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PI3Kγ Exacerbates Lipid Overload-induced Insulin Resistance in H9c2 Cardiomyocytes

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Abstract

Diabetic cardiomyopathy is a heart disease in diabetic patients, resulting from the metabolic disorder in cardiomyocytes. The molecular mechanism underlying diabetic cardiomyopathy is largely unknown. Our preliminary investigation finds that phosphatidylinositol-3 kinase gamma (PI3Kγ) is upregulated in diabetic hearts, implying that PI3Kγ may participate in the development of diabetic cardiomyopathy. This study aims at demonstrating the role of PI3Kγ in lipid Overload-induced lipotoxicity during diabetic cardiomyopathy. We successfully constructed a pcDNA4-p110γ plasmid and transfected it into H9c2 cardiomyocytes to overexpress p110γ, the catalytic subunit of PI3Kγ. PI3Kγ overexpression reduced H9c2 viability in basal level and aggravated fatty acid Overload-induced cell death. Mechanismly, fatty acid overload induced insulin resistance in H9c2 cardiomyocytes, which was further exacerbated by PI3Kγ overexpression. In summary, we demonstrate that PI3Kγ overexpression is involved in lipid over-induced insulin resistance in H9c2 cardiomyocytes. PI3Kγ may play a pivotal role in the development of diabetic cardiomyopathy.

Keywords

Diabetic Cardiomyopathy; Insulin Resistance; Phosphatidylinositol-3 Kinase; PI3Kγ.

1. Introduction

Cardiovascular diseases are the most severe complications and the leading causes of death in diabetic patients. Diabetic cardiomyopathy is a heart disease in diabetic patients defined by the existence of abnormal myocardial structure and function, but in the absence of other cardiac risk factors, including coronary artery disease and hypertension [1]. It is mainly characterized by cardiac hypertrophy, fibrosis, and alteration of the structure and function of cardiomyocytes. Diabetic cardiomyopathy could directly lead to heart failure and also exacerbates cardiac injury induced by other risk factors, such as ischemia [2,3]. In recent years, cardiovascular diseases accompanied with diabetes have received great attention. However, the molecular mechanisms underlying diabetic cardiomyopathy are still largely unkown. The complex environment changes in diabetic patients, including hyperlipidemia and hyperglycemia, may trigger abnormal cardiomyocyte metabolism, which in turn causes cellular stresses, such as endoplasmic reticulum stress, inflammation, reactive oxygen species (ROS) and mitochondrial dysfunction. These cellular stresses ultimately lead to myocardial stiffness, cardiac hypertrophy and myocardial fibrosis [4].

The phosphoinositide 3-kinases (PI3Ks)-protein kinase B (PKB, also named Akt) signaling axes is the key molecular pathway downstream insulin signaling. Insulin can bind and activate insulin receptor (IR), which consequently stimulates PI3Ks. Activated PI3Ks catalyze the production of the important intracellular second messenger, phosphatidylinositol (3,4,5)-trisphosphate (PI(3,4,5)P3, also named PIP3) on plasma membrane. PIP3 can recruit PH

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domain containing proteins, such as PDK1 and Akt, to the plasma membrane and further promote the phosphorylation of Akt at Ser473 and Thr308. Akt is a central protein kinase that activates or inhibits a series of downstream substrates involved in cell survive, proliferation, protein synthesis and metabolism [5]. Inhibition the activity of PI3Ks leads to decrease of Akt function and results in cycle arrest and apoptosis, whereas hyperactivation of PI3K-Akt signaling may result in abnormal cell growth and diseases, such as cancers.

Class I PI3Ks include PI3Kα, PI3Kβ, PI3Kγ and PI3Kδ isoforms [6]. Among them, PI3Kα and PI3Kβ are ubiquitously expressed and considered as the major PI3K isoforms that function downstream insulin signaling. Deficiency of these two isoforms results in embryonic lethality [7].In contrast, PI3Kδ and PI3Kγ are only expressed in special cell types. PI3Kγ is highly expressed in leukocytes, but can also be upregulated by stresses in cardiomyocytes. In the heart, the catalytic subunit of PI3Kγ, p110γ, is upregulated under multiple stresses, including pressure overload [8], myocardial infarction [9] and anti-cancer drugs [10], and triggers maladaptive cardiac remodeling and, ultimately, heart failure. These works suggest that hyper-activation of PI3Kγ is detrimental in the heart.

To investigate whether PI3Kγ is also involved in the development of diabetic cardiomyopathy, we performed bioinformatic analysis of differentially expressed genes (DEGs) on diabetic cardiomyopathy database (GSE26887). We found that PI3Kγ is the only PI3K isofroms that are upregulated in hearts from diabetic patients (data not shown), suggesting that PI3Kγ isoforms are involved in the development of diabetic cardiomyopathy. In this study, we investigated the role of PI3Kγ in fatty acid Overload-induced lipotoxicity, which dominantly contributes to the development of diabetic cardiomyopathy.

2. Methods and Materials

2.1. Plasmid Construction

Full-length of human *pik3cg* CDS sequences were cloned from cDNA library and ligated into the pcDNA4-HisMax plasmid vector, using EcoRI and BamHI restriction endonucleases. Sequencing results confirmed that the sequence 100% matched with the CDS of *pik3cg* transcript variant 2 (NM_002649.3).

2.2. Transfection

PI3Kγ overexpression in H9c2 cells was achieved by plasmid transfection with lipofectamine 3000 (Thermo Fisher Scientific), according to manufacturer's protocol. In briefly, H9c2 cells were seeded with antibiotic-free medium and grew to reach 70%-90% confluence when transfection. Plasmid was mixed with P3000 reagent in Opti-MEM medium, before added to Lipofectamine 3000 reagent with Opti-MEM medium. The above mix was mantained at room temperature for 15 min to allow the formation of liposome-encapsulated DNA, before added to cultured cells. Cells were cultured for additional 8 hours or overnight before replaced with flesh medium and for another 24-48 hours to allow the overexpression of PI3Kγ.

2.3. Cell Treatment

H9c2 cells with PI3Kγ overexpression or control cells were seeded in 6-cm dish or 96-well plates for experiments. After attachment, cells were treated with different concentration of palmitic acid (PA) for 24-48 hours. Cells were subjected to MTT assays to detect cell viability. To assess insulin resistance, PA-treated H9c2 cells were incubated with 10μg/ml insulin for 5min. Cells were collected and subjected to Western blot analysis.

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2.4. Cell Viability Assays

PA-treated H9c2 cells were incubated with MTT (0.5 mg/ml) for 4 hours. Cells were then lyzed with DMSO to dissolve the crystals and subjected to absorbance measurement under 490nm. OD650nm measurement was setted as backgroud.

2.5. Western Blot

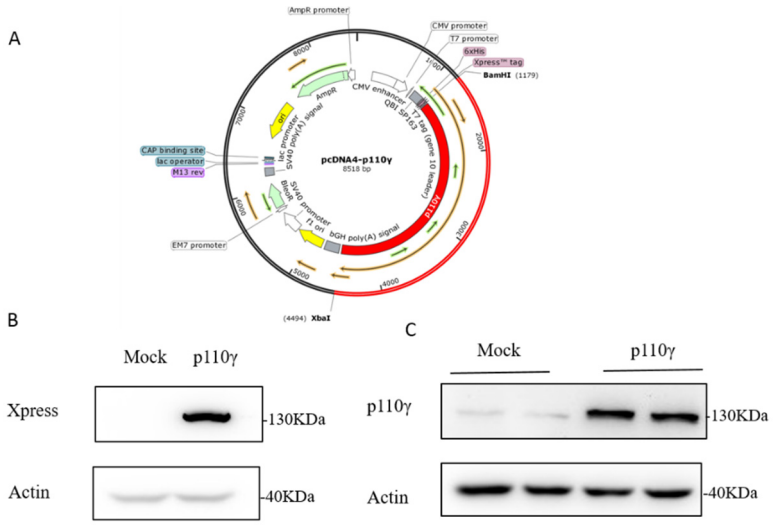
PA-treated H9c2 cells were washed with PBS and collected with a cell scraper. Cells were further lysed with cell lysis buffer, containing protease inhibitors, for 10 min. After centrifuging at 13000rpm for 10min, the supernatant was collected. Samples were either subjected to protein quantification with BCA or mixed with laemmily buffer and boiled at 95℃ for 5min before use. Protein samples were subjected to SDS-PAGE electrophoresis and transfered to PVDF membranes. PVDF membranes that contain the interested proteins were blocked with 5% milk or BSA diluted in TBS-Tween-20 (TBST) for 1h and incubated with primary antibodies overnight at 4°C. After washing with TBST for three times, 5min, membranes were further incubated with secondary antibodies at room temperature for 1h. Membranes were washed with TBST for three times and subjected to ECL development to detect the protein signal.

2.6. Statistical Analysis

Prism software (GraphPad software Inc) was used for statistical analysis. P values were calculated with 2-way ANOVA, followed by Bonferroni post hoc test. Data are presented as mean±SEM, and Data are presented as means ± SEM. $P<0.05$ was considered statistically significant.

3. Results

3.1. p110γ Overexpression in H9c2 Cardiomyocytes



(A) CDS of p110γ mRNA were cloned and inserted into pcDNA4-HisMax backbone to construct the pcDNA4-p110γ plasmid. (B-C) p110γ overexpression in H9c2 cells validated by Western blot with (B) anti-Xpress-tag and (C) anti-p110γ antibodies.

Figure 1. pcDNA4-p110γ plasmid construction and p110γ overexpression in H9c2 cells

To validate the role of PI3Kγ in diabetic cardiomyopathy, we firstly constructed the pcDNA4-p110γ by cloning the CDS coding sequence of p110γ, the catalytic unit of PI3Kγ, and inserted it into the pcDNA4-HisMax plasmid backbone. Xpress-tag was fused with p110γ to track the p110γ expression (Figure 1A). pcDNA4-p110γ (p110γ) or pcDNA4-HisMax control (Mock) was transfected into H9c2 cardiomyocytes with lipofectamine 3000. 48h after transfection, p110γ

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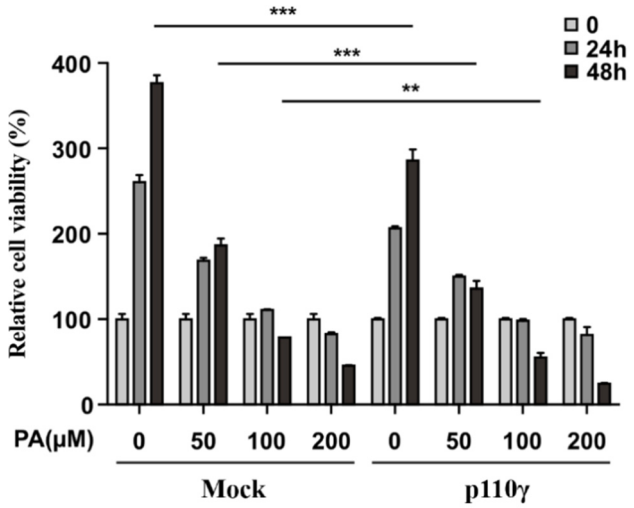
expression was validated by western blot. Xpress-tag labling clearly showed that p110γ was upregulated in H9c2 cells after pcDNA4-p110γ transfection (Figure 1B). This is further confirmed with anti-p110γ antibody, demonstrating that p110γ is expressed in baseline, but significantly upregulated after pcDNA4-p110γ transfection (Figure 1C).

3.2. PI3Kγ Upregulation Exacerbates Fatty Acid Overload-induced Cardiomyocyte Injury

Hyperlipidemia during diabetes is a key reason that results in insulin resistance in organs, including the heart. Increased levels of lipids in the heart could lead to myocardial injury. We used palmitic acid (PA), a key component of saturated fatty acids, to mimic the lipid-overload environment in cardiomyocytes. H9c2 were treated with different concentration of PA (50-200μM) for 24-48h. Cell viability was measured with MTT assay. PA induced H9c2 injury in a concentration-dependent manner, with 50μM PA leaded to around 50% of cell death in 48h (Figure 2). Interestingly, PI3Kγ activation inhibited cell proliferation in baseline and significantly promoted PA-induced cell death. This result implies that PI3Kγ plays a pivotal role during lipid Overload-induced cardiomyocyte injury.



H9c2 cells were transfected with pcDNA4-p110γ (p110γ) or pcDNA4-HisMax (Mock). 24h after transfection, cells were treated with indicated concentration of palmitic acid (PA) for 24-48h. Cell viability was measured by MTT assay **p<0.01,***p<0.001, Mock vs p110γ

Figure 2. PI3Kγ upregulation exacerbates fatty acid Overload-induced cardiomyocyte injury in H9c2 cells

3.3. PI3KΓ Overexpression Promotes Insulin Resistance in Fatty Acid-Treated Cardiomyocytes

Insulin directly activates the insulin receptor (IR) and the PI3K-Akt signal axes to regulate the downstream proteins that involved in glucose uptake and utilization. Thus, PI3K-Akt is the central pathway that functions downstream insulin signaling. PI3Kα and PI3Kβ are considered as the dominant isoforms that participate in this process. In contrast, the role of PI3Kγ in insulin signaling is still unclear.

Lipid Overload-induced cardiomyocyte insulin resistance is a key reason that invovled in diabetic cardiomyopathy. As PI3Kγ overexpression exacerbates PA-induced cell death (Figure 2), we would like to know whether PI3Kγ upregulation influences lipid Overload-induced insulin resistance. pcDNA4-p110γ (p110γ)- or pcDNA4-HisMax4 (Mock)-transfected H9c2 cells were treated with 50μM PA for 48h, followed by insulin treatment for 5 mins. Insulin rapidly stimulated Akt phosphorylation at Ser473 and Thr308 in 5 mins. However, Akt activation is

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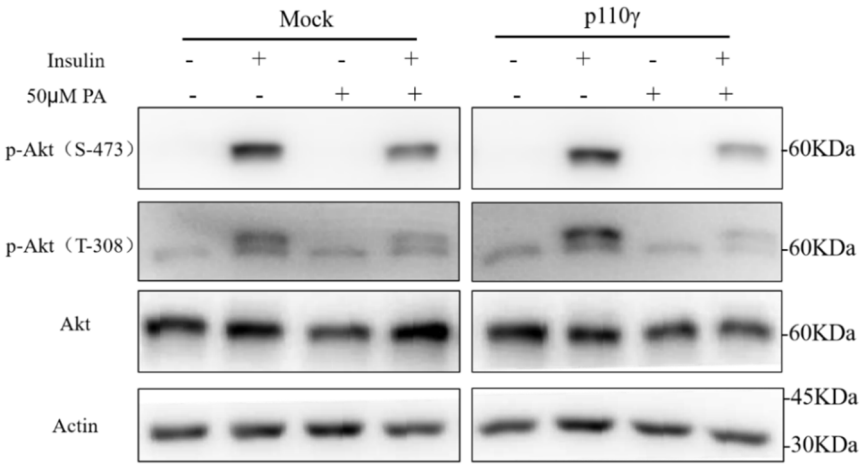
dampened in PA-treated H9c2 cells, validating that fatty acid overload induces insulin resistance in cardiomyocytes (Figure 3). Interestingly, when PI3Kγ is upregulated in H9c2 cardiomyocytes, PA-induced blunt of Akt phosphorylation, in response of insulin treatment, was further enhanced (Figure 3). This result revealed that PI3Kγ overexpression exacerbates lipid Overload-induced insulin resistance in cardiomyocytes.



Western blot validation of Akt phosphorylation in H9c2 cells treated with 50μM PA for 48h and receiving insulin (10μg/ml, 5min) treatment

Figure 3. PI3Kγ overexpression promotes insulin resistance in fatty acid-overloaded cardiomyocytes

4. Discussion

In this study, we demonstrated that PI3Kγ is detrimental during the development of diabetic cardiomyopathy. Overexpression of p110γ, the catalytic subunit of PI3Kγ, significantly exacerbates fatty acid Overload-induced cardiomyocyte death and insulin resistance. These results imply that targeting PI3Kγ may provide a potential strategy to limit lipid Overload-induced cardiomyocyte lipotoxicity.

PI3K isoforms are differently expressed in the system. Among them, PI3Kα and PI3Kβ are ubiquitously expressed. Depletion of either PI3Kα or PI3Kβ results in embryonic lethality, suggesting a fundamental role of these two isoforms in cell survive. In contrast, PI3Kδ and PI3Kγ are only expressed in special cell types. PI3Kδ is mainly expressed in leukocytes and thus are essential for immuno responses. PI3Kγ is also expressed in leukocytes, such as macrophages and neutrophils. PI3Kγ controls the migration and infiltration of leukocytes in tissues, thus contributing to the progress of inflammation. In additional, PI3Kγ is also expressed in cardiomyocytes and can be activated by multiple cardiac stress, such as pressure overload, myocardial infarction and anti-cancer drugs. Thus, PI3Kγ could be a potential molecular target for the therapy of heart disease.

The present study further adds evidence that PI3Kγ is also involved in the development of diabetic cardiomyopathy. Overexpression of PI3Kγ exacerbates fatty acid Overload-induced cardiac insulin resistance and cell death. However, the limitation of this study is that strategies with PI3Kγ inhibition, either with genetic silencing or small molecular inhibitors, is still missing. In the future, we should further validate whether inhibition of PI3Kγ could attenuate fatty acid Overload-induced lipotoxicity. In summary, we provide clue that PI3Kγ activation is detrimental in the development of diabetic cardiomyopathy.

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References

[1] Dillmann WH. Diabetic Cardiomyopathy. Circ Res. 2019;124:1160–1162.

[2] Bugger H, Abel ED. Molecular mechanisms of diabetic cardiomyopathy. Diabetologia. 2014;57:660–671.

[3] Boudina S, Abel ED. Diabetic cardiomyopathy revisited. Circulation. 2007;115:3213–3223.

[4] Kaur N, Guan Y, Raja R, et al. Mechanisms and Therapeutic Prospects of Diabetic Cardiomyopathy Through the Inflammatory Response. Front Physiol. 2021;12:694864.

[5] Vadlakonda L, Dash A, Pasupuleti M, et al. The Paradox of Akt-mTOR Interactions. Front Oncol. 2013;3:165.

[6] Vanhaesebroeck B, Guillermet-Guibert J, Graupera M, et al. The emerging mechanisms of isoform-specific PI3K signalling. Nat Rev Mol Cell Biol. 2010;11:329–341.

[7] Vanhaesebroeck B, Ali K, Bilancio A, et al. Signalling by PI3K isoforms: insights from gene-targeted mice. Trends Biochem Sci. 2005;30:194–204.

[8] Damilano F, Franco I, Perrino C, et al. Distinct effects of leukocyte and cardiac phosphoinositide 3-kinase γ activity in pressure Overload-induced cardiac failure. Circulation. 2011;123:391–399.

[9] Siragusa M, Katare R, Meloni M, et al. Involvement of phosphoinositide 3-kinase gamma in angiogenesis and healing of experimental myocardial infarction in mice. Circ Res. 2010;106:757–768.

[10] Li M, Sala V, De Santis MC, et al. Phosphoinositide 3-Kinase Gamma Inhibition Protects From Anthracycline Cardiotoxicity and Reduces Tumor Growth. Circulation. 2018;138:696–711.

[11] Vanhaesebroeck B, Ali K, Bilancio A, et al. Signalling by PI3K isoforms: insights from gene-targeted mice. Trends Biochem Sci. 2005;30:194–204.

[12] Damilano F, Franco I, Perrino C, et al. Distinct effects of leukocyte and cardiac phosphoinositide 3-kinase γ activity in pressure Overload-induced cardiac failure. Circulation. 2011;123:391–399.