit a cardiac injury similar to wild-type animals³⁴. This suggests a protective role of PI3K γ in myocardial ischemia-reperfusion injury in mice, mediated via a kinase-independent mechanism. The data shown in Fig. 5 suggest that PI3K γ , but not PI3K α , plays a major role in ROS-induced cell death. Notably, we first found that PI3K γ interacts directly with PIK3IP1 to regulate programmed cell death.

PIK3IP1 was recently identified as a transmembrane protein that shares homology with the PI3K regulatory subunit p85⁹. This protein directly interacts with p110, the catalytic subunits of PI3K, and regulates PI3K activity through its p85-like domain. Previous studies showed that PIK3IP1 could bind to class IA PI3K subtype such as PI3K α and PI3K β ⁹. Our data now demonstrate that class IB PI3K subtype PI3K γ can also interact with PIK3IP1 (Fig. 5), suggesting class IB regulatory subunit p101 or p84 could also share the homology with PIK3IP1.

PIK3IP1 is abundantly expressed in many tissues, including the heart, liver, brain, and lungs. Overexpression of PIK3IP1 in hepatocytes leads to inhibition of PI3K signaling and a suppression of hepatocyte carcinoma development¹⁰. PIK3IP1 is involved in the PI3K pathway, which is associated with many cellular functions, such as T cell activation, carcinogenesis, and apoptosis³⁵. DeFrances *et al.* (2012) reported that silencing PIK3IP1 increases PI3K activity in basal conditions³⁶. We previously reported the function of PIK3IP1 in physiological cardiac hypertrophy¹¹, but the function of PIK3IP1 in the heart under pathological conditions remained unknown. In the present study, PIK3IP1 overexpression inhibited cardiomyocyte cell death via downregulating the PI3K-AKT signaling (Fig. 2), while PIK3IP1 KD exhibited the opposite effect (Fig. 3). According to our unpublished data, using the regression model^{37,38}, the expression level of PIK3IP1 is markedly downregulated by trans-aortic constriction (TAC), but rapidly increased after the release of TAC. This suggests that PIK3IP1 expression is dynamically changed by blood pressure. Interestingly, PIK3IP1 expression was also decreased upon MI initiation, but was reversed with increasing MI duration (unpublished data), further supporting the idea that PIK3IP1 is closely related to the pathophysiological condition of the heart.

The use of an ET_A receptor antagonist exerts beneficial effects in chronic heart failure, as evidenced by a reduction in infarct size, improved reperfusion, coronary flow, and protection during ischemic-reperfusion injury^{39–41}. Results of studies blocking ET_B receptors have been inconsistent. Our data, showing inhibition of ROS-induced cardiac apoptosis by an ET_A antagonist (BQ-123), but not by an ET_B antagonist (BQ-788) (Fig. 4), suggest that ET_A is important for the signaling cascade, but not ET_B. Our Co-IP data also showed that ET_A, but not ET_B could interact with PIK3IP1 (Fig. 4, Supplementary Fig. 1). However, further studies will be required to clarify the molecular basis of the interactions. Furthermore, the results displayed in Fig. 5 indicate that PI3K γ , but not PI3K α , can mainly modulate ET_A in the signaling cascade. Taken together, our results provide evidence that ET_A, PI3K γ , and PIK3IP1 are the major interacting partners involved in ROS-induced cardiac cell death.

In conclusion, the present study provides evidence to suggest that ROS-induced cardiac cell death is mediated by the ET_A-PIK3IP1-PI3K γ axis. The molecular model presented in Fig. 6 suggests that under normoxic conditions, PIK3IP1 expression is relatively high. This is due to the lower inhibition by ET_A; hence, subdued PI3K γ activity is retained because of the intense inhibition by PIK3IP1. On the other hand, under hypoxic conditions, when ROS is generated from mitochondria, the signaling direction is reversed,

and the likelihood of programmed cell death increases. Thus, the present study provides evidence that PIK3IP1 plays an essential role in regulating pathogenic signals in the heart.

Materials And Methods

2.1. Chemicals and antibodies

All plastic materials, including cell culture vessels, flasks, spreader, and multi-well plates were purchased from BD Falcon Labware (Franklin Lakes, NJ, USA) and Corning (Corning, NY, USA). BQ-123, BQ-788, IPI-549 and LY294002 were purchased from Sigma-Aldrich (St. Louis, MO, USA). H₂O₂ was dissolved in distilled water and BQ-123, BQ-788, LY294002 or IPI-549 was dissolved in 0.01% DMSO to make the stock solutions. The primary antibodies used were as follows: anti-PDK1 (#3062; Cell Signaling Technology, Danvers, MA, USA), anti-phospho AKT (#4060; Cell Signaling Technology), anti-AKT (#9272; Cell Signaling Technology), anti-PI3K α (#4249; Cell Singnaling Technology, Denver, MA, USA), anti-PI3K γ (sc-7177; Santa Cruz Biotechnology), anti-PIK3IP1 (#16826-1-AP; Proteintech, Rosemont, IL, USA), anti-ET-1 (ab117757; Abcam, Cambridge, UK), anti- HIF-1 α (# 14179; Cell Signaling Technology, Danvers, MA, USA) anti- α -tubulin (sc-5286; Santa Cruz Biotechnology).

2.2. Cell culture and treatment

H9c2 rat cardiomyocyte-derived cells were obtained from American Type Culture Collection (ATCC, Manassa, VA, USA). Cells were routinely maintained in Dulbecco's modified Eagle's medium (ATCC) supplemented with 10% heat-inactivated foetal bovine serum (Gibco BRL, Gaithersburg, MD, USA) and 1% antibiotics (WelGENE, Gyeongsan, Korea). All cultured cells were incubated at 37°C in a humidified atmosphere containing 5% carbon dioxide. Cells were fed with fresh culture medium two to three times per week and sub-cultured at 80% confluence.

2.3. siRNA transfection and chemical treatment

H9C2 cells were placed in a serum-free medium without antibiotics for 24 h prior to siRNA transfection. Cells were then transfected with 25 nM siRNA for PIK3IP1 and negative control (Bioneer, Daejeon, Korea) using DharmaFECT-1 reagent (Dharmacon, Lafayette, CO, USA) according to the manufacturer's instructions. After 48 h, cells were treated with H₂O₂ (200 nM) or with blank for 3 h or 24 h, respectively. The mRNA and protein expression levels were measured by qRT-PCR and western blot analysis, respectively.

2.4. Transient transfection of PIK3IP1-overexpressing clone

PIK3IP1-overexpressing clone was purchased from GenScript (Piscataway, NJ, USA), and was constructed using the pcDNA3.1+/C-(K)-DYK vector. DAN construct was dissolved in distilled water, stored in small aliquots at -20°C and used for subsequent experiments. H9C2 cells were transfected with PIK3IP1-overexpressing (PIK) and pcDNA3.1 control (Vec) using DharmaFECT-1 reagent (Dharmacon) according to the manufacturer's instructions. After 24 h, cells were treated with H_2O_2 , and protein expression was measured by western blot analysis using anti-DYKDDDDK antibodies.

2.5. Protein extraction and western blot analysis

Total cell lysates were prepared by scraping the cells in 200 μL 1X RIPA lysis buffer (Invitrogen, Eugene, OR, USA). After determining the protein concentration using a BCA protein assay kit (Pierce, Thermo Fisher Scientific, Waltham, MA, USA), proteins (10–30 μg) were mixed with 5X sample buffer, incubated at 95°C for 5 min, and loaded onto 8-12% sodium dodecyl sulphate-polyacrylamide gels. Proteins separated on the gels were transferred to polyvinylidene fluoride membranes. The membranes were blocked using 5% skim milk (BD Life Sciences, Franklin Lakes, NJ, USA) or 5% bovine serum albumin (BSA; Sigma-Aldrich) in TBST (0.1% Tween 20 in Tris-buffered saline; 137 mmol/L NaCl, and 20 mmol/L Tris/HCl, pH 7.4) for 1 h at room temperature and incubated at 4°C overnight with primary antibodies. After washing with TBST three times, membranes were probed with horseradish peroxidase-conjugated anti-goat immunoglobulin G (IgG), anti-mouse IgG, and anti-rabbit IgG (Cell Signaling Technology) for 1 h. The membranes were washed three times with TBST for 1 h and developed with ECL Advance Western Blotting Detection Kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK) using an ImageQuant LAS 4000 mini (GE Healthcare). Quantitation was performed with ImageJ software (NIH Image). The values for the test proteins were normalised to the corresponding values for glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Nuclear extracts were prepared from siNC or siPIK-transfected H9C2 cells for nuclear translocation analysis of NFAT using nuclear/cytosol fractionation kits (BioVision, Milpitas, CA, USA).

2.6. TUNEL assay

DNA fragmentation was detected in situ using a TUNEL assay kit (In Situ Cell Death Detection Kit, TMR red; Roche Applied Science, Penzberg, Germany) as described previously⁴³. Briefly, 48 hours after transfection with siRNA for PIK3IP1 and negative control, PIK3IP1-overexpressing (PIK) and pcDNA3.1 control (Vec), H9C2 cells were treated with H_2O_2 (200 μM) for 24 h. Cells were then fixed with 4% paraformaldehyde in PBS for 1 h at room temperature, washed with PBS, and permeabilized with 0.2% triton X-100 for 2 min on ice. Nuclear staining was performed with Hoechst (Molecular Probes, Eugene, OR, USA). The percentage of apoptotic nuclei was analyzed using Image J software (NIH Image).

2.7. Co-immunoprecipitation

The cells were lysed in 400 µl of NP-40 buffer with protease inhibitors and immunoprecipitated with Dynabeads protein G (Invitrogen, 10003D) The experiment was continued according to the manufacturer's instructions, beginning with the addition of 10 µL of antibodies or 10 µL of rabbit IgG to create the Co-IP bead complex by using a magnetic rack and added to the lysate. Each lysate (500 µL) was added to the PIK3IP1 antibody or rabbit IgG-Dynabead complex and incubated for 10 min. The bound proteins were eluted and the samples were subjected to western blotting.

2.8. Statistics

Statistical analyses were performed with the Prism 8 software (GraphPad software, SanDiego, CA, USA). All data are shown as mean $\pm$ standard deviation (SD) for each group. For statistical comparison of the two groups, an unpaired Student's t-test was used; for multiple comparisons, one-way analysis of variance (ANOVA) with Bonferroni correction was used. All experiments were performed at least three times. A value of $P < 0.05$ was considered statistically significant.

Abbreviations

AKT (protein kinase B)

AMI (accute myocardial infarction)

BAX (bcl-2-like protein 4)

c-Casp3 (cleaved caspase-3)

Co-IP (co-immunoprecipitation)

ET-1 (endothelin-1);

ET_A (endothelin A receptor)

ET_B (endothelin B receptor)

GPCR (G protein-coupled receptor);

H₂O₂ (hydrogen peroxide)

HIF-1_α (hypoxia-inducible factor-1_α)

MI (myocardial infarction)

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

PK1 (phosphoinositide-dependent kinase-1)

PI3K (phosphoinositide 3-kinase);

PIK3IP1 (phosphatidylinositol-3-kinase interacting protein 1);

ROS (reactive oxygen species);

TAC (trans-aortic constriction)

Declarations

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Author Contributions

J.H.P. designed the study, make experiments, and wrote the paper. K.J.N. and J.Y.L. make experiments. Y.J.Y., W.J.P., C.C. participated in planning and discussion, and provided resources. D.H.K. designed the study, supervised experiments and wrote the paper.

Additional Information

Supplementary information accompanies this paper at

Competing Interests: The authors declare no competing interests.

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Figures

Figure 1

H₂O₂-induced apoptosis of H9c2 cardiomyocytes is accompanied by decreased PIK3IP1 and Bcl2 expression, but increased HIF-1α, ET-1, BAX, and cleaved caspase-3 expression.

(A) H9c2 cardiomyocytes were treated with the indicated concentrations of H₂O₂ for 24h. Cell viability was measured using the MTT assay. (B) DNA fragmentation as an indication of programmed cell death was detected by TUNEL assay, and cells were counterstained with DAPI for nuclei staining. The scale bar

represents 200 μm . The graph on the right shows the significantly increased TUNEL positive cells by H_2O_2 treatment.(C) The protein expression levels of HIF-1 α , ET-1, PIK3IP1, BAX, Bcl-2, cleaved caspase-3 (c-Casp3) in the control (ctrl) and in the H_2O_2 -treated samples were measured by western blotting. The gel band intensities were normalized by GAPDH for quantifications and shown on the right. The results are presented as means $\pm$ SEM; N=3-5; statistical significance is shown as *P <0.05 relative to Control groups (ctrl).

Figure 2

PIK3IP1 overexpression inhibits H₂O₂-induced cell death via downregulating p110 α , p110 γ , p-AKT, and cleaved caspase-3 expression. H9c2 cardiomyocytes were transfected with pcDNA3.1 (Vec) or pcDNA3.1-PIK3IP1 (PIK) for 24h, and subsequently treated with or without H₂O₂ (200 μ M). (A) The transfected vector expression was detected using anti-DYKDDDDK tag antibody. The total PIK3IP1 amounts were detected by western blot analysis and normalized by GAPDH. (B) The protein levels of p110 α , p110 γ , and p-AKT, and cleaved caspase-3 were measured by western blotting. P-AKT was normalized by total AKT, and p110 α , p110 γ and cleaved caspase-3 were normalized by GAPDH. (C) The DNA fragmentation as an indication of programmed cell death were detected by TUNEL assay and the cells were counterstained with DAPI (nuclei staining). The scale bar represents 200 μ m. The results for data displayed in bar charts are presented as means $\pm$ SEM; N=3-5; Statistical significance is shown as *P <0.05 relative to Vec group. #P <0.05 relative to Vec treated with H₂O₂.

Figure 3

PIK3IP1 knockdown increased H₂O₂-induced cell death via upregulating p110α, p110γ, p-AKT, and cleaved caspase-3 expression.

H9c2 cells were transfected with siPIK (si-PIK3IP1) or siNC (negative control) for 24 h (25 nM each), and subsequently treated with or without H₂O₂ (200 μM). (A) To check the PIK3IP1 knockdown, the protein

expression level of PIK3IP1 was examined by the western blotting. GAPDH was used as an internal control. (B) The protein levels of p110 α , p110 γ , and p-AKT, and cleaved caspase-3 were measured by western blotting using the relevant antibodies as described in **Materials and Methods**. P-AKT was normalized by total AKT, and p110 α , p110 γ and cleaved caspase-3 normalized by GAPDH. (C) The DNA fragmentation as an indication of programmed cell death was detected by TUNEL assay, and the cells were counterstained with DAPI (nuclei staining). The scale bar represents 200 μ m. The combined results displayed in bar charts are presented as mean $\pm$ SEM; N=3-5; Statistical significance is shown as *P <0.05 relative to Vec group. #P <0.05 relative to Vec treated with H₂O₂.

Figure 4

H₂O₂ induced cardiomyocyte cell death via activating ET_A-PIK3IP1 binding.

(A) H9c2 cells were treated with H₂O₂ (200 μM) for 24h after pretreatment with BQ-123 (10 mM) or BQ-788 (10 mM) or DMSO. Fragmentation was detected by TUNEL assay and cells were counterstained with DAPI for nuclei staining. The scale bar represents 200 μm. (B) H9c2 cells were transfected with siPIK (si-

PIK3IP1) or siNC (negative control) for 24 h (25 nM each), and subsequently treated with or without BQ-123 (10 mM) for 3h. The protein expression levels of PIK3IP1 and cleaved caspase-3 were measured by western blotting. (C) Co-immunoprecipitation (Co-IP) assay using H9c2 cells were detected by western blotting using the anti-PIK3IP1 and anti-ETA antibodies. The mock IP negative control was performed by incubating cell extracts with G Dynabeads coupled with rabbit IgG. Results are presented as mean $\pm$ SEM; N=3-5; Statistical significance is shown as *P <0.05 relative to control group (Ctrl or siNC). #P <0.05 relative to H₂O₂ treated group. \$P<0.05 relative to siPIK group.

Figure 5

H_2O_2 induces H9c2 cell death through the ET_A -PIK3IP1-PI3Ky axis.

(A) H9c2 cells were treated with H_2O_2 (200 μ M) for 3h after pretreatment with BQ-123 (10 mM) or BQ-788 (10 mM) or DMSO. The protein expression levels of p110 α and p110 γ were measured by western blotting. GAPDH was used as an internal control. (B) Cells were treated with H_2O_2 for 3h after pretreatment with 20

μ M LY294002 (LY) or 10 μ M of IPI-549 (IPI). The expression levels of p110 α , p110 γ , and p-AKT, HIF-1 α and cleaved caspase-3 were measured by western blotting. P-AKT was normalized by total AKT, and p110 α , p110 γ and cleaved caspase-3 normalized by GAPDH. (C) Co-immunoprecipitation (Co-IP) assay using H9c2 cell lysates were detected by western blot using the anti-PIK3IP1 and anti-p110 γ antibodies. The mock IP negative control were performed by incubating cell extracts with G Dynabeads coupled with rabbit IgG. Results are presented as mean $\pm$ SEM; N=3-5; Statistical significance is shown as *P <0.05 relative to control group. #P <0.05 relative to H₂O₂ treated group.

Figure 6

The proposed model shows that the anti-ischemic effects of PIK3IP1 in reducing the likelihood of cardiac cell death are mediated through its interactions with the ET_A-PI3Kγ-AKT axis.

Supplementary Files

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