Published in final edited form as:

Circ Res. 2023 March 17; 132(6): 690–703. doi:10.1161/CIRCRESAHA.122.321735.

Hypoxia Sensing of β-Adrenergic Receptor Is Regulated by Endosomal PI3Kγ

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Abstract

Background—Impaired beta-adrenergic receptor (β1 and β2AR) function following hypoxia underlies ischemic heart failure/stroke. Activation of phosphoinositide 3-kinase γ (PI3Kγ) by βAR leads to feedback regulation of the receptor by hindering βAR dephosphorylation through inhibition of protein phosphatase (PP2A). However, little is known about PI3Kγ feedback mechanism in regulating hypoxia-mediated β1 and β2AR dysfunction and cardiac remodeling.

Methods—HEK 293 cells or mouse adult cardiomyocytes and C57BL/6 (WT) or PI3Kγ knockout (KO) mice were subjected to hypoxia. Cardiac plasma membranes and endosomes were isolated and evaluated for β1 and β2AR density and function, PI3Kγ activity and β1 and β2AR-associated PP2A activity. Metabolic labeling was performed to assess β1 and β2AR phosphorylation and epinephrine/norepinephrine levels measured post-hypoxia.

Results—Hypoxia increased β1 and β2AR phosphorylation, reduced cAMP and led to endosomal accumulation of phosphorylated β2ARs in HEK 293 cells and WT cardiomyocytes. Acute hypoxia in WT mice resulted in cardiac remodeling and loss of adenylyl cyclase activity associated with increased β1 and β2AR phosphorylation. This was agonist-independent as plasma and cardiac epinephrine and norepinephrine levels were unaltered. Unexpectedly, PI3Kγ activity was selectively increased in the endosomes of HEK 293 cells and WT hearts post-hypoxia. Endosomal β1- and β2AR-associated PP2A activity was inhibited upon hypoxia in HEK 293 cells and WT hearts showing regulation of βARs by PI3Kγ. This was accompanied with phosphorylation of endogenous inhibitor of protein phosphatase 2A (I2PP2A) whose phosphorylation by PI3Kγ inhibits PP2A. Increased β1 and β2AR-associated PP2A activity, decreased βAR phosphorylation, and normalized cardiac function was observed in PI3Kγ KO

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Disclosures: None

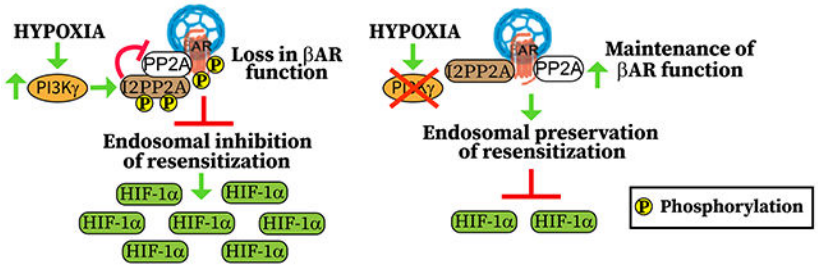
Online Supplement: Expanded Supplementary Methods and Materials, Online Supplementary Table 1, Online Figs S1–S5 and Figure Legends, Online References 31–35.

mice despite hypoxia. Compared to WT, PI3Kγ KO mice had preserved cardiac response to challenge with β1AR-selective agonist dobutamine post-hypoxia.

Conclusions—Agonist-independent activation of PI3Kγ underlies hypoxia sensing as its ablation leads to reduction in β1- and β2AR phosphorylation and amelioration of cardiac dysfunction.

BioRxiv Submission: https://www.biorxiv.org/content/10.1101/2020.09.17.301903v1.

Grahpical Abstract



Keywords

PI3Kγ; HIF-1α; β2AR; cardiac dysfunction; Cell Signaling/Signal Transduction; Mechanisms; Heart Failure

Introduction

Oxygen is a key currency driving the sustenance of cells as it plays a central role in metabolism and respiration[1]. Eukaryotes have developed an efficient and rapid oxygen sensing system through the hypoxia-inducible factors (HIFs) that are master transcription factors [2]. HIF-1α isoform is the most well studied member of the HIF family represented by HIF -1, -2 and -3. HIF-1α is stabilized in hypoxia and dimerizes with HIF-1β to form a potent transcription factor that drives the hypoxia response [2, 3]. Our previous study has shown that HIF-1α stabilization seems to be dependent GRK2-mediated on beta-adrenergic receptor (βAR) phosphorylation [4].

βARs play a key role in cardiac function [5], wherein their sustained dysfunction due to phosphorylation is associated with deleterious cardiac remodeling/heart failure [5, 6]. Activation of βAR family members, β1AR or β2AR by agonist leads to phosphorylation by G-protein coupled receptor kinase 2 (GRK2) [7] that recruits β-arrestin promoting endocytosis of phosphorylated βARs[8]. Phosphorylated βARs undergo protein phosphatase 2A (PP2A)-mediated dephosphorylation (resensitization) in the endosomes before recycling back to the plasma membrane [8]. We have shown that phosphoinositide 3-kinase γ (PI3Kγ) inhibits PP2A by phosphorylating an endogenous inhibitor of PP2A (I2PP2A) which robustly binds to PP2A inhibiting its activity [9]. Thus, agonist activation leads to inhibition of PP2A activity by PI3Kγ leading to impaired resensitization resulting in accumulation of phosphorylated βARs. However, less is understood about the mechanistic underpinnings of hypoxia-mediated agonist-independent β1- and β2AR phosphorylation.

Since PI3Kγ inhibits dephosphorylation of βARs, we tested whether hypoxia activates PI3Kγ in an agonist-independent manner, in contrast to its agonist-mediated activation [10]. Thus, inhibition of β1- and β2AR dephosphorylation *ie* resensitization could account for accumulation of phosphorylated βARs during hypoxia. Furthermore, we investigated whether acute hypoxia *per se* alters cardiac function in C57BL/6 wild type (WT) mice by impairing resensitization. Finally, PI3Kγ knock out (PI3Kγ KO) mice were used to evaluate whether cardiac function is preserved and resensitization normalized despite hypoxia.

Methods

Data Availability

All data and supporting materials have been provided with the published article. Furthermore, the authors state that all the supporting data are available within the article and are detailed in the online supplementary files containing Material and Methods (that includes the study design) and Major resources. Material requests are available from the corresponding author, upon reasonable request.

Experimental Animal:

C57BL/6 wild type (WT) mice and PI3Kγ knock out (PI3Kγ KO) mice in the C57BL/6 background [11] of either sex 8-12 weeks of age were subjected to hypoxia (10% O2) [12] or normoxia for 20 hours. FLAG-β1AR transgenic (Tg) mice (expression of FLAG-β1AR under α/MHC promoter [13]), WT or PI3Kγ KO mice (age 8-12 weeks) were used to isolate primary cardiomyocytes. Animal studies were performed in accordance with institutional and national guidelines and regulations, as approved by Cleveland Clinic IACUC.

Cell Culture:

FLAG-β2AR expressing HEK 293 (β2AR expressing HEK 293) cells were maintained and seeded as described previously [14]. Cells were serum starved and treated with normoxia or hypoxia or stimulated with 10μM ISO (Sigma-Aldrich) for 10 min in normoxia and are detailed in supplementary methods.

Primary adult cardiomyocyte isolation and hypoxia treatment:

Adult mouse cardiomyocytes were isolated as described previously [9, 14]. Isolated cardiomyocytes were plated onto 10μg/ml laminin (Invitrogen) coated dishes in plating media for 1 h. After 1 h, the plating media was replaced by culture medium and cardiomyocytes subjected to normoxia or hypoxia for 1.5 h and used for biochemical experiments (detailed in supplementary methods).

Isolation of Plasma Membranes and Endosomes:

Plasma membranes and endosomes were isolated as described previously [9]. Plasma membrane and endosomal fractions were validated by immunoblotting with anti- Na^+K^+ ATPase or anti-Rab5 antibody (see supplementary methods) respectively, and were used for experiments.

Confocal microscopy:

Confocal microscopy was performed as described by us [9] for β2AR expressing HEK 293 cells. While adult cardiomyocytes were plated onto to chamber slides coated with laminin. Anti-phospho-β2AR 355/356 [15] (p-β2AR) antibody was used to visualize p-β2AR (details in supplementary methods). Cells were visualized by line excitation at 488 and 568 nm for green and red fluorescence, and quantitation was performed using IMAGE PRO PLUS7 (Media Cybernetics, Inc) followed by plot profile analysis using ImageJ software.

Western immunoblotting:

Standard procedure for western immunoblotting were performed [14]. The proteins were resolved on SDS-PAGE, transferred to PVDF (BIO-RAD) membrane and immunoblotted using primary anti-bodies (supplementary methods). Anti-phospho-I2PP2A antibody was generated in-house at Cleveland Clinic hybridoma core, validated and has been described in our recent publication [16].

Adult cardiomyocyte metabolic labeling to evaluate βAR phosphorylation:

Metabolic labeling of primary adult cardiomyocytes was modified from our previous studies [9, 14] and is detailed in supplementary methods. Plating medium of the adult cardiomyocytes were replaced by phosphate free MEM media containing 10μg/ml BSA and 100 μCi/ml of $^{[32]}$P-inorganic phosphate, and subjected to normoxia or hypoxia for 1.5 h. DOB stimulation was used as positive control. After normoxia, hypoxia or DOB stimulation, the cells were lysed and receptors immunoprecipitated using anti-FLAG antibody or alprenolol-conjugated beads that binds to β1- and β2AR [4], and phosphorylation visualized by autoradiography.

Phosphatase assay:

PP2A phosphatase activity was measured using phosphatase assay kit following manufacturer's protocol as previously described by us with minor modifications [17] and is detailed in supplementary methods. FLAG-β2ARs from HEK 293 were immunoprecipitated using anti-FLAG antibody, while cardiac β1- and β2ARs were immunoprecipitated using alprenolol-conjugated beads and subjected to malachite green phosphatase assay.

βAR Density, Adenylyl Cyclase Activity, and cAMP and lipid assays:

All these assays have been described by us in multiple publications [9, 14, 18] and detailed in supplementary methods.

Immunohistochemistry:

Cardiac samples were placed in fresh 4% paraformaldehyde at room temperature for 24 h and processed through xylene exchange and embedded into paraffin blocks as described in our previous study [19]. Paraffin slides (5 μm thickness) were subsequently stained with H&E and imaged using a Slide Scanner-Aperio AT2 (Leica Biosystems).

Echocardiography:

M-mode echocardiography was performed on anesthetized 8-12 weeks old mice using a VEVO 2100 (VisualSonics) as previously described [17]. Details are provided in the supplementary methods including the DOB challenge following hypoxia to assess acute cardiac response.

Statistical analysis:

Results are expressed as means ± SD. Multiple comparisons were performed with the non-parametric tests, wherein Mann-Whitney test was performed for comparing two independent sample data sets. Kruskal-Wallis test with Dunn's correction was used for data sets with multiple comparisons. All calculations were performed with GraphPad Prism software version 9.4. P=0.05 or less was considered to be statistically significant.

Results

Hypoxia mediates non-canonical agonist-independent β2AR dysfunction:

To test whether hypoxia *per se* alters βAR function independent of agonist, β2AR expressing HEK 293 cells (FLAG-β2AR expressing HEK 293 cells) were serum starved and subjected to hypoxia for 0, 3 or 6 hours (h). Immunoblotting of cell lysates with anti-phospho-β2AR (p-β2AR) antibody showed significant phosphorylation of β2AR by 6 h [Fig. 1A & B (left panel) & Supplementary Fig. 1A]. HIF-1α (marker for hypoxia) also showed significant stabilization by 6 h [Fig. 1A] reflecting an association of β2AR phosphorylation with HIF-1α accumulation. Therefore, we have used 6 h hypoxia treatment in the HEK cellular studies. GRK2 was upregulated consistent with our previous studies [16] underlying β2AR phosphorylation [Fig. 1A & B (right panel)]. Confocal microscopy showed significant phosphorylation of β2AR with hypoxia as visualized by anti-p-β2AR antibody (green) [Fig. 1C] and measured by fluorescent intensity/cell [Fig. 1D]. While, anti-FLAG staining showed both plasma membrane and cytosol distribution of β2ARs in normoxia and hypoxia [Supplementary Fig. 1B]. Also, punctate FLAG-staining in cytoplasm was observed with hypoxia-treated cells compared to normoxia [Supplementary Fig. 1B, arrows, panels 4 & 6] reflecting enrichment of phosphorylated β2ARs.

Given that hypoxia mediates β2AR phosphorylation, βAR function was assessed by cAMP levels and adenylyl cyclase activity. Significant reduction in cAMP was observed following hypoxia [Fig. 1E]. To determine whether G-protein coupling is impaired, in vitro isoproterenol (βAR agonist, ISO)-stimulated adenylyl cyclase activity was performed on plasma membrane and endosomal fractions. Adenylyl cyclase activity was significantly reduced with hypoxia compared to normoxia at the plasma membrane [Supplementary Fig. 2A] and endosomes [Supplementary Fig. 2A]. The loss in adenylyl cyclase activity was higher in the endosomes showing that hypoxia impairs endosomal βAR function. Since hypoxia causes β2AR phosphorylation, we tested whether β-arrestin-2 participates in the hypoxia-mediated regulation of βAR function by using β-arrestin-2 GFP-β2AR double-stable HEK 293 cells that were subjected to hypoxia or ISO (as a positive control). ISO treatment resulted in significant recruitment of β-arrestin-2 GFP (green) to phosphorylated β2ARs (red) [Supplementary Fig. 2B, confocal panels 5, 6 & 8] and

measured by β-arrestin-2 GFP clearance from cytosol [Supplementary Fig. 2C, plot profile analysis]. Although hypoxia promotes phosphorylation of β2ARs (red) [Supplementary Fig. 2C, panels 10 & 12], no significant changes in β-arrestin-2 GFP recruitment was observed [Supplementary Fig. 2B & C] showing unique regulation of β2ARs by hypoxia.

As hypoxia leads to phosphorylated β2ARs in the cytosol [Fig. 1C], we tested whether hypoxia promotes internalization of β2ARs. β2AR expressing HEK 293 cells were pretreated with internalization blockers (sucrose and β-cyclodextrin [9]), subjected to hypoxia and β2AR internalization assessed by confocal microscopy. ISO treatment as control showed phosphorylation of β2ARs (green) decorating the plasma membrane due to inhibition of internalization [Supplementary Fig. 2D, confocal panels 4 & 6, & right panel, cumulative data]. In contrast, phosphorylated β2ARs were observed in the cytosol upon hypoxia [Supplementary Fig. 2D, confocal panels 7 & 9, & right panel cumulative data] despite internalization blockers. This suggests that hypoxia-mediated phosphorylation of β2ARs could be independent of β2AR internalization. To further validate whether hypoxia plays a role in β2AR internalization, $^{[125]}$I-Cyanopindalol radio-ligand binding was performed on plasma membrane and endosomal fractions from β2AR expressing HEK 293 cells following hypoxia. Radio-ligand binding showed no differences of β2AR distribution at the plasma membrane or endosomal fractions following normoxia or hypoxia [Fig. 1F]. This shows that hypoxia mediates phosphorylation of β2ARs independent of internalization potentially promoting endosomal receptor phosphorylation [Supplementary Fig. 2D]. This reflects mechanisms beyond the canonical agonist-mediated regulation of β2AR phosphorylation and internalization. To determine whether hypoxia mediated phosphorylation of β2AR is agonist-independent, β2AR expressing HEK 293 cell lysates and medium (the media was concentrated (see methods)) were assessed for epinephrine or norepinephrine (catecholamine) levels following hypoxia. There was no statistical difference in the catecholamine levels from cell lysates subjected to hypoxia or normoxia (catecholamine levels in media were below the detection limits (kit sensitivity range ~ nanograms) ([Supplementary Fig. 2E]. These observations suggest that hypoxia-mediated phosphorylation of β2AR is agonist- and internalization-independent, facilitating an increase in phosphorylated β2ARs.

Activation of PI3Kγ by hypoxia inhibits endosomal protein phosphatase 2A (PP2A) activity impairing β2AR resensitization:

Since hypoxia mediates β2AR phosphorylation that is agonist as well as internalization independent, receptor phosphorylation was evaluated at the plasma membrane and endosomes from β2AR expressing HEK 293 cells following hypoxia. Significant β2AR phosphorylation was observed in the endosomal fraction of the cells subjected to hypoxia compared to normoxia, while differences at the plasma membrane were statistically not significant [Fig. 2A]. As phosphorylated β2ARs are observed post-hypoxia in the endosomes, we tested whether dephosphorylation/resensitization mediated by protein phosphatase 2A (PP2A) [20] is impaired in response to hypoxia. β2AR-associated phosphatase activity was measured by immunoprecipitating FLAG-β2AR from plasma membrane (PM) and endosomes as a measure of resensitization. While, β2AR-associated phosphatase activity at the PM was not statistically significant [Fig. 2B, left panel], there

was significant reduction in β2AR-associated phosphatase activity in the endosomes [Fig. 2B, right panel].

Despite reduced PP2A activity there was no difference in the expression of PP2A following hypoxia [Fig. 2C] suggesting inhibition of PP2A activity rather than changes in expression underlies its response to hypoxia. Since PI3Kγ is known to inhibit PP2A activity by phosphorylating the endogenous inhibitor of PP2A (I2PP2A) [20], immunoblotting was performed to assess I2PP2A phosphorylation using a new in-house generated anti-phospho-I2PP2A antibody. Although total I2PP2A levels did not change [Fig. 2C], significant increase in I2PP2A phosphorylation was observed upon hypoxia [Fig. 2C]. This shows that hypoxia inhibits β2AR-associated PP2A activity impairing resensitization leading to phosphorylated β2ARs in the endosome accounting for β2AR dysfunction. As PI3Kγ regulates I2PP2A phosphorylation [20], PI3Kγ was immunoprecipitated from the PM and endosomal fractions to measure lipid kinase activity. PI3Kγ activity was significantly increased in the endosomal fractions following hypoxia compared to normoxia [Fig. 2D, right panel], while no significant differences were observed at the PM [Fig. 2D, left panel]. These shows that hypoxia activates PI3Kγ inhibiting PP2A activity leading to impaired resensitization resulting in hypoxia-mediated β2AR phosphorylation and associated receptor dysfunction.

Hypoxia leads to βAR dysfunction and is associated with endosomal phosphorylated β2ARs in adult cardiomyocytes:

Although β2AR is expressed in the heart, β1AR is the dominant receptor and a key determinant of cardiac outcomes. To directly test whether hypoxia causes β1AR phosphorylation, we used the transgenic (Tg) mouse line with cardiomyocyte-specific expression of FLAG-β1AR [13]. Since commercial antibodies are not available to assess β1AR phosphorylation, metabolic labeling was performed on adult cardiomyocytes isolated from FLAG-β1AR Tg mice using [^{32}P] inorganic phosphate (Pi). Adult cardiomyocytes underwent metabolic labeling under hypoxia condition for 1.5 h. β1AR-selective agonist dobutamine (DOB) stimulation was used as positive control. Following hypoxia, β1AR was immunoprecipitated from cardiomyocytes using anti-FLAG antibody and phosphorylated β1ARs assessed by autoradiography. Significant phosphorylation of β1AR was observed following hypoxia compared to normoxia treated adult cardiomyocytes [Fig. 3A, upper and lower panel]. Also, marked phosphorylation was observed with treatment of β1AR-selective agonist, DOB [Fig. 3A, upper and lower panel]. As β2ARs are also expressed in the hearts, adult cardiomyocytes isolated from C57BL/6 (WT) mice were subjected to hypoxia, and β2AR phosphorylation was visualized by confocal microscopy. β2AR phosphorylation was evaluated using anti-phospho-β2AR antibody (green) and measured by fluorescent cell intensity. Confocal microscopy showed moderate, yet significant phosphorylation of β2AR as reflected by increased green fluorescence in response to hypoxia [Fig. 3B, panel 4 & Fig. 3C] compared to normoxia [Fig. 3B, panel 1 & Fig. 3C]. To determine whether βAR phosphorylation post-hypoxia occurs at PM and/or endosomes, we assessed β2AR phosphorylation due to availability of commercial antibody recognizing phosphorylated β2ARs. PM and endosomal fractions were isolated from adult cardiomyocytes following hypoxia and immunoblotted with anti-phospho-β2AR antibody. Significant phosphorylation

of β2ARs was observed in the endosomal fractions of adult cardiomyocytes subjected to hypoxia compared to normoxia [Fig. 3D and right panel]. The differences in the β2AR phosphorylation was not significant at the PM fraction post-hypoxia [Fig. 3D and right panel]. Anti-Na^+K^+ ATPase and anti-Rab5 antibodies were used as PM and endosomal markers [Fig. 3D, lower panels]. As hypoxia causes βAR phosphorylation, cAMP level was assessed as a measure of βAR function. There was significant decrease in cAMP levels post-hypoxia compared to normoxia suggesting that hypoxia mediates βAR dysfunction in conditions of acute hypoxia [Fig. 3E].

Hypoxia causes cardiac remodeling and is associated with β1 and β2AR dysfunction:

Given our observation that acute hypoxia causes β1 and βAR dysfunction, we evaluated whether short term acute hypobaric hypoxia could lead to cardiac remodeling in mice. C57BL/6 (WT) mice were subjected to hypoxia for a short period of 20 h [24] which unexpectedly showed cardiac remodeling as assessed by echocardiography [Fig. 4A & Supplementary Table 1] showing mild, yet significant reduction in % ejection fraction (%EF), % fractional shortening (%FS) [Fig. 4A & right panels] and wall thickness [Supplementary Table 1]. Also, there was marked increase in heart weight to body weight ratio (HW/BW) post-hypoxia [Supplementary Fig. 3A] reflecting cardiac remodeling that is further supported by the H & E staining [Fig. 4B]. To measure the molecular markers of cardiac remodeling, RNA was isolated from the WT mice post-normoxia/hypoxia and RT-PCR was performed for ANF, BNP and βMHC. While, differences were not significant in ANF or BNP expression post-hypoxia [Supplementary Fig. 3B & C], there was significant increase in βMHC post-hypoxia [Supplementary Fig. 3D] suggesting a dichotomy in the response. Since βARS acutely regulate cardiac contraction, β2AR phosphorylation was assessed as an underlying measure of dysfunction due to availability of anti-phospho-β2AR antibody. Immunoblotting showed significant increase in β2AR phosphorylation following hypoxia [Fig. 4C & cumulative data right panel]. This was associated with HIF-1α stabilization [Fig. 4C & cumulative data right panel] showing hypoxic effects in the heart. As β1AR and β2AR are expressed in the heart, function of both β1 and β2AR were assessed by adenylyl cyclase activity. Adenylyl cyclase activity was significantly reduced at baseline and upon in vitro ISO (agonist for both β1 and β2AR) stimulation showing reduced G-protein coupling [Fig. 4D]. To further selectively assess for β1AR function, adenylyl cyclase activity was performed by using β1AR-selective agonist DOB after pre-treatment of the membranes with β2AR-specific antagonist ICI 181,551. Adenylyl cyclase activity was significantly reduced at baseline and upon DOB-stimulation [Supplementary Fig. 3E] upon hypoxia. To determine whether increased βAR phosphorylation upon hypoxia is associated with changes in β-agonist epinephrine (Epi) and/or norepinephrine (Norepi) (catecholamines) levels, catecholamines were measured in the plasma and the hearts. The differences in the catecholamine levels were not significant either in plasma or the cardiac lysates post-hypoxia compared to normoxia [Supplementary Fig. 3F & G]. These findings show that short term acute hypoxia causes adverse cardiac remodeling characterized by significant phosphorylation/dysfunction of βARs that is independent of agonist.

Hypoxia activates PI3Kγ to inhibit endosomal protein phosphatase 2A (PP2A):

As our studies in adult cardiomyocytes and mice show that hypoxia leads to phosphorylation of βARs, studies were performed to assess whether hypoxia selectively inhibits resensitization in the hearts. PM and endosomes were isolated from the hearts following hypoxia of the WT mice and PI3Kγ was immunoprecipitated and subjected to lipid kinase activity. Significant increase in lipid kinase activity was observed in endosomes compared to PM as measured by generation of phosphoinositide mono-phosphate (PIP) [Fig. 5A, cumulative data right panel]. To test whether activated PI3Kγ phosphorylates the endogenous I2PP2A, cardiac endosomal fractions were immunoblotted with anti-p-I2PP2A antibody. There was significant phosphorylation of I2PP2A with hypoxia compared to normoxia with no significant difference in total I2PP2A [Fig. 5B, left- upper and middle panels; cumulative data right panel]. Also, there was no difference in the level of PP2A at the cardiac endosomes following hypoxia [Fig. 5B, left- lower most panel]. To test whether βAR-associated PP2A activity is altered, alprenolol-conjugated beads were used to immunoprecipitated β1- and β2ARs and the beads were subjected to measurement of PP2A activity. There was significant decrease in βAR-associated PP2A activity following hypoxia [Fig. 5C] with no significant difference in co-immunoprecipitating PP2A [Fig. 5C, **lower panel**]. This shows that reduced PP2A activity underlies the endosomal enrichment of phosphorylated βARs.

PI3Kγ regulates cardiac function and HIF-1α stabilization following hypoxia:

Since activation of PI3Kγ in response to hypoxia mediates inhibition of βAR resensitization that may underlie cardiac dysfunction, PI3Kγ KO mice were subjected to hypoxia. Interestingly, echocardiography showed that cardiac function was preserved in PI3Kγ KO mice post-hypoxia [Fig. 6A, upper panel] as measured by %FS and %EF [Fig. 6A, cumulative data-lower panel] with no significant changes in HW/BW ratio [Supplementary Fig. 4A]. To further test whether the preservation of cardiac function is associated with altered βAR function, cardiac lysates were immunoblotted with anti-p-β2AR antibody. While significant β2AR phosphorylation was observed in WT upon hypoxia that was abrogated in the PI3Kγ KO despite hypoxia [Fig. 6B, upper top panel and lower left panel]. Unexpectedly, there was also significant reduction in accumulation of HIF-1α in the PI3Kγ KO mice compared to WT despite hypoxia [Fig. 6B, cumulative data-lower right panel]. Since hypoxia upregulates GRK2 in cells, GRK2 levels were assessed in PI3Kγ KO mice post-hypoxia. In contrast to WT type mice [Fig. 6B, cumulative data-lower middle panel], GRK2 levels did not increase in PI3Kγ KO mice despite hypoxia [Fig. 6B, cumulative data-middle panel]. As PI3Kγ KO mice had no significant changes in β2AR phosphorylation upon hypoxia, studies were performed to evaluate whether βAR resensitization is preserved in these mice. Cardiac endosomes were isolated from PI3Kγ KO mice following hypoxia and immunoblotted with anti-p-I2PP2A antibody. In contrast to the WT [Fig. 5B], there was no significant increase in I2PP2A phosphorylation in the PI3Kγ KO cardiac endosomes despite hypoxia [Fig. 6C, cumulative data-right panel]. Also, there was no difference in the total I2PP2A [Fig. 6C] or PP2A [Fig. 6C]. To test whether PP2A activity is preserved in PI3Kγ KO mice, β1- and β2ARs were immunoprecipitated with alprenolol-conjugated beads and associated PP2A activity was measured. There was

no inhibition of βAR-associated PP2A activity in the PI3Kγ KO cardiac endosomes [Supplementary Fig. 4B] compared to WT [Fig. 5C] following hypoxia. This suggests that βAR resensitization is preserved due to absence of PI3Kγ unlocking the inhibition of βAR-associated PP2A activity in the endosomes. To evaluate whether βAR resensitization is preserved in the PI3Kγ KO mice, cardiac endosomes were isolated after hypoxia and in vitro DOB (β1AR agonist) -stimulated adenylyl cyclase activity measured. Consistent with normalized endosomal PP2A activity, adenylyl cyclase activity was preserved in PI3Kγ KO cardiac endosomes even after hypoxia [Supplementary Fig. 4C]. Since hypoxia does not alter cardiac catecholamine levels, we determined whether hypoxia *per se* would alter βAR distribution. Radio-ligand binding studies were performed on cardiac PM and endosomes (Endo) from WT and PI3Kγ KO mice post-hypoxia. Interestingly, the βAR densities were not significantly different either at the PM or the endosomes following hypoxia compared to normoxia in both the WT and PI3Kγ KO mice [Supplementary Fig. 4D]. This supports the premise that hypoxia initiates non-canonical agonist-independent βAR dysfunction by inhibition of resensitization through impairment of PP2A activity.

To directly determine whether state of βAR phosphorylation is regulated by PI3Kγ in response to hypoxia, primary adult cardiomyocytes were isolated from WT and PI3Kγ KO mice and metabolic labeling was performed under hypoxia condition. β1- and β2AR were immunoprecipitated using alprenolol-conjugated beads and receptor phosphorylation assessed by autoradiography. Significant phosphorylation of βARs was observed in the WT adult cardiomyocytes upon hypoxia [Fig. 6D, cumulative data-right panel] which was significantly reduced in the PI3Kγ KO adult cardiomyocytes [Fig. 6D, cumulative data-right panel]. Ponceau staining was performed to assess input [Fig. 6D, left lower panel]. These data shows that PI3Kγ plays a key role in determining the state of βAR phosphorylation in response to hypoxia, and consequently cardiac dysfunction and HIF-1α stabilization.

PI3Kγ regulates acute cardiac function in response to hypoxia:

Since hypoxia causes βAR phosphorylation by inhibiting resensitization, we tested whether hypoxia alters cardiac functional outcomes to acute challenge with β1AR-agonist DOB. WT and PI3Kγ KO mice underwent echocardiography post -normoxia or -hypoxia and were administered DOB to evaluate acute real-time effects on cardiac function post-hypoxia. Consistent with our observation in Fig. 4A, cardiac dysfunction was observed in WT post-hypoxia [Fig. 7A, panel 2; Supplementary Fig. 5A & B], which was preserved in PI3Kγ KO mice [Fig. 7A, panel 6; Supplementary Fig. 5A & B]. WT or PI3Kγ KO mice subjected to normoxia responded to DOB stimulation by significantly increasing cardiac contraction [Fig. 7A, panel 3 or 7] as measured by %FS and %EF [Supplementary Fig. 5A & B]. DOB-mediated cardiac contraction was significantly impaired in WT mice post-hypoxia [Fig. 7A, panel 4 & Supplementary Fig. 5A & B] compared to WT normoxia mice [Fig. 7A, panel 3 & Supplementary Fig. 5A & B]. In contrast, PI3Kγ KO mice showed preserved DOB response despite hypoxia exposure [Fig. 7A, panel 8 & Supplementary Fig. 5A & B]. To compare the cardiac contractile response to DOB administration post-hypoxia, relative change in cardiac functional outcome was measured as a ratio of hypoxia (%EF or %FS) response to their respective normoxia (%EF or %FS) measurements. Evaluation of relative cardiac contractile function measured as a ratio of hypoxia/normoxia showed

significantly impaired responses in the WT mice compared to the PI3Kγ KO mice either in presence [Fig. 7B, dark grey circles] or the absence [Fig. 7B, light grey circles] of DOB. These observations show that efficient resensitization in the PI3Kγ KO mice results in normalized β1AR function despite hypoxia leading to preserved contractile response to DOB compared to WT mice. This suggests that acute hypoxia may pre-dispose heart towards deleterious outcomes as it may not be able to meet the acute metabolic demands of the body immediately post-acute hypoxia.

Discussion

Our studies show that hypoxia causes agonist-independent β1- and β2AR phosphorylation non-canonically regulating the receptor function that underlies cardiac dysfunction. Exposure of β2AR expressing HEK 293 cells or adult cardiomyocytes to hypoxia resulted in β1 and β2AR phosphorylation with reduced cAMP. Increased βAR phosphorylation was associated with GRK2 upregulation, HIF-1α stabilization with no changes in catecholamines reflecting agonist-independence. Along with minimal changes in the dynamics of β2AR internalization, hypoxia led to increased endosomal β2AR phosphorylation. This inhibition of β2AR-associated PP2A activity in the endosomes leads to impaired βAR resensitization in cells and the hearts. Hypoxia increases endosomal PI3Kγ activity resulting in elevated phosphorylation of its substrate I2PP2A [9]. Phosphorylated I2PP2A now binds to PP2A and inhibits PP2A activity impairing resensitization of βARs. Subjecting WT mice to acute hypoxia resulted in cardiac remodeling with significant β1- and β2AR phosphorylation and dysfunction as measured by reduced adenylyl cyclase activity. Consistently, cardiac function and β1- and β2AR phosphorylation was normalized in PI3Kγ KO mice with reduced HIF-1α accumulation despite hypoxia. Acute challenge of PI3Kγ KO mice with β1AR-selective agonist DOB resulted in preserved cardiac response post-hypoxia, which was impaired in the WT mice. This shows that acute hypoxia leads to activation of endosomal PI3Kγ that inhibits PP2A activity impairing βAR resensitization leading to phosphorylation of βARs in the endosomes. These studies show a yet to be appreciated role of PI3Kγ in regulating hypoxia responses including βAR function and HIF-1α stabilization.

Our findings show that in cellular as well as in vivo studies, acute hypoxia *per se* causes βAR phosphorylation independent of agonist as there were no changes in catecholamines. Increased βAR phosphorylation observed in our studies, in part explains the loss in G-protein coupling of βARs observed in the rat hearts following long term hypoxia [21, 22]. In this regard, our study shows that even short-term acute hypoxia is sufficient to cause molecular changes including βAR dysfunction that in part, could underlie cardiac remodeling. Hypoxia mediated phosphorylation of β2ARs was associated with GRK2 upregulation, but is not accompanied with changes in βAR density/distribution between plasma membrane and endosomes. This shows that hypoxia could mediate βAR phosphorylation in selective cellular compartments as reflected by endosomal phosphorylation of βARs by GRK2 which may by-pass the classical need of Gβγ subunits for recruitment as observed for intra-cellular substrates of GRK2 [14]. The increase in endosomal phosphorylation of β2ARs with minimal changes at the plasma membranes gives a snap-shot of early molecular events mediated by hypoxia.

It is known that endosomal βARs can be activated by agonist [23, 24] like intracellular βAR activation mediates calcium transients in cardiomyocytes [25]. This suggests that endosomal dephosphorylated βARs can be engaged by agonists resulting in measurable cellular events. Since hypoxia results in increased endosomal βAR phosphorylation, hypoxia could selectively inhibit endosomal receptor signaling. Although hypoxia leads to GRK2 upregulation and βAR phosphorylation, the accumulation of endosomal phosphorylated βARs shows impairment in the counter-balancing dephosphorylation mediated by PP2A. Indeed, selective inhibition of endosomal β2AR-associated PP2A activity was observed in our cellular and mouse studies with hypoxia. Our previous studies showed a role for βARs in HIF-1α stabilization [4] and the current study demonstrates that hypoxia engages intracellular βARs to mediate HIF-1α stabilization. More importantly this is associated adaptive response to hypoxia including cardiac remodeling.

Inhibition of βAR-associated PP2A activity upon hypoxia suggests that it impairs βAR resensitization leading to endosomal phosphorylated βARs. We have previously shown that PI3Kγ inhibits PP2A activity at the βAR complex as PI3Kγ is recruited to the agonist-activated β1- and β2AR. PI3Kγ now phosphorylates the inhibitor of PP2A (I2PP2A) that now binds to PP2A inhibiting its activity [9]. Hypoxia leads to loss in endosomal PP2A activity and is associated with increased PI3Kγ activity reflecting a role of PI3Kγ-I2PP2A axis in PP2A inhibition. The engagement of this pathway by hypoxia was identified by using: a) an in-house generated anti-phospho-I2PP2A antibody showing increased endosomal phosphorylation of I2PP2A to hypoxia, and b) the use of PI3Kγ KO mice that displayed preserved βAR and cardiac function despite hypoxia. In contrast, to the Gβγ-mediated recruitment of PI3Kγ [18], the selective increase in the endosomal PI3Kγ activity upon hypoxia suggests a Gβγ-independent non-canonical regulation of PI3Kγ. Thus, hypoxia activates PI3Kγ through, yet to be understood mechanisms that is outside the scope of the current studies.

Long term hypoxia leads to cardiac remodeling including cardiac hypertrophy and pulmonary hypertension [22, 26], while our studies on acute hypoxia shows mild, yet significant left ventricular remodeling. Importantly, short term hypoxia in C57BL/6 mice not only leads to HIF-1α stabilization reflecting a hypoxic milieu, but is also associated with acute inhibition of βAR resensitization. Importantly, this is reversed in the PI3Kγ KO mice as assessed by dobutamine challenge. This reflects that unlocking the inhibition of PP2A activity to restore βAR resensitization may provide short-term beneficial outcomes as PI3Kγ KO mice have higher PP2A activity [9, 17]. This suggests a key role for PI3Kγ in regulating βAR phosphorylation and HIF-1α stabilization consistent with our cellular studies showing the role of βAR phosphorylation for HIF-1α stabilization [27]. Additionally, there was pronounced cardiac dysfunction at baseline following hypoxia compared to DOB challenge in WT mice reflecting, yet to be determined role of PI3Kγ in regulating baseline cardiac function.

The unexpected observation that short term acute hypoxia leads to inhibition of resensitization impairing βAR function resulting in cardiac remodeling and dysfunction has significant clinical implications. Understanding the mechanistic underpinnings of cardiac dysfunction with acute hypoxia is important beyond ischemic heart disease. This is because

of increasing recognition that patients with chronic obstructive pulmonary disease (COPD) or sleep apnea develop cardiovascular complications [28, 29] but less is known about the underlying mechanisms. In this context, >50% of COPD patients experience nocturnal hypoxia which could directly affect βAR function and cardiac outcomes primarily through hypoxia which is different than ischemia. While, classically ischemia is associated with hypoxia, inflammation and loss in nutrients [28, 29], it is however, important to note that hypoxia would be the most instantaneous acute stress the heart would experience with ischemia. Therefore, signaling pathways activated by acute hypoxia *per se* could be critical early determinants of cardiac functional outcomes. In recognition, our study shows that acute hypoxia non-canonically activates PI3Kγ inhibiting PP2A activity impairing βAR resensitization. This underlies β1- and β2AR phosphorylation, HIF-1α accumulation [Fig. 7C] associated with cardiac remodeling. Thus, preserving βAR resensitization in acute hypoxia would maintain βAR signaling/cardiac function. This would permit the heart to meet the metabolic demands of body by reducing the pre-disposition to deleterious outcomes including stroke due to dysfunctional βARs [30].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Dr. Sadashiva Karnik for feedback and insightful thoughts during the development and progression of the project. These studies were, in part supported by NIH R01 HL089473, HL128382 and small instrumentation grant S10ODO21561 (SVNP).

Sources of Funding:

NIH: HL089473, HL128382, S10ODO2321561

Non-standard Abbreviations and Acronyms

βAR	beta-adrenergic receptor
PI3Kγ	phosphoinositide 3-kinase γ
HEK	human embryonic kidney
HIF-1α	hypoxia-inducible factor-1 alpha
GRK2	G-protein coupled receptor kinase 2
PP2A	protein phosphatase 2A
I2PP2A	inhibitor of PP2A
ISO	isoproterenol
FS	fractional shortening
EF	ejection fraction

PM	plasma membrane
Endo	Endosomes
Epi	Epinephrine
Norepi	Norepinephrine
DOB	Dobutamine
Nor	Normoxia
Hyp	Hypoxia

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Novelty and Significance

What is known?

1. Hypoxia is a key factor in ischemia contributing to heart failure and stroke, whereas impaired beta-adrenergic receptor (βAR) function can underlie deleterious cardiac events.
2. Although ischemia reduces βAR function, the mechanistic underpinnings for ischemia-mediated βAR dysfunction are not known.
3. Phosphoinositide 3-kinase γ (PI3Kγ) is traditionally activated by an agonist at the plasma membrane leading to decreased βAR resensitization through inhibition of protein phosphatase 2A (PP2A) activity.

What New Information Does This Article Contribute?

1. Acute hypoxia leads to agonist-independent βAR phosphorylation and endosomal accumulation of βARs with loss in adenylyl cyclase activity underlying cardiac remodeling.
2. Hypoxia activates PI3Kγ in an agonist-independent manner inhibiting endosomal βAR-associated PP2A activity impairing βAR resensitization.
3. In contrast to wild type (WT) control mice, PI3Kγ knockout (PI3Kγ KO) mice subjected to hypoxia showed reduced βAR phosphorylation and HIF-1α accumulation, and increased β2AR-associated PP2A activity accompanied by preserved cardiac function.
4. Challenging PI3Kγ KO mice with the β1AR-selective agonist dobutamine post-hypoxia resulted in a sustained cardiac response that was impaired in WT mice.

Hypoxia is the most proximate acute stress encountered by the heart during ischemia. βARs represented by β1 and β2ARs are critical regulators of cardiac function. Given the central role of β1 and β2ARs in cardiac function, signaling pathways activated by these receptors in response to acute hypoxia could be early determinants of cardiac functional outcomes. However, less is known about the mechanisms underlying hypoxia-mediated β1 and β2AR dysfunction that lead to deleterious cardiac remodeling. Acute hypoxia mediates agonist-independent β1 and β2AR phosphorylation and endosomal activation of PI3Kγ accompanied by deleterious cardiac remodeling. Endosomal activation of PI3Kγ inhibits β1 and β2AR-associated PP2A activity impairing βAR resensitization leading to accumulation of phosphorylated βARs. Exposure of PI3Kγ KO mice to acute hypoxia resulted in preserved cardiac function, reduced βAR phosphorylation associated with normalized β2AR-associated PP2A activity, uncovering a unique role for PI3Kγ in hypoxia sensing and cardiac dysfunction. Accordingly, challenging wild type mice post-hypoxia with dobutamine resulted in an impaired cardiac response that was normalized in PI3Kγ KO mice. Our study suggests that preserving βAR resensitization by targeting the PI3Kγ pathway would maintain βAR signaling and cardiac function, thereby permitting the heart to meet the metabolic demands of the body following ischemia.

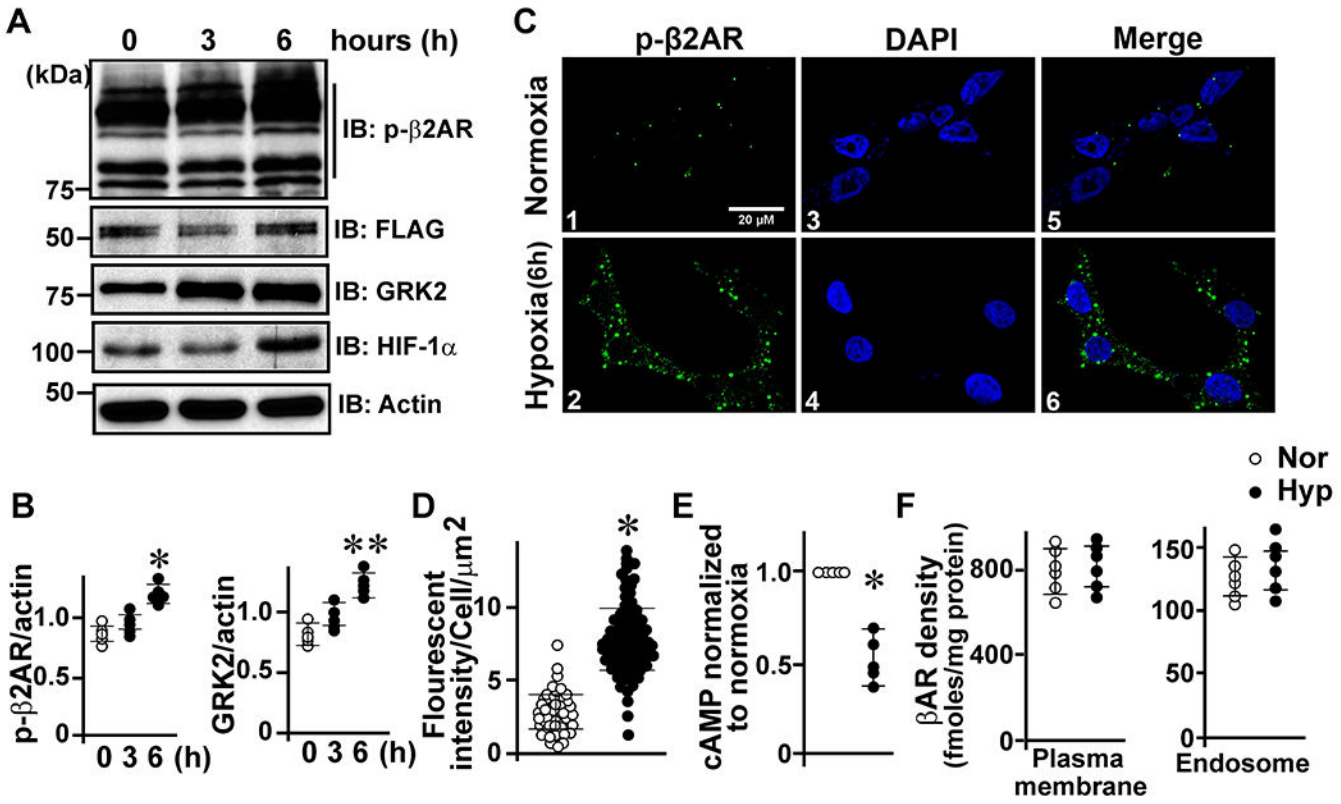

Figure 1 - Hypoxia leads to increased β2AR phosphorylation and dysfunction:

A, Total lysates (80 μg) from serum starved β2AR expressing HEK 293 cells following 0, 3 and 6 h of hypoxia (2% oxygen) were immunoblotted with anti-phospho-β2AR (p-β2AR) antibody (upper panel). The immunoblots were probed with anti-GRK2 and anti-HIF-1α (molecular surrogate for hypoxia) antibodies. FLAG and actin were used as loading controls (n=5). **B,** Data analyzed by Kruskal-Wallis test with Dunn's correction for multiple comparisons. *P=0.0016 or **P=0.0021 vs. 0 h (normoxia). **C,** Phosphorylation of β2ARs was visualized by confocal microscopy in the β2AR expressing HEK 293 cells stained with anti-p-β2AR antibody (green) after 6 h normoxia or hypoxia. Nucleus was visualized by DAPI (blue). Scale, 20 μm. **D,** Fluorescent intensity of phosphorylated β2ARs/cell. (n=4/~60-80 cells/experiment). Mann-Whitney test was performed on the data. *P=0.00213 vs. normoxia. **E,** cAMP levels were measured in β2AR expressing HEK 293 cells following 6 h of hypoxia or normoxia (n=5). *P=0.0079 vs normoxia (Mann-Whitney test). **F,** Plasma membrane and endosomes were isolated from β2AR expressing HEK 293 cells post-hypoxia or normoxia and were subjected to $^{[125]}$I-Cyanopindolol radio-ligand binding (n=6). Data analyzed by Mann-Whitney test. Not Significant (NS). Normoxia - Nor; Hypoxia - Hyp.

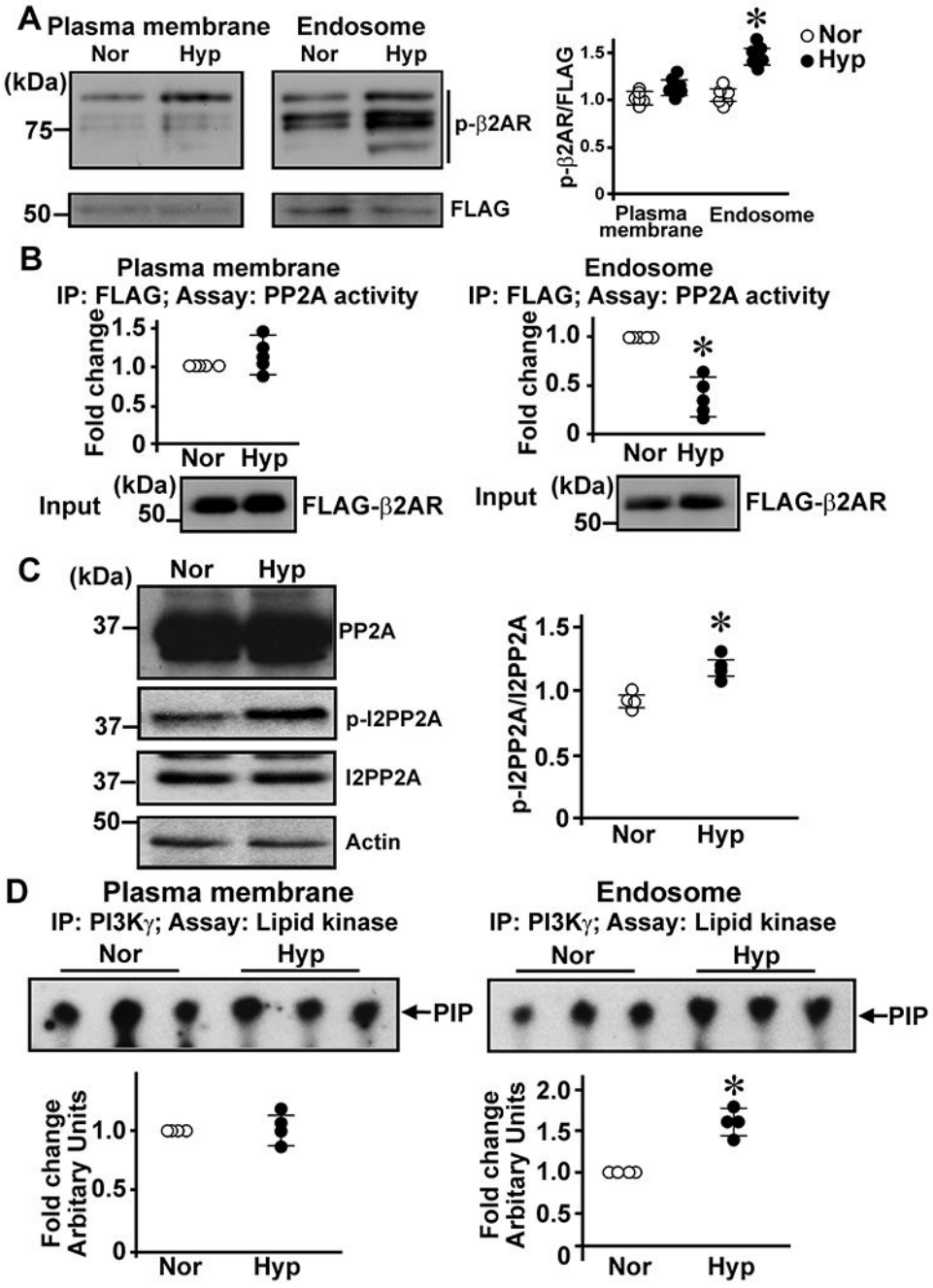

Figure 2 - Hypoxia activates PI3Kγ to inhibit endosomal β2AR-associated PP2A activity impairing β2AR resensitization:

A, Plasma membrane (50 μg) or endosomes (50 μg) from β2AR expressing HEK 293 cells were immunoblotted with anti-p-β2AR antibody and anti-FLAG following hypoxia (n=5). Data analyzed by Mann-Whitney test. *P=0.00835 vs normoxia. **B,** FLAG-β2AR was immunoprecipitated (IP) from plasma membrane (50 μg) (left) or endosomes (50 μg) (right) and associated PP2A activity measured in the FLAG immunoprecipitates (n=6). Plasma membrane, NS; Endosomes, *P=0.00212 vs. normoxia (analyzed by Mann-Whitney

test). Lower panel: FLAG input. **C,** Immunoblotting performed on total lysates following hypoxia to detect PP2A, phospho-I2PP2A (p-I2PP2A) and total I2PP2A. Actin was used as loading control. I2PP2A was used for normalization (n=4). Data analyzed by Mann-Whitney test. *P=0.03252 vs. normoxia. **D,** PI3Kγ was immunoprecipitated from plasma membrane or endosomes of β2AR expressing HEK 293 cells following hypoxia and subjected to in vitro lipid kinase activity and lipid products resolved on TLC plates (upper panel) (n=4). Data analyzed by Mann-Whitney test. Plasma membrane, NS; Endosomes, *P=0.01272 vs. normoxia.

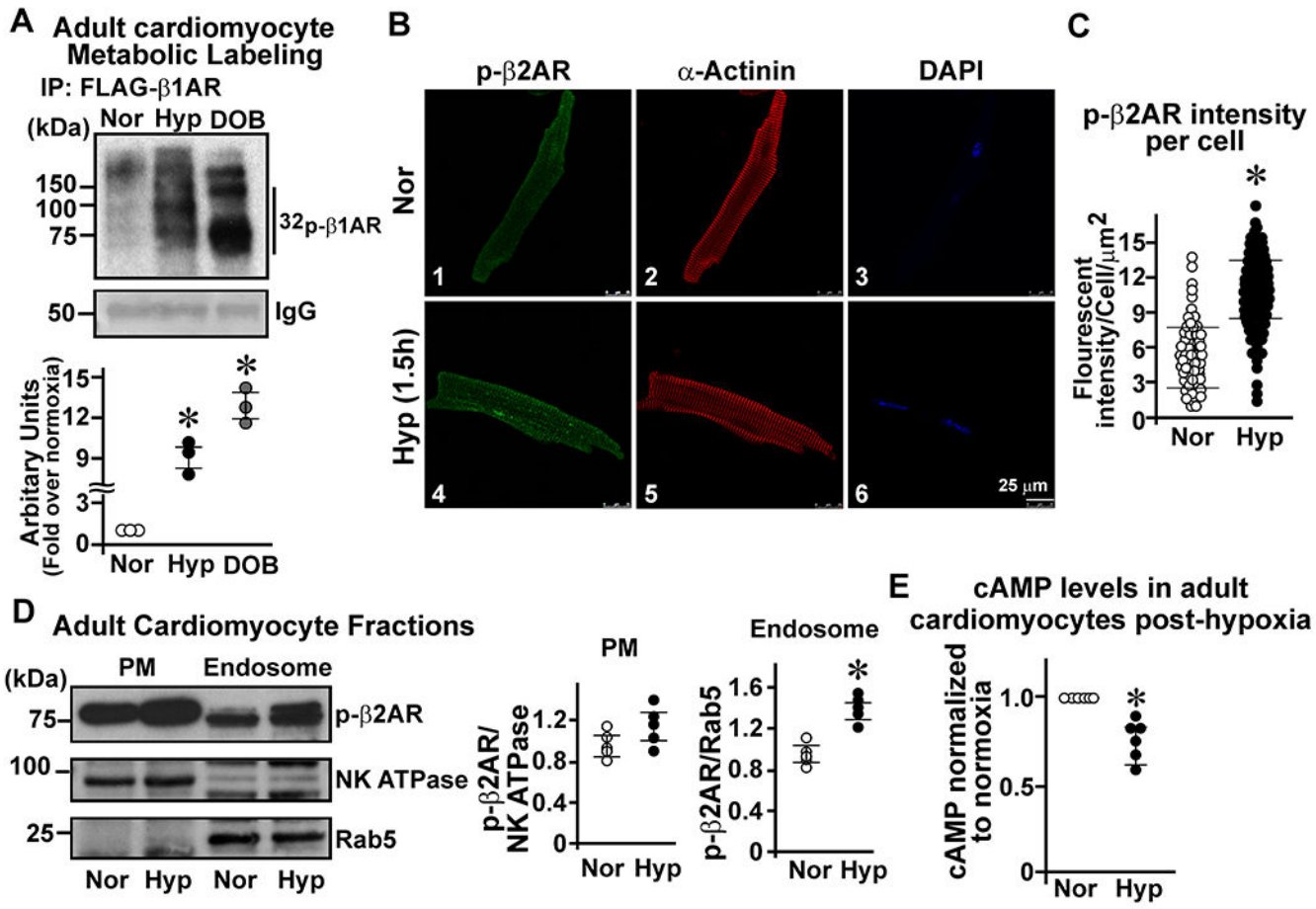

Figure 3- Hypoxia leads to β1 and β2AR phosphorylation in adult cardiomyocytes:

A, Adult cardiomyocytes isolated from mice with cardiomyocyte-specific overexpression of FLAG-β1AR were metabolically labeled with $^{[32]}$P and subjected to Normoxia or Hypoxia (1.5 h). Dobutamine (DOB) (10 μM) stimulation was used as a positive control. Labelled β1AR was evaluated by auto-radiography following immunoprecipitation of FLAG (n=3). *P=0.00112 vs. normoxia (Kruskal-Wallis test with Dunn's correction). **B,** Confocal microscopy of adult cardiomyocytes isolated from C57BL/6 (WT) mice stained with anti-p-β2AR antibody (green) or anti-α-actinin antibody (red) after 1.5-hours of Normoxia or Hypoxia. Nucleus was visualized by DAPI (blue) staining. Scale, 25 μm. **C,** Fluorescent intensity assessment (n=4,~30-50 cells/experiment). Data analyzed by Mann-Whitney test. *P=0.00512 vs. normoxia. **D,** Plasma membrane (PM) and endosomes isolated from Normoxia or Hypoxia treated WT adult cardiomyocytes were immunoblotted with anti-p-β2AR antibody. Na^+K^+ ATPase was used as PM marker and Rab5 as an endosomal marker (n=5). Data was analyzed by Mann-Whitney test. Plasma membrane, NS; Endosomes, *P=0.03165 vs. normoxia. **E,** cAMP was measured in the primary adult cardiomyocytes from WT mice after Hypoxia and normalized to Normoxia (n=6). Data was analyzed by Mann-Whitney test. *P=0.0022 vs normoxia.

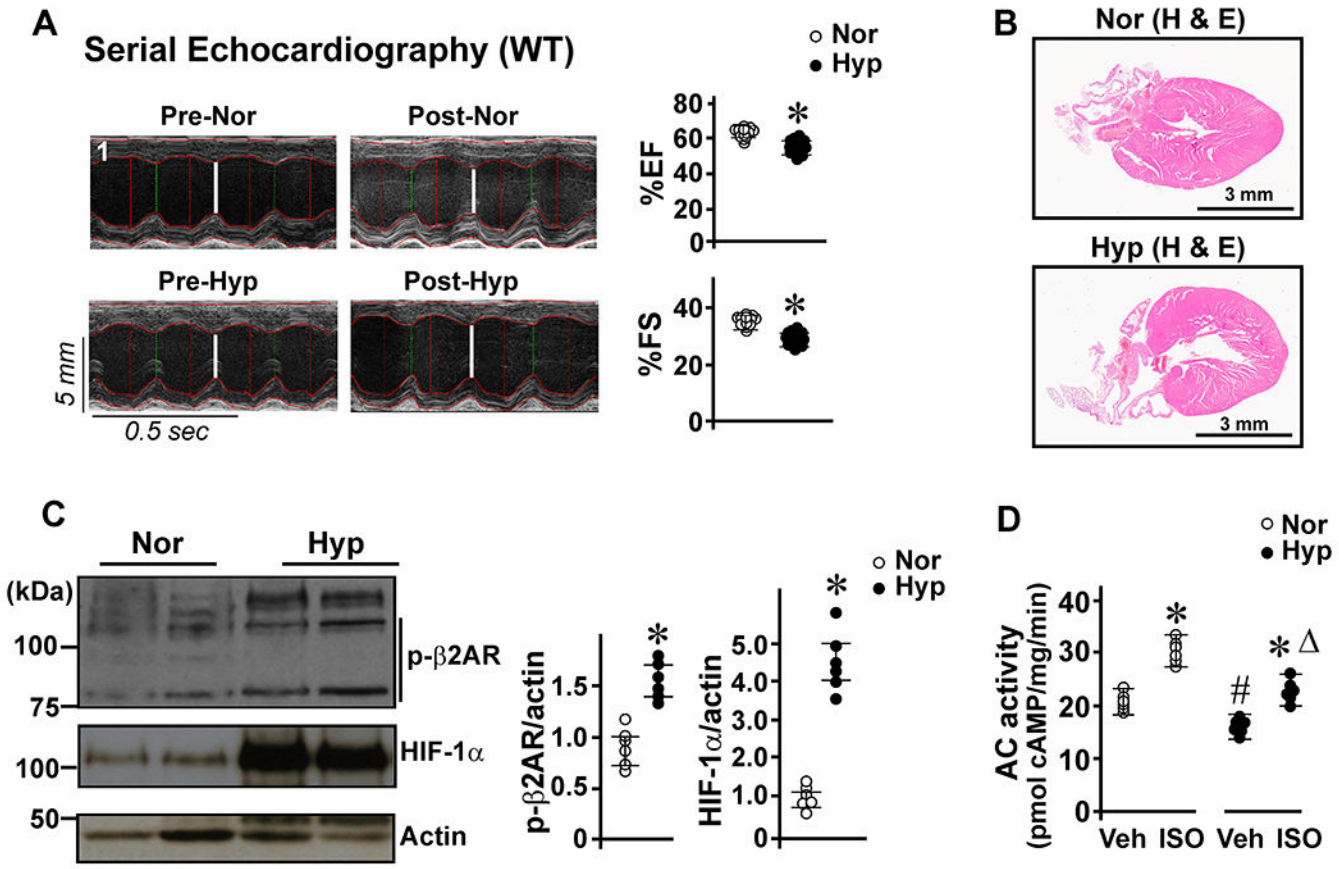

Figure 4- Acute hypobaric hypoxia in WT mice leads to adverse cardiac remodeling associated with βAR dysfunction:

A, M-mode echocardiography was performed pre- and post-Hypoxia (20 h) or Normoxia, (n=12) (left 4 panels). Mann-Whitney test was performed on the two independent data sets (Hypoxia vs. Normoxia). % ejection fraction (%EF), *P=0.009 vs. Normoxia; % fractional shortening (%FS), *P=0.0036 vs. Normoxia. **B,** Heart sections from mice subjected to Normoxia or Hypoxia were stained with H & E to evaluate remodeling (n=4). Scale bar (3 mm). **C,** Cardiac lysates (100 μg) were immunoblotted with anti-p-β2AR antibody (upper panel). The blots were probed with anti-HIF-1α (middle) antibody. Actin was used as a loading control, (n=6). Data analyzed by Mann-Whitney test. *P=0.006 vs. normoxia (p-β2AR); *P=0.00121 vs. normoxia (HIF-1α). **D,** In vitro ISO-stimulated adenylyl cyclase activity measured in the cardiac membranes following Hypoxia or Normoxia (n=6). Data was analyzed using Kruskal-Wallis test with Dunn's correction for multiple comparisons. P values were calculated by two tailed test. *P=0.0327 vs basal (Normoxia and Hypoxia); $^{\#}$P= 0.0452 vs. basal normoxia; $^{\Delta}$P= 0.0312 vs. ISO normoxia.

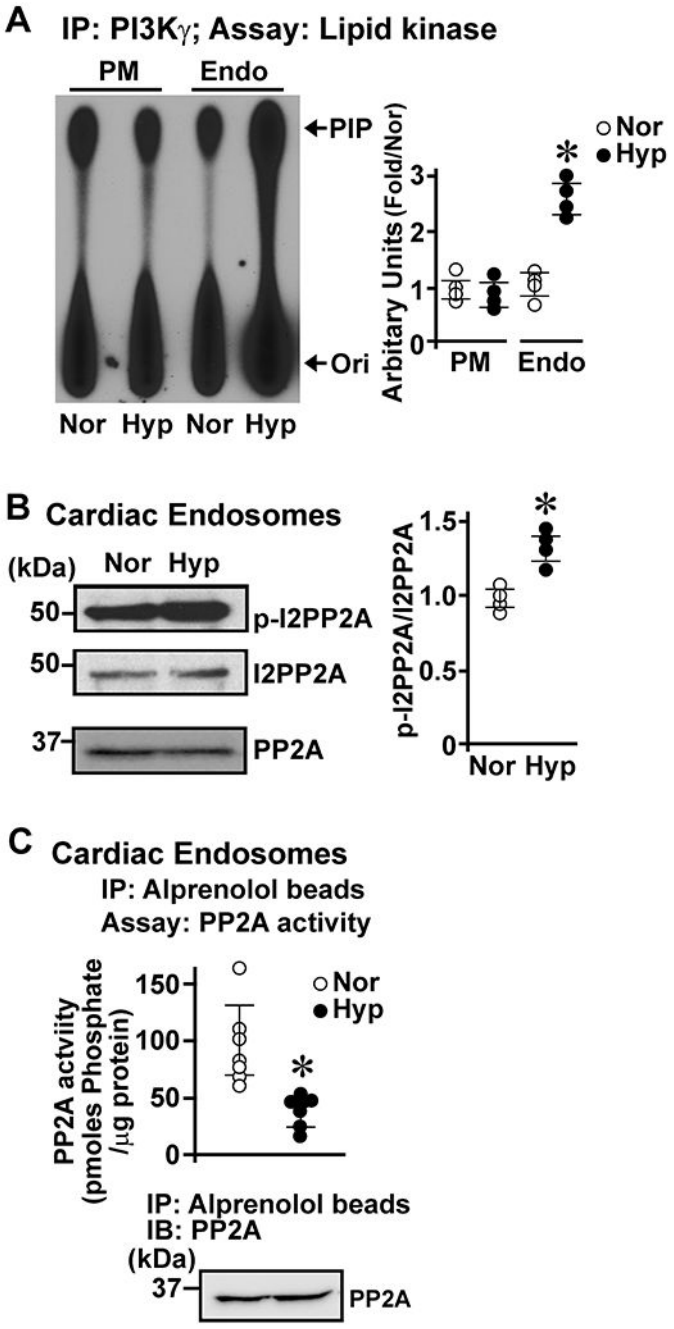

Figure 5 - Hypoxia in WT mice activates cardiac endosomal PI3Kγ accompanied by inhibition of βAR-associated PP2A activity:

A, PI3Kγ was immunoprecipitated from plasma membrane (PM) or endosomes (Endo) isolated from WT hearts post-normoxia or -hypoxia and immunoprecipitates subjected to lipid kinase activity (n=4). Data was analyzed by Kruskal-Wallis test with Dunn's correction for multiple comparison. *P=0.0062 vs normoxia (PM or Endo) and Hypoxia (PM). **B,** Immunoblotting performed on cardiac endosomes following hypoxia to assess phospho-I2PP2A (p-I2PP2A), total I2PP2A and PP2A (n=4). *P=0.00852 vs. normoxia

(data analyzed by Mann-Whitney test). **C,** To evaluate endogenous βAR-associated PP2A activity, alprenolol-conjugated beads were used to immunoprecipitate endosomal βARs and subjected to malachite green PP2A activity assay (n=7). Data was analyzed by Mann-Whitney test.*P=0.0165 vs. normoxia. Lower panel: Immunoblotting for co-immunoprecipitating PP2A with alprenolol beads.

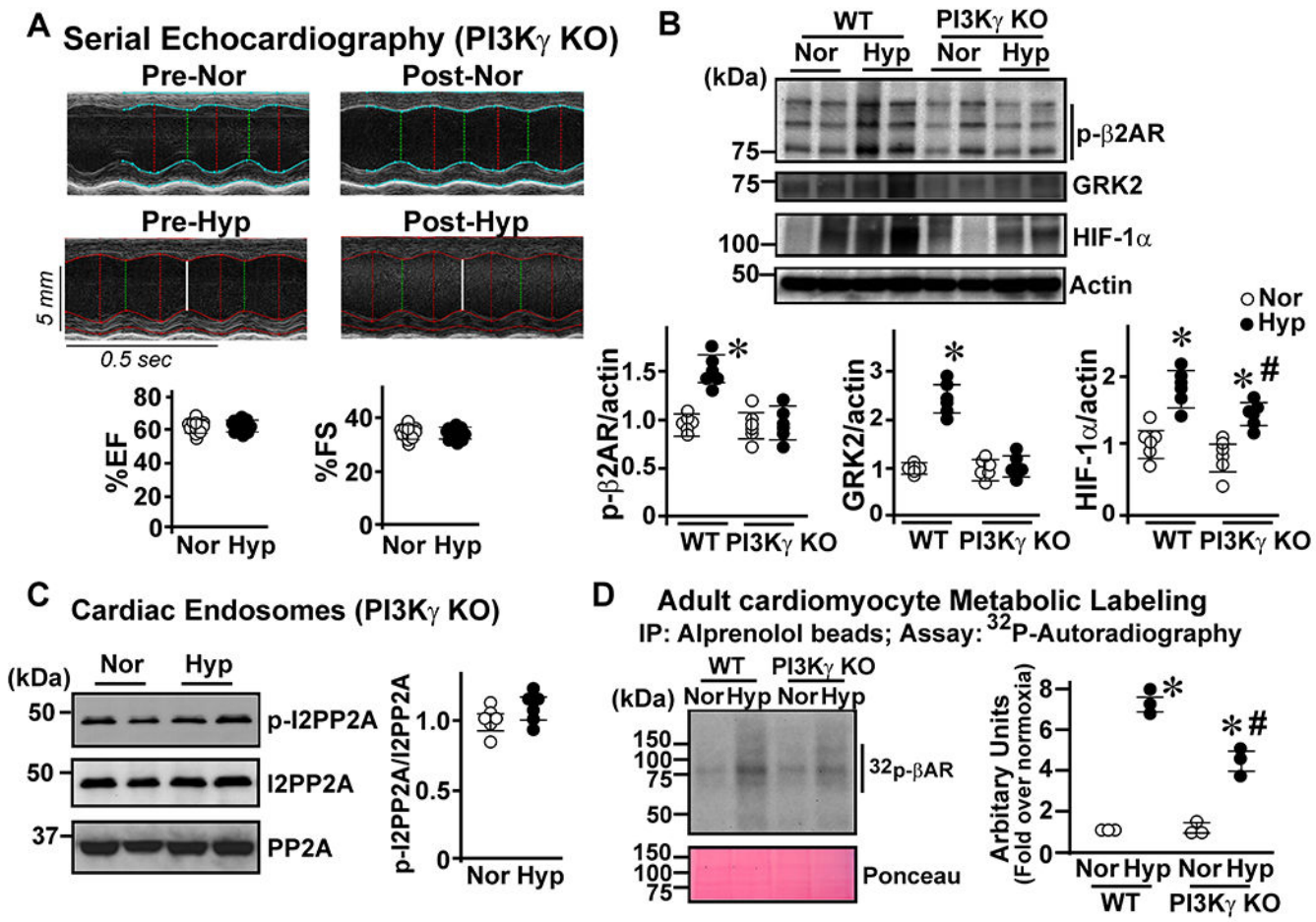

Figure 6 - PI3Kγ regulates hypoxia-mediated β2AR phosphorylation and HIF-1α stabilization:

A, M-mode echocardiography was performed pre- and post- normoxia or hypoxia on PI3Kγ KO mice. Lower panels: Cumulative data (n=12); %EF (left) and %FS (right). **B,** Cardiac lysates (100 μg) were immunoblotted with anti-p-β2AR antibody, anti-GRK2 and anti-HIF-1α. Actin was used as loading control (n=6). Data was analyzed by Kruskal-Wallis test with Dunn's correction for multiple comparison. p-β2AR (left panel, *P=0.0126 vs. WT normoxia and PI3Kγ KO (normoxia or hypoxia); GRK2 (middle panel, *P=0.00512 vs. WT normoxia and PI3Kγ KO (Normoxia or Hypoxia), and for HIF-1α (right panel, *P=0.0076 vs. normoxia; #P=0.0374 vs. WT hypoxia). **C,** Immunoblotting performed on cardiac endosomes following hypoxia to evaluate p-I2PP2A, total I2PP2A and PP2A, (n=6). **D,** Primary adult cardiomyocytes from WT or PI3Kγ KO mice underwent [32]P-metabolic labeling followed by hypoxia. Endogenous βARs were immunoprecipitated using alprenolol-conjugated beads and βAR phosphorylation assessed by autoradiography (n=3). Data was analyzed by Kruskal-Wallis test with Dunn's correction. *P=0.00312 vs. respective normoxia; #P=0.0081 vs. WT hypoxia. P

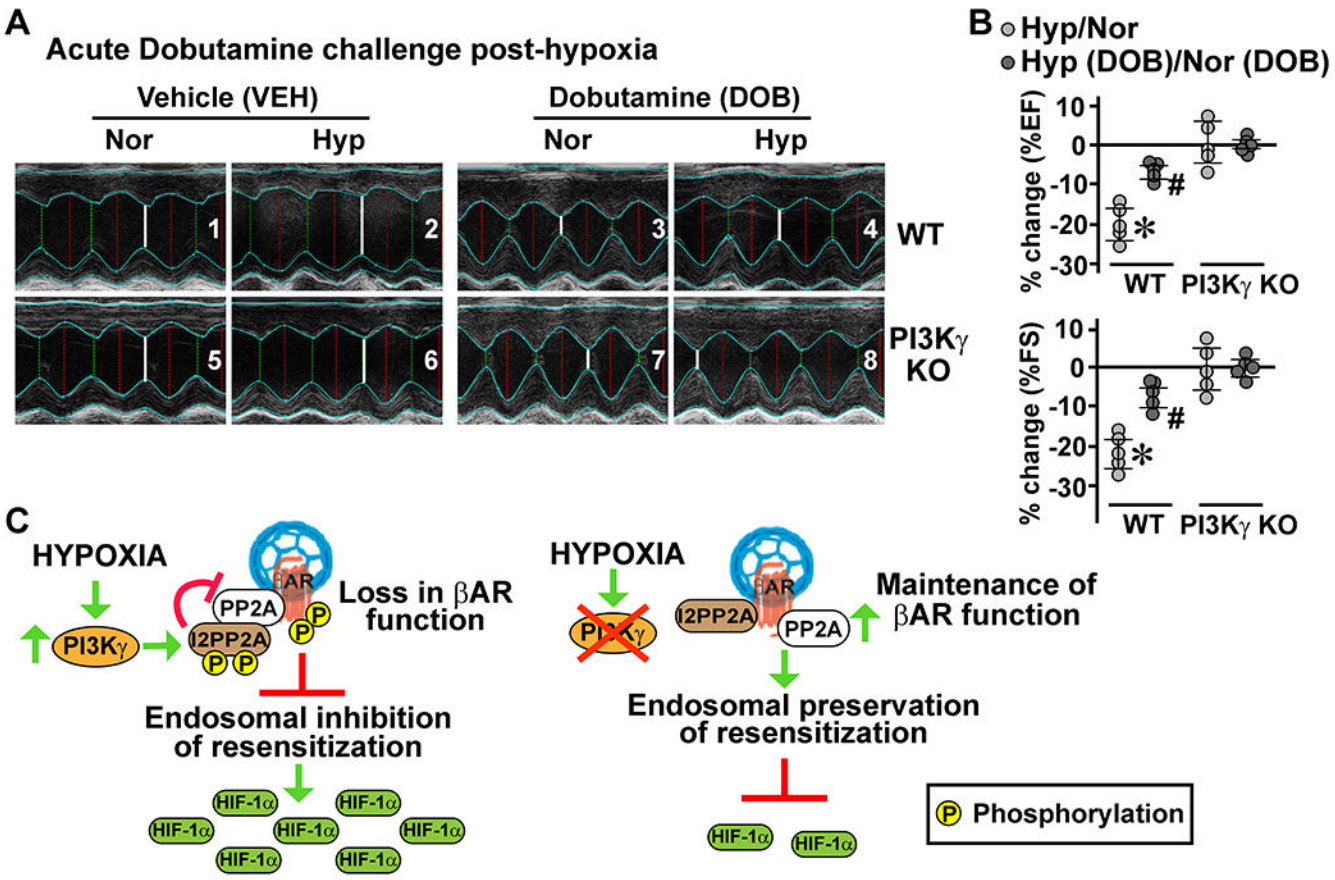

Figure 7 - PI3Kγ regulates acute cardiac response post-hypoxia:

A, M-mode echocardiography was performed pre- and post-hypoxia on WT or PI3Kγ KO mice. To test whether hypoxia alters acute cardiac response, WT or PI3Kγ KO mice were challenged with Dobutamine (DOB) or Vehicle (VEH) (saline) and cardiac functional response assessed by M-mode echocardiography (n=5). **B,** Upper Panel- relative change in cardiac function as measured by ratio of %EF in hypoxia over their respective normoxia values in WT or PI3Kγ KO. Data was analyzed by Kruskal-Wallis test with Dunn's correction with multiple comparisons Light grey circle: Hypoxia/Normoxia for WT or PI3Kγ KO. *P=0.008 vs. Hypoxia/Normoxia PI3Kγ KO. Dark grey circle: Hypoxia (DOB)/Normoxia (DOB) for WT or PI3Kγ KO. #P=0.0046 vs. Hypoxia (DOB)/Normoxia (DOB) PI3Kγ KO. Lower panel- ratio of %FS in hypoxia over respective normoxia values in WT or PI3Kγ KO. Light grey: Hypoxia/Normoxia for WT or PI3Kγ KO. *P=0.0116 vs. Hypoxia/Normoxia PI3Kγ KO. Dark grey: Hypoxia (DOB)/Normoxia (DOB) for WT or PI3Kγ KO. #P=0.0168 vs. Hypoxia (DOB)/Normoxia (DOB) PI3Kγ KO. **C,** Schematic illustration: Our studies show that hypoxia non-canonically activates endosomal PI3Kγ leading to increased endosomal βAR phosphorylation associated with HIF-1α stabilization. Elevated PI3Kγ inhibits endosomal PP2A activity through phosphorylation of I2PP2A impairing βAR resensitization. Studies with PI3Kγ KO mice show that ablation of PI3Kγ results in preservation of PP2A activity leading to efficient βAR dephosphorylation maintaining resensitization. However, it also unexpectedly leads to loss in HIF-1α accumulation. This

suggests that cardiomyocytes initiate a dichotomous response to hypoxia, wherein activation of beneficial HIF-1α pathway is associated with loss in βAR function by enhancing endosomal phosphorylation. Therefore, understanding the mechanistic underpinnings of HIF-1α stabilization to βAR phosphorylation could provide insight into developing therapeutics that allow for selective HIF-1α stabilization, while simultaneously maintaining βAR function to preserve cardiac outcomes with acute hypoxia.