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Enhanced Phosphoinositide 3-Kinase (p110α) Activity Prevents Diabetes–Induced Cardiomyopathy and Superoxide Generation in a Mouse Model of Diabetes

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Abstract

Aims/hypothesis: Diabetic cardiomyopathy is characterized by diastolic dysfunction, oxidative stress, fibrosis, apoptosis and pathological cardiomyocyte hypertrophy. Phosphoinositide 3-kinase[PI3K(p110α)] is a cardioprotective kinase but its role in the diabetic heart is unknown. The aim of this study was to assess whether PI3K(p110α) plays a critical role in the induction of diabetic cardiomyopathy, and whether increasing PI3K(p110α) activity in the heart can prevent the development of cardiac dysfunction in a setting of diabetes. Methods: Type 1 diabetes was induced with streptozotocin in adult male cardiac-specific transgenic mice with increased PI3K(p110α) activity [constitutively active PI3K(p110α), caPI3K], decreased PI3K(p110α) activity [dominant negative PI3K(p110α), dnPI3K] and non-transgenic(Ntg) mice for twelve weeks. Cardiac function, histological and molecular analyses were performed. Results: Diabetic Ntg mice displayed diastolic dysfunction, increased cardiomyocyte size, increased expression of atrial and B-type natriuretic peptides(ANP, BNP), fibrosis, apoptosis, as well as increased superoxide generation, and increased protein kinase C β2(PKCβ2), p22phox and apoptosis signal-regulating kinase 1(ASK1) expression. Diabetic dnPI3K mice displayed an exaggerated cardiomyopathy phenotype compared with diabetic Ntg mice. In contrast, diabetic caPI3K mice were protected against diastolic dysfunction, pathological cardiomyocyte hypertrophy, fibrosis, and apoptosis. Protection in diabetic caPI3K mice was associated with attenuation of LV superoxide generation, attenuated ANP, BNP, PKCβ2, ASK1 and p22phox expression, and elevated Akt. Further, in cardiomyocyte-like cells, increased PI3K(p110α) activity suppressed high glucose-induced superoxide generation and enhanced mitochondrial function. Conclusions/interpretation: These results demonstrate that reduced PI3K activity accelerates the development of diabetic cardiomyopathy, and that enhanced PI3K(p110α) activity can prevent adverse cardiac remodeling and dysfunction in a setting of diabetes.

Keywords: PI3K; left ventricular function; fibrosis; hypertrophy; myocardium; diabetes; NADPH oxidase, reactive oxygen species, PKCβ2.
**Abbreviations**

ANP    atrial natriuretic peptide
ASK1   apoptosis signal-regulating kinase 1
BNP    B-type natriuretic peptide
dP/dt<sub>max</sub>, dP/dt<sub>min</sub> maximal rates of rise and fall of LV pressures
E/A    ratio of initial (E) and second (A) blood flow velocities
FS     fractional shortening
GHB    glycated haemoglobin
H&E    hematoxylin and eosin
HG     high glucose
HW     heart weight
IVRT   isovolumic relaxation time
LV     left ventricular
LVEDP  LV end-diastolic pressure
LV PW  LV posterior wall thickness
LVSP   LV systolic pressure
NRVM   neonatal rat ventricular myocytes
PI3K(p110α) phosphoinositide 3-kinase, p110α isoform
PKCβ2  protein kinase C, β2 isoform
ROS    reactive oxygen species
STZ    streptozotocin
TFAM   mitochondrial transcription factor A
TL     tibial length
UCP3   uncoupling protein 3
Introduction

The incidence of diabetes is rising globally and is predicted to affect 439-472 million adults by 2030[1, 2]. Diabetes is associated with increased mortality and morbidity, attributed largely to cardiovascular and kidney disease[3, 4]. Diabetic cardiomyopathy, both independent of and exacerbated by co-existent atherosclerosis, coronary disease and renal dysfunction, is characterized by structural and functional remodeling, including diastolic dysfunction, fibrosis, apoptosis and pathological cardiomyocyte hypertrophy[3, 4]. Oxidative stress, an imbalance between reactive oxygen species(ROS) generation and endogenous antioxidant capacity, is triggered in cardiomyocytes by hyperglycemia. This contributes to the development and progression of diabetic cardiomyopathy[5-8]. The superoxide generating enzyme NADPH oxidase is considered a major source of ROS in the heart[9].

Current anti-diabetic agents do not consistently reduce cardiovascular risk, and some may even increase it[10, 11]. Thus, new therapeutic strategies to protect the heart in a setting of diabetes are greatly needed. We have previously demonstrated that increased activation of phosphoinositide 3-kinase[PI3K(p110α)] in the heart is protective in pathological settings in which the heart is subjected to a localized cardiac insult such as aortic-constriction (pressure overload) or coronary artery ligation (myocardial infarction)[12-14]. However, whether PI3K(p110α) could protect the heart against diabetic cardiomyopathy due to global hyperglycemia was unknown. Furthermore, PI3K has previously been linked to ROS inhibition in the kidney[15] but whether PI3K could regulate cardiac ROS had not been elucidated.

Multiple signaling proteins/cascades are dysregulated in the diabetic heart, including ERK1, STAT3 and Akt(key downstream target of PI3K)[4, 16-19]. A critical step in developing better therapeutics is to understand which signaling events represent key mechanisms in the development of cardiomyopathy in a setting of hyperglycemia. The goal of the current study was to assess whether PI3K(p110α) plays a critical role in the induction of diabetic cardiomyopathy, and whether
increasing PI3K(p110α) activity in the mouse heart prevents the development of diabetes-induced left ventricular (LV) remodeling and dysfunction.

Methods

Mouse models-The Alfred Medical Research and Education Precinct Animal Ethics Committee approved animal care and experimentation. Cardiomyocyte-specific transgenic mice (Tg, FVB/N background) with increased PI3K(p110α) activity [constitutively active PI3K(p110α), caPI3K] or decreased PI3K(p110α) activity [dominant negative PI3K(p110α), dnPI3K] were originally generated and provided by S Izumo (BIDMC, Boston, USA)[20]. caPI3K-Tg have elevated cardiac PI3K(p110α) activity, normal cardiac function and hearts that are 20% larger (physiological hypertrophy) than non-transgenic (Ntg)[20]. dnPI3K-Tg have reduced cardiac PI3K(p110α) activity, normal cardiac function, and hearts that are 20% smaller than Ntg.

Male caPI3K-Tg, dnPI3K-Tg and Ntg were randomly allocated into diabetic and non-diabetic groups. At 6-7 weeks of age, mice received five consecutive daily intraperitoneal injections of streptozotocin (STZ, 55mg/kg body weight, in 0.1mol/l citrate buffer, pH 4.5; Sigma Aldrich, St Louis MO) to induce diabetes or five consecutive daily intraperitoneal injections of citrate buffer vehicle of equivalent volume (non-diabetic group)[21]. Diabetes was confirmed by measurements of blood glucose every two weeks from saphenous vein whole blood using a glucometer (ACCU-CHEK Advantage; Roche, Basel, Switzerland). The upper limit of detection for blood glucose readings was 33.3mmol/L; readings at this level were entered as 33.3mmol/L and confirmed by subsequent submandibular bleed from conscious mice for assessment of final plasma glucose (Austin Pathology Service, Heidelberg, Vic). Mice with blood glucose levels exceeding 28mmol/L were considered diabetic. Diabetes progressed untreated for 12 weeks. At the end-point of the study (≈18-19 weeks of age) whole blood was collected by cardiac puncture after cardiac catheterization for analysis of glycated haemoglobin (GHB; Austin Pathology Service).
LV function-Echocardiography (two-dimensional M-mode and Doppler flow) was performed in anesthetized mice (ketamine/xylazine/atropine: 100/10/1.2 mg/kg i.p.) prior to catheterization and mouse dissection (~18-19 weeks of age) utilizing a Philips iE33 ultrasound machine with a 15MHz linear array transducer as described [21]. LV posterior wall thickness (LVPW), LV chamber dimensions and fractional shortening (FS) were assessed. LV filling was assessed on transmitral Doppler echocardiography, on the ratio of initial (E) and second (A) blood flow velocities (E/A ratio), E velocity deceleration time, and isovolumic relaxation time (IVRT).

Hemodynamic parameters were examined after 12 weeks of diabetes by catheterization. Arterial pressures, LV systolic pressure (LVSP), LV end-diastolic pressure (LVEDP), maximal rates of rise and fall of LV pressures (dP/dt_max, dP/dt_min) and heart rate were measured in anesthetized mice (ketamine/xylazine/atropine: 100/10/1.2 mg/kg i.p.) using a 1.4-Fr Millar MIKRO-TIP catheter and a Powerlab System (ADInstruments) [21].

Tissue collection, histological and immunohistochemical analyses-Following cardiac catheterization, heart, lung and tibia were collected [21]. Ventricular sections (4 μm) were stained with hematoxylin and eosin (H&E) for assessment of cardiomyocyte width, or 0.1% picrosirius red for assessment of collagen deposition as described [21]. Apoptosis was detected in paraffin-embedded ventricular sections (4 μm) by TUNEL staining (CardioTACS™ In Situ Apoptosis Detection Kit, Trevigen, Maryland, USA). Apoptotic nuclei stained blue and were quantitated as a percentage of non-apoptotic nuclei (counter-stained red with nuclear fast red) and expressed as a fold-change of non-diabetic Ntg.

For immunohistochemical examination of p22^phox, paraffin-embedded ventricular sections (4 μm) were deparaffinized, rehydrated and antigen retrieval was induced by heat (100°C) in a citrate buffer (10mmol/l citric acid, 0.05% Tween-20, pH 6.0). Endogenous peroxidase activity was quenched [3%H_2O_2(v/v)], sections blocked (15 μl/ml normal goat serum in 1%BSA), and
stained with p22phox (Santa Cruz Biotechnology Inc: sc-20781, 1:100) overnight followed by biotinylated secondary goat α-rabbit IgG (Vector Laboratories, 1:200). After exposure to VECTASTAIN® Elite ABC avidin-biotinylated horseradish peroxidase complex (Vector Laboratories, CA, USA) the chromogenic reaction was carried out with Sigma-FAST® diaminobenzidine tablets (Sigma-Aldrich). Sections were counterstained with hematoxylin. Positive p22phox stained brown and was graded by a blinded observer as follows: score 0-negative stain, score 1-weak, score 2-moderate, score 3-strong/intense.

*Superoxide generation*- Generation of superoxide was assessed in fresh LV tissue, neonatal rat ventricular myocytes (NRVM) or H9c2 cells using lucigenin-enhanced chemiluminescence, as described[22]. Primary NRVM [22] or H9c2 cells (Cell Bank Australia) were plated for 72h or 18h respectively, in 96 well Optiview plates (50,000 & 5000 cells/well, respectively, 37°C/5%CO2), serum-deprived for ~18h, pre-treated with/without a specific PI3K (p110α) inhibitor (A66[23], 1μmol/l), IGF1 (10nmol/l), and/or tempol (100μmol/l) for 24h prior to 24h treatment with/without H2O2 (100μmol/l) in high glucose (HG, 25mmol/l) or low glucose (LG, 5.56mmol/l) media as specified.

*Mitochondrial function*- H9c2 cells were seeded into Seahorse plates (10,000 cells/well in HG: 25mmol/l), serum-deprived overnight, treated with IGF1 (10nmol/l), and/or A66 (10μmol/l) for 24h, and mitochondrial function assessed (Seahorse XF analyser). Following basal respiration measurements, cells were sequentially treated with (all 1μmol/l): oligomycin (ATP synthase inhibitor), carbonylcyanide p-trifluoromethoxyphenylhydrazone (FCCP, proton ionophore) and antimycin A/rotenone combination injection (inhibitors of complex I and III), and changes in respiration recorded. Treatments were analysed as ten replicates, over two independent experiments (plates) and data pooled to give average values for each treatment. Basal respiration, proton leak,
ATP turnover, spare respiratory capacity and maximal respiratory capacity were calculated from these analyses.

*RNA and protein extraction*-RNA and protein were extracted from frozen ventricle samples as previously described[21].

*Northern Blot Analysis*—Northern blot analysis was performed as reported[20]. Total RNA(10μg) was electrophoresed in 1.3% denaturing formaldehyde-agarose gels and blotted onto Hybond™-N membranes(Amersham Biosciences). Membranes were probed with atrial natriuretic peptide(*Anp*), B-type natriuretic peptide(*Bnp*), *p22phox*, apoptosis signal-regulating kinase 1(*Ask1*), uncoupling protein 3(*Ucp3*), *Pim1*, mitochondrial transcription factor A(*Tfam*), and *Gapdh*-radiolabeled probes. Details of Northern probes are presented in electronic supplementary material.

*Western blotting*—For pAKT, blots were probed with anti-phospho AKT antibody(Ser473, Cell Signaling:9271,1:500) followed by anti-AKT antibody(Cell Signaling:9272,1:1000). For BAX and BCL2, blots were probed with anti-BAX antibody(Cell Signaling:2772,1:1000) followed by anti-BCL2 antibody(Cell Signaling:2876,1:1000). For protein kinase C β2(*PKCβ2*) and UCP3, blots were probed with anti-PKCβ2(Santa Cruz Biotechnology Inc., sc-210,1:200) and anti-UCP3 antibody(Affinity BioReagents:PA1-065,1:1000), both followed by anti-GAPDH antibody(Santa Cruz Biotechnology Inc., sc-32233,1:5000).

*Statistical analysis*-Results are presented as mean±standard error. Differences between groups were compared using one-way ANOVA followed by the Fisher’s protected least significant difference, unless otherwise specified. P<0.05 was considered significant.
Results

**Induction of diabetes in Ntg and PI3K Tg:** STZ induced a comparable degree of diabetes in Ntg, dnPI3K and caPI3K mice. Blood glucose, plasma glucose and GHB levels increased to a similar extent in STZ-treated mice from each genotype compared with citrate treated non-diabetic controls (Table 1). No genotype-dependent differences in glycemia, body weight or tibial length(TL) were observed (Table 1).

**Increased PI3K(p110α) activity prevents the diabetes-induced increase in cardiomyocyte size, fibrosis and apoptosis:** Cardiomyocyte hypertrophy, fibrosis and apoptosis all contribute to the pathogenesis and progression of diabetic cardiomyopathy[24, 25]. Induction of diabetes in Ntg mice for 12 weeks had no impact on heart weight (see HW/TL, Table 1) but was associated with increased cardiomyocyte width(≈13%; Fig 1a-c), increased *Anp* and *Bnp* cardiac gene expression(markers of pathological hypertrophy and/or cardiac stress; Fig 1d), cardiac fibrosis (Fig 2a), and increased apoptosis (Fig 2b). A diabetes-induced increase in cardiomyocyte size in the absence of a parallel increase in heart size is consistent with previous findings[21], and can be attributed to increased cell death in the diabetic heart (Fig 2b)[26].

Under basal/non-diabetic conditions, caPI3K-Tg displayed physiological cardiac hypertrophy associated with larger cardiomyocytes compared to Ntg, but no increase in *Anp* or *Bnp* expression as previously reported [20](Table 1, see HW, HW/TL; Fig 1a,b&d). In contrast, non-diabetic dnPI3K-Tg had smaller hearts, smaller cardiomyocytes, and elevated *Anp* and *Bnp* expression[20](Table 1, Fig 1a,b&d). Neither caPI3K-Tg or dnPI3K-Tg showed evidence of fibrosis or apoptosis under basal/non-diabetic conditions (Fig 2a&b). Diabetes had no effect on HW/TL in caPI3K-Tg or dnPI3K-Tg (Table 1). However, PI3K activity had a significant impact on cardiomyocyte size, fibrosis and apoptosis in a setting of diabetes that differed from diabetic Ntg. Expression of the dnPI3K transgene was associated with an exaggerated diabetes-induced increase in cardiomyocyte size compared with Ntg(≈35% in dnPI3K vs ≈13% in Ntg), whereas expression of
the caPI3K transgene prevented any significant increase in cardiomyocyte size in a setting of diabetes (Fig 1a-c). There was no increase in Anp or Bnp expression in hearts of diabetic caPI3K-Tg, consistent with the suggestion that increased PI3K activity prevented pathological cardiomyocyte growth (Fig 1d). Furthermore, fibrosis, apoptosis, and the BAX/BCL2 ratio(apoptotic marker) were significantly increased in hearts of diabetic dnPI3K-Tg but not diabetic caPI3K-Tg (Fig 2a-c). Increased apoptosis in diabetic dnPI3K-Tg is likely to account for the absence of an increase in HW/TL despite the increase in cardiomyocyte size in diabetic dnPI3K-Tg. Protection against fibrosis and apoptosis in caPI3K diabetic hearts was associated with enhanced AKT phosphorylation (Fig 2d). pAKT/total AKT was not elevated in Ntg or dnPI3K hearts under non-diabetic or diabetic conditions. Gene expression of Pim1(a kinase that mediates protection downstream of AKT) was elevated in Ntg and caPI3K hearts in response to diabetes, but not dnPI3K (Fig 2e).

Enhanced PI3K(p110α) activity protects the heart against diabetes-induced cardiac dysfunction. Non-diabetic caPI3K-Tg had thicker LV walls than non-diabetic Ntg and dnPI3K-Tg, reflecting the development of physiological hypertrophy(Table 2, see LVPW), as previously shown[20]. In contrast, dnPI3K-Tg had thinner walls than Ntg(Table 2). At comparable heart rates, there were no significant differences in systolic or diastolic function in non-diabetic Ntg, caPI3K-Tg or dnPI3K-Tg(Table 2). The model of diabetes utilized in the current study was previously shown to induce diastolic but not systolic dysfunction[21], consistent with the clinical context of the diabetic heart[3, 4]. In the current study, diabetes did not suppress fractional shortening in Ntg mice; interestingly fractional shortening was significantly higher in diabetic caPI3K-Tg than diabetic Ntg and diabetic dnPI3K-Tg(Table 2). Aortic systolic pressure and LV systolic pressures were lower in diabetic Ntg and diabetic dnPI3K-Tg compared with their non-diabetic counterparts, but not diabetic caPI3K-Tg(Table 2). dP/dt\text{max}(marker of LV systolic function) was not different between the 6 groups(Table 2).
Diabetic Ntg displayed diastolic dysfunction as shown by reduced LV dP/dt\(_{\text{min}}\) (≈10%, Fig 3a) and elevated LVEDP (≈87%, Fig 3b). The increased LVEDP was exaggerated in diabetic dnPI3K-Tg compared to non-diabetic dnPI3K-Tg (≈119%), and was greater than that found in diabetic Ntg (Fig 3b). There was also a fall in dP/dt\(_{\text{min}}\) in diabetic dnPI3K-Tg compared with non-diabetic dnPI3K-Tg (Fig 3a). By contrast, there were no significant differences in dP/dt\(_{\text{min}}\) or LVEDP between diabetic caPI3K-Tg and non-diabetic caPI3K-Tg (Fig 3a&b). Diastolic function was also assessed by measuring transmitral valve flow velocities during early and late diastolic filling. This was the first study to investigate the impact of the dnPI3K transgene on transmitral flow, under either basal or diabetic conditions. Non-diabetic dnPI3K-Tg displayed abnormal patterns of mitral flow (Fig 3c). As a result, E/A ratios in dnPI3K mice were very distinct from that observed in non-diabetic Ntg and caPI3K-Tg (Fig 3d). The reason for this novel functional phenotype is currently unclear and requires further investigation. We demonstrate however, that diabetes was associated with a fall in E/A ratio in Ntg and dnPI3K-Tg, but not caPI3K-Tg (Fig 3d). Furthermore, diabetes was associated with a prolongation in deceleration time and IVRT in Ntg and dnPI3K-Tg, but a smaller or no rise in caPI3K-Tg (Fig 3e&f). Collectively, these data suggest that the caPI3K transgene can preserve diastolic function in a setting of diabetes.

**Increased PI3K(p110\(\alpha\)) activity prevents superoxide generation in a setting of diabetes and can improve mitochondrial function:** Hyperglycemia promotes ROS production in many tissues, contributing to the pathogenesis of multi-organ damage in diabetes[7]. The superoxide-generating NADPH oxidase is a key source of ROS in the heart[27, 28]. Diabetes induced an increase in cardiac superoxide generation by approximately 90% in Ntg compared with non-diabetic Ntg (Fig 4a). Interestingly, superoxide was increased to a similar degree in non-diabetic dnPI3K-Tg and there was no further increase in a setting of diabetes. By contrast, superoxide generation was similar in hearts from non-diabetic Ntg and caPI3K-Tg, and there was no increase in hearts of diabetic caPI3K-Tg (Fig 4a). A large body of evidence has demonstrated that hyperglycemia leads to
activation of PKCβ2 in the heart and subsequent activation of NADPH oxidase[29]. PKCβ2 protein expression was increased in diabetic Ntg hearts compared with non-diabetic Ntg hearts(Fig 4b), increased further in diabetic dnPI3K hearts, but not significantly increased in diabetic caPI3K hearts (Fig 4b). Gene expression of p22phox (membrane-associated subunit of NADPH oxidase) and Ask1 [ROS-sensitive mitogen-activated protein kinas kinase kinase] was also elevated in hearts of diabetic Ntg in comparison with non-diabetic Ntg (Fig 4b). As observed with LV superoxide generation, p22phox and Ask1 expression levels were elevated in hearts of dnPI3K-Tg regardless of diabetic status, but were not increased in hearts from diabetic caPI3K-Tg(Fig 4b). Immunohistochemistry analysis of p22phox expression was consistent with gene expression data (Fig 4c).

Superoxide generation was also assessed in NRVM and the cardiomyoblast H9c2 cell line to assess whether acute activation of PI3K(p110α) with IGF1 (upstream regulator) would inhibit HG-induced superoxide (as occurred in the caPI3K heart), and whether acute inhibition of PI3K(p110α) with a specific inhibitor(A66) would increase superoxide (as occurred in the dnPI3K heart). In NRVM, HG induced an increase in superoxide compared with LG, which was blunted with IGF1 (Fig 4d, left panel), whereas A66 in a setting of HG increased superoxide which was prevented with tempol (superoxide dismutase mimetic; Fig 4d, right panel). The more robust H9c2 cell line was studied under HG conditions with and without H2O2. The addition of H2O2 was designed to mimic the chronic impact of HG causing mitochondrial dysfunction and subsequent release of mitochondrial ROS(e.g. H2O2). Under HG conditions, H2O2 and A66(alone or in combination) all increased superoxide generation and this was blunt by tempol(Fig 4e, left panel). Furthermore, IGF1 treatment suppressed superoxide generation induced by HG with H2O2(Fig 4e, right panel).

Differential NADPH-driven superoxide generation in PI3K-Tg(Fig 4a) indicates that PI3K regulates extra-mitochondrial ROS. To assess whether PI3K has the potential to regulate mitochondrial function and mitochondrial ROS production we examined expression of Tfam (essential for mitochondrial transcription and replication) and Ucp3/UCP3 (mitochondrial anion
carrier protein that can limit mitochondrial ROS production[30]). Tfam gene expression was lower in hearts of non-diabetic dnPI3K compared with non-diabetic Ntg and there was no change with diabetes (Fig 5a). By contrast, Tfam expression was higher in caPI3K mice than dnPI3K mice in non-diabetic and diabetic conditions (Fig 5a). Ucp3 gene and UCP3 protein expression tended to increase in a setting of diabetes in each of the genotypes but was only significantly higher in caPI3K diabetic mice (Fig 5b&c). To more directly examine whether activation of PI3K can affect mitochondrial function, we measured the respiratory profile of IGF1-treated H9c2 cells under HG conditions with or without the specific PI3K(p110α) inhibitor, A66. IGF1 treatment was associated with increased basal respiration, uncoupled respiration, ATP turnover, and a trend for increased maximal respiration (P<0.06); these changes were all prevented by the PI3K(p110α) inhibitor (Fig 5d).

**Discussion**

The development of improved therapeutics for patients with diabetic cardiomyopathy will require a comprehensive understanding of the critical mechanisms responsible for the induction and transition to cardiomyopathy in a setting of hyperglycemia. To our knowledge, this is the first study to show a direct causal role of decreased PI3K activity exacerbating diabetic cardiomyopathy and increased PI3K activity preventing diabetic cardiomyopathy. Another key finding from this study is *in vivo* evidence that PI3K(p110α) regulates superoxide generation in the mouse heart under basal and diabetic settings. The cardiac protection observed in a setting of enhanced PI3K(p110α) activation was associated with increased AKT phosphorylation, Tfam and Ucp3/UCP3 expression, and suppression of LV superoxide generation, PKCβ2, p22phox and Ask1 expression.

Utilizing dnPI3K-Tg with depressed PI3K(p110α) activity, we have demonstrated that PI3K(p110α) is critical for protecting the heart against dysfunction and remodeling in a setting of type 1 diabetes. By contrast, increasing PI3K(p110α) activity utilizing caPI3K-Tg, provided
protection against diastolic dysfunction and myocardial remodeling in diabetic mice. LV diastolic dysfunction is one of the earliest manifestations of diabetic cardiomyopathy, presenting prior to the onset of systolic dysfunction[26, 31, 32]. Diastolic dysfunction is characterized by abnormal LV relaxation and filling, associated with elevated LVEDP, depressed E/A ratio, and increased deceleration time[25]. Contributing factors include pathological cardiomyocyte hypertrophy, increased cell death and fibrosis. Previous studies have reported each of these features in the STZ-induced type 1 diabetic mouse model[21, 26, 33]. In the current study, diabetes in Ntg and dnPI3K-Tg was associated with depressed E/A ratio, and increased LVEDP, deceleration time, IVRT, myocyte size, apoptosis and fibrosis compared with non-diabetic mice. LVEDP, fibrosis, and apoptosis were further elevated in diabetic dnPI3K-Tg compared with diabetic Ntg. By contrast, diabetes was not associated with changes in LVEDP, E/A ratio, IVRT, myocyte size, fibrosis or apoptosis in caPI3K-Tg.

Oxidative stress contributes to the cardiovascular complications associated with diabetes[34]. NADPH oxidase generates superoxide and is considered a major source of ROS in the heart[9, 35]. In the current study, NADPH-driven superoxide generation was increased in diabetic Ntg hearts compared with non-diabetic Ntg hearts, and this was accompanied by increased expression of a membrane-associated subunit of NADPH oxidase(p22phox). The p22phox NADPH subunit was the focus of this study because it was the only subunit which was differentially regulated in the hearts of PI3K-Tg under control/sham conditions based on previous microarray data[12]. Interestingly, superoxide generation and p22phox in the current study were higher in hearts from non-diabetic dnPI3K compared with non-diabetic Ntg, and remained elevated in a setting of diabetes. By contrast, there was no increase in superoxide or p22phox expression in hearts of non-diabetic and diabetic caPI3K-Tg. Findings in the diabetic setting were consistent with those obtained in NRVM and H9c2 cells under HG conditions. In a setting of HG, superoxide generation was enhanced in cells when PI3K(p110α) was inhibited with A66, and blunted when PI3K(p110α) was activated with IGF1. The superoxide dismutase mimetic, tempol, attenuated A66-induced
superoxide generation. Collectively, these data suggest that increased PI3K(p110α) activity mediates protection, at least in part, via attenuation/prevention of superoxide generation.

Our assessment of NADPH-driven superoxide generation indicates that PI3K regulates extra-mitochondrial ROS. Tissue availability constraints precluded direct measurements of mitochondrial ROS. However, to assess the potential contribution of mitochondria we examined cardiac Tfam and Ucp3/UCP3 expression, and performed a mitochondrial function test in H9c2 cells in HG conditions. Tfam and Ucp3/UCP3 expression were elevated in hearts of diabetic caPI3K mice, and acute activation of the IGF1-PI3K(p110α) pathway in H9c2 cells enhanced mitochondrial function in a setting of HG. Collectively, this could contribute to the protection observed in diabetic caPI3K mice. UCP3 can protect muscle cells against mitochondrial ROS and oxidative damage[30], and overexpression of Tfam protected the heart against mitochondrial respiratory defects and cardiac dysfunction in a setting of myocardial infarction[36]. In addition, it was previously shown that isolated mitochondria from caPI3K mice (basal conditions) had increased mitochondrial enzymatic activity, associated with increased fatty acid oxidative capacity[37].

Under basal/non-diabetic conditions, dnPI3K-Tg mice have normal cardiac function despite increased cardiac expression of p22phox and Ask1, decreased Tfam, and increased LV superoxide generation. We recently reported that genes encoding key components of the Z-disc are also depressed in hearts of dnPI3K mice[38]. This suggests that under normal conditions the dnPI3K heart can compensate for these abnormalities, but cannot under conditions of stress such as diabetes; resulting in accelerated cardiomyopathy. Consistent with this hypothesis, cardiac-specific Ask1-Tg show no evidence of pathology under basal conditions but display a more severe pathological phenotype (cardiac dysfunction, increased fibrosis and apoptosis) in response to pressure overload or ischemia-reperfusion injury[39]. Further, basal blood pressure was normal in Nox2-Tg despite increased vascular ROS production and increased p22phox protein. Differences in blood pressure compared with wildtype mice were only apparent in response to an insult (angiotensin II infusion)[40].
Based on data presented in the current study, together with previous reports in the literature, we have assembled a schematic highlighting mechanisms via which increased PI3K(p110α) could mediate protection in a setting of diabetes (Fig 6). It is well recognised that hyperglycemia causes activation of PKCβ2 in the heart and subsequent activation of NADPH oxidase[29]. In the current study, PKCβ2 and p22phox increased in diabetic Ntg hearts but not diabetic caPI3K hearts. PKCβ2 can phosphorylate p22phox[41], and we previously demonstrated that the caPI3K transgene can blunt cardiac pathology and PKCβ2 protein expression in PKCβ2-Tg[42]. Superoxide generation, Ask1 and apoptosis were elevated in hearts of diabetic Ntg and dnPI3K-Tg, but not diabetic caPI3K-Tg. Increased pAKT was maintained in hearts of diabetic caPI3K-Tg, and AKT was previously shown to directly inhibit ASK1 in response to oxidative stress[43]. Further, AKT1 or deficiency of Ask1 protects the heart against cardiac pathology[44-46]. Thus, PI3K(p110α) is likely to mediate protection, in part, by activation of AKT. NADPH oxidase-induced ROS production can lead to mitochondrial dysfunction and increased mitochondrial ROS. Increased expression of Ucp3/UCP3 and Tfam in diabetic caPI3K hearts, together with enhanced mitochondrial function in IGF1-treated H9c2 cells in HG may suggest that PI3K(p110α) is able to enhance mitochondrial function in a setting of diabetes. However, this will require further investigation.

Limitations and future research: Limited tissue availability precluded a thorough assessment of apoptosis mechanisms (mitochondrial vs extramitochondrial), other NADPH oxidase subunits (activation and translocation e.g. Nox2 previously implicated in a setting of hyperglycemia[47]), as well as activity of PKCβ2, PIM1 and ASK1. Such assessment will be important to more comprehensively understand why the dnPI3K heart progresses to cardiomyopathy in a setting of diabetes but not under basal conditions, despite elevated expression of some ROS-related genes and superoxide in both settings. While results obtained from NRVM +/- the PI3K(p110α) inhibitor were similar to those obtained in hearts from diabetic dnPI3K-Tg, it is acknowledged that pharmacological inhibitors are generally less specific than genetic tools, and the metabolism of...
NRVM is different from adult cells. Caution should also be taken when comparing results from \textit{in vitro}, \textit{ex vivo} and \textit{in vivo} studies, as mechanisms may differ. The isolation of mitochondria from PI3K-Tg mice under diabetic conditions will be important to comprehensively examine of the role of PI3K(p110α) in regulating mitochondrial function and mitochondrial ROS.

The current study suggests that increasing PI3K(p110α) activity in the heart could prevent LV superoxide generation and diabetic cardiomyopathy. We recently generated a recombinant adeno-associated viral(rAAV) vector containing caPI3K that selectively transduced cardiac muscle, minimizing the concern of PI3K’s tumorigenic potential in other cell types\cite{48}. rAAV6-caPI3K improved cardiac function in a mouse model of pressure overload and AAV-based therapies have entered clinical trials in heart failure patients\cite{49, 50}. Future studies will be required to assess whether rAAV6-caPI3K can improve function in a setting of diabetic cardiomyopathy.

In summary, we have shown that increasing PI3K(p110α) in the heart of STZ-induced type 1 diabetic mice provided protection against diastolic dysfunction, cardiomyocyte hypertrophy, cardiac fibrosis, and apoptosis. By contrast, decreasing PI3K(p110α) in type 1 diabetic mice was associated with accelerated cardiomyopathy. Enhanced PI3K(p110α) activity prevented the diabetes-induced increase in LV superoxide generation, PKCβ2, p22\textsuperscript{phox} and \textit{Ask1} expression. Furthermore, activation of PI3K(p110α) in cardiomyocyte-like cells was able to suppress HG-induced superoxide generation and enhance mitochondrial function. Thus, therapies that target the cardiac PI3K(p110α) pathway may represent a potential strategy for the prevention and treatment of cardiomyopathy in diabetic patients.

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Duality of interest

The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement

RHR contributed to the design of the study, data acquisition, analysis and interpretation of data, and revising it critically for important intellectual content. JL, KH, BCB and DH contributed to the design of some experiments, data acquisition and interpretation, and manuscript revision. HK, YKT, GS, NC, CQ and EJHB contributed to data acquisition and interpretation, and manuscript revision. KJD and XJD contributed to design of the study, data interpretation and revising it critically for important intellectual content. JRM contributed to the design of the study, data acquisition, analysis and interpretation of data, and drafting the article. All authors have approved the final version of the manuscript.
References


Table 1. Systemic & morphological characteristics of Ntg and PI3K non-diabetic and diabetic mice

<table>
<thead>
<tr>
<th></th>
<th>Ntg non-diabetic</th>
<th>Ntg diabetic</th>
<th>dnPI3K non-diabetic</th>
<th>dnPI3K diabetic</th>
<th>caPI3K non-diabetic</th>
<th>caPI3K diabetic</th>
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</thead>
<tbody>
<tr>
<td>Numbers (n)</td>
<td>11</td>
<td>12</td>
<td>7</td>
<td>7</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>Blood glucose (mmol/l)</td>
<td>11.3 ± 0.3</td>
<td>33.2 ± 0.1 *</td>
<td>11.3 ± 0.5</td>
<td>33.3 ± 0.0 *</td>
<td>11.0 ± 0.7</td>
<td>32.8 ± 0.6 *</td>
</tr>
<tr>
<td>Plasma glucose (mmol/l)</td>
<td>8.6 ± 0.4 (n=6)</td>
<td>33.3 ± 1.7 * (n=10)</td>
<td>7.9 ± 0.9 (n=5)</td>
<td>34.1 ± 1.7 * (n=6)</td>
<td>8.4 ± 0.3</td>
<td>31.2 ± 2.1 *</td>
</tr>
<tr>
<td>GHB (%)</td>
<td>3.8 ± 0.5 (n=6)</td>
<td>9.3 ± 0.3 * (n=10)</td>
<td>3.9 ± 0.7 (n=5)</td>
<td>8.9 ± 0.3 * (n=5)</td>
<td>3.2 ± 0.3</td>
<td>9.9 ± 0.4 *</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>32.9 ± 1.3</td>
<td>29.8 ± 0.8</td>
<td>31.2 ± 1.6</td>
<td>29.3 ± 0.8</td>
<td>30.2 ± 1.3</td>
<td>28.7 ± 1.0</td>
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<tr>
<td>Tibial length (mm)</td>
<td>16.7 ± 0.1</td>
<td>16.5 ± 0.1</td>
<td>16.6 ± 0.2</td>
<td>16.2 ± 0.2</td>
<td>16.3 ± 0.3</td>
<td>16.2 ± 0.2</td>
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<tr>
<td>Heart weight (mg)</td>
<td>133.1 ± 3.4</td>
<td>122.3 ± 4.2</td>
<td>105.0 ± 7.2 †</td>
<td>98.9 ± 2.3 †</td>
<td>147.2 ± 6.0 ‡§</td>
<td>149.3 ± 6.4 †‡</td>
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<tr>
<td>Lung weight (mg)</td>
<td>148.0 ± 3.8</td>
<td>155.3 ± 4.6</td>
<td>155.6 ± 8.8</td>
<td>153.0 ± 6.9</td>
<td>140.7 ± 4.5</td>
<td>139.5 ± 5.9</td>
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<tr>
<td>HW/TL (mg/mm)</td>
<td>7.96 ± 0.18</td>
<td>7.41 ± 0.23</td>
<td>6.31 ± 0.38 †</td>
<td>6.10 ± 0.10 †</td>
<td>9.05 ± 0.31 †‡</td>
<td>9.19 ± 0.31 †‡</td>
</tr>
</tbody>
</table>

GHB, glycated haemoglobin; HW, heart weight; TL, tibial length

*P<0.05 vs non-diabetic of the same genotype, †P<0.05 vs Ntg non-diabetic, ‡P<0.05 vs dnPI3K non-diabetic, §P=0.06 vs Ntg non-diabetic
Table 2. Echocardiographic & catheterization analyses of heart size and function

<table>
<thead>
<tr>
<th></th>
<th>Ntg non-diabetic</th>
<th>Ntg diabetic</th>
<th>dnPI3K non-diabetic</th>
<th>dnPI3K diabetic</th>
<th>caPI3K non-diabetic</th>
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<td>M-mode echo</td>
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<td></td>
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<tr>
<td>n=11</td>
<td>n=11</td>
<td>n=6</td>
<td>n=7</td>
<td>n=6</td>
<td>n=8</td>
<td></td>
</tr>
<tr>
<td>LVPW (mm)</td>
<td>0.88 ± 0.02</td>
<td>0.80 ± 0.03</td>
<td>0.74 ± 0.03 *</td>
<td>0.75 ± 0.04 *</td>
<td>1.07 ± 0.04 *</td>
<td>0.97 ± 0.02 *†‡§</td>
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<tr>
<td>LVDD (mm)</td>
<td>3.79 ± 0.07</td>
<td>3.83 ± 0.07</td>
<td>3.74 ± 0.11</td>
<td>3.69 ± 0.19</td>
<td>3.68 ± 0.07</td>
<td>3.75 ± 0.10</td>
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<tr>
<td>LVSD (mm)</td>
<td>2.35 ± 0.05</td>
<td>2.36 ± 0.07</td>
<td>2.47 ± 0.12</td>
<td>2.36 ± 0.21</td>
<td>2.21 ± 0.11</td>
<td>2.07 ± 0.12</td>
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<td>FS (%)</td>
<td>38 ± 1</td>
<td>38 ± 2</td>
<td>34 ± 2</td>
<td>37 ± 3</td>
<td>40 ± 2</td>
<td>45 ± 2 *‡§§</td>
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<tr>
<td>Heart rate (bpm)</td>
<td>426 ± 13</td>
<td>450 ± 13</td>
<td>410 ± 20</td>
<td>373 ± 9 *†</td>
<td>426 ± 26</td>
<td>436 ± 10 §</td>
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<tr>
<td>Catheterization</td>
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<td>n=8</td>
<td>n=11</td>
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<td>n=6</td>
<td>n=6</td>
<td>n=7</td>
<td></td>
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<tr>
<td>Heart rate (bpm)</td>
<td>327 ± 7</td>
<td>344 ± 5</td>
<td>309 ± 10</td>
<td>334 ± 20</td>
<td>323 ± 21</td>
<td>368 ± 14</td>
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<td>Ao SBP (mmHg)</td>
<td>98 ± 3</td>
<td>84 ± 3 †</td>
<td>101 ± 8</td>
<td>77 ± 2 *†</td>
<td>94 ± 4</td>
<td>87 ± 2 *</td>
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<tr>
<td>LVSP (mmHg)</td>
<td>99 ± 3</td>
<td>95 ± 1</td>
<td>95 ± 6</td>
<td>78 ± 2 *†</td>
<td>96 ± 3</td>
<td>92 ± 2 §</td>
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<td></td>
<td>6610 ± 249</td>
<td>7266 ± 200</td>
<td>6784 ± 762</td>
<td>6432 ± 618</td>
<td>7401 ± 503</td>
<td>7906 ± 297</td>
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<tr>
<td>dP/dt\text{max} (mmHg/s)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>dP/dt\text{min} (mmHg/s)</td>
<td>5330 ± 163</td>
<td>4686 ± 185 †</td>
<td>5196 ± 434</td>
<td>4315 ± 270 *†</td>
<td>5335 ± 323</td>
<td>5222 ± 227 §</td>
</tr>
</tbody>
</table>

Echo, echocardiography; LVPW, left ventricular posterior wall; LVDD, LV diastolic dimension; LVSD, LV systolic dimension; FS, fractional shortening; Ao SBP, aortic systolic blood pressure; LVSP, left ventricular systolic pressure. *P<0.05 vs Ntg non-diabetic, †P<0.05 vs non-diabetic of the same genotype, ‡P<0.05 vs Ntg diabetic, §P<0.05 vs dnPI3K diabetic.
FIGURE LEGENDS

Figure 1. The diabetes-induced increase in cardiomyocyte width is exaggerated in dnPI3K mice and attenuated in caPI3K mice. (a) Representative LV sections stained with H&E from non-diabetic and diabetic Ntg, dnPI3K and caPI3K mice (magnification ×400) (b) Quantitative analysis of cardiomyocyte width. (c) Percent increase in cardiomyocyte width in comparison to non-diabetic mice of the same genotype. b & c, n=5-11 per group. (d) Representative Northern blot (left panel) showing gene expression of Anp and Bnp in hearts from Ntg, dnPI3K (dnP) and caPI3K (caP). Gapdh was used to normalize for RNA loading. Quantitative analyses (right panel; n=4-6 per group). Mean values for non-diabetic Ntg were normalized to 1. White bars, non-diabetic; black bars, diabetic. *P<0.05 vs non-diabetic of the same genotype, †P<0.05 vs non-diabetic Ntg, ‡P<0.05 vs diabetic Ntg, §P<0.05 vs diabetic dnPI3K, ¶P≤0.05 vs non-diabetic Ntg (unpaired t test), ¶P=0.05 vs diabetic Ntg (unpaired t test).

Figure 2. PI3K(p110α) provides protection against fibrosis and apoptosis. (a) Representative LV sections from non-diabetic and diabetic Ntg, dnPI3K and caPI3K mice showing collagen deposition/fibrosis in red (upper panel, sirius-red stain; magnification ×200). Quantitative analysis of collagen area/total ventricular area (lower panel, n=4-13 per group). (b) Representative LV sections labeled with TUNEL staining (upper panel). Positively stained apoptotic cells appear dark blue (indicated by arrow); magnification ×200. Quantitation of positively-stained apoptotic cells/negatively-stained non-apoptotic cells (lower panel), fold non-diabetic Ntg, n=4-7 per group. (c) Representative Western blot showing BAX (upper panel) and BCL2 (lower panel) in hearts of non-diabetic (ND) and diabetic (D) mice. Quantitative analysis (right panel) of BAX/BCL2 ratio. Mean values for non-diabetic Ntg were normalized to 1 (n=3-4 per group). (d) Representative Western blot showing pAKT and
total AKT (upper panel). Quantitative analysis of AKT phosphorylation normalized to total AKT (lower panel; n=3-6 per group). (c) Representative Northern blot (upper panel) showing gene expression of *Pim1* and *Gapdh*. Quantitative analyses (lower panel; n=3-4 per group). White bars, non-diabetic; black bars, diabetic. *P<0.05 vs non-diabetic of the same genotype, †P<0.05 vs diabetic Ntg, ‡P<0.05 vs diabetic dnPI3K, §P<0.05 vs non-diabetic and diabetic Ntg and dnPI3K.

**Figure 3.** PI3K(p110α) provides protection against diastolic dysfunction. (a) Maximal rate of fall of LV pressure (dP/dt\text{min}) and (b) LV end-diastolic pressure (LVEDP) assessed by catheterization. A & B, n=5-11 per group. (c) Representative mitral flow patterns from pulsed wave Doppler echocardiography. (d) E/A wave ratio, (e) deceleration time, and (F) IVRT. C-F, n=4-10 per group. White bars, non-diabetic; black bars, diabetic. *P≤0.05 vs non-diabetic of the same genotype, †P<0.05 vs diabetic Ntg, ‡P<0.05 vs diabetic dnPI3K, §P<0.05 vs non-diabetic and diabetic Ntg.

**Figure 4.** PI3K(p110α) protects against diabetes-induced superoxide generation. (a) LV superoxide generation assessed by lucigenin-enhanced chemiluminescence expressed as relative light units per second per mg(RLU/s/mg). (b) Upper panel, Western blot showing PKCβ2 and GAPDH in hearts of non-diabetic (ND) and diabetic (D) mice. Quantitative analysis of PKCβ2 normalized to GAPDH (n=4-5 per group). Lower panel, representative Northern blot showing gene expression of *p22^{phox}* (n=4-6 per group) and *Ask1* (n=5-6 per group) from Ntg, dnPI3K (dnP) and caPI3K (caP). *Gapdh* was used to normalize for RNA loading. Quantitative analyses (right panel). Mean values for non-diabetic Ntg were normalized to 1. (c) Immunohistochemical (IHC) examination of p22^{phox} in LV sections.
Representative staining (left panel, p22phox stains brown, magnification ×100) and semi-quantitative analysis (right panel, IHC score. n=3 per group). For panels a-c: white bars, non-diabetic; black bars, diabetic. *P<0.05 vs non-diabetic Ntg, #P<0.05 vs non-diabetic Ntg (unpaired t test), †P<0.05 vs non-diabetic dnPI3K, ‡P<0.05 vs diabetic Ntg, §P<0.05 vs diabetic dnPI3K. (d) Superoxide generation in NRVM under low glucose (LG) and high glucose (HG) conditions treated with IGF1, A66 [PI3K(p110α) inhibitor], and tempol (superoxide dismutase mimetic), n=4-5 per group. *P<0.05 vs LG, †P<0.05 vs HG, ‡P<0.05 vs LG and HG, §P<0.05 vs HG+A66. (e) Superoxide generation in H9c2 cells under HG conditions treated with H2O2, A66, tempol, and IGF1 (n=5-7 per group). *P<0.05 vs control, †P<0.05 vs A66+H2O2, ‡P<0.05 vs H2O2. d) and e) statistics performed using one-way repeated measures ANOVA followed by pairwise multiple comparison (Student-Newman-Keuls). Values relative to LG or control (100%) as shown.

**Figure 5.** Enhanced PI3K(p110α) activity increases Tfam and Ucp3/UCP3 expression and improves mitochondrial function. (a) and (b) Representative Northern blots (left panels) showing gene expression of Tfam and Ucp3 in hearts of non-diabetic (ND) and diabetic (D) mice. Gapdh was used to normalize for RNA loading. Quantitative analyses (right panel; n=3-4 per group). (c) Representative Western blot showing UCP3 and GAPDH (left panel). Quantitative analysis (right panel; n=3 per group). For panels a-c: white bars, non-diabetic; black bars, diabetic. Mean values for non-diabetic Ntg were normalized to 1 for panels a-c. *P<0.05 vs non-diabetic Ntg, †P<0.05 vs non-diabetic and diabetic dnPI3K, ‡P<0.05 vs non-diabetic of the same genotype, §P<0.05 vs diabetic dnPI3K. (d) Trace of mean data showing oxygen consumption rate (OCR) throughout a mitochondrial function test (left panel; A=oligomycin, B=FCCP, C&D=antimycin A and rotenone) in H9c2 cells in HG conditions. Circles, control (HG alone); squares, A66 treated; upward triangles, IGF1 treated; downward
triangles, IGF1 and A66. Quantitation (right panel). UC Resp=uncoupled respiration, ATP=ATP turnover, Max Resp=maximal respiration. White bars, control (HG alone); black bars, A66; grey bars, IGF1; hash bars, IGF1 and A66. *P<0.05 vs control, †P<0.05 vs IGF1.

**Figure 6.** Proposed mechanisms via which PI3K(p110α) could mediate protection in a setting of high glucose. High glucose activates PKCβ2 in the heart, leading to activation of NADPH oxidase. ROS (e.g. superoxide, O₂⁻) produced by NADPH oxidase causes mitochondrial dysfunction and increased mitochondrial ROS, which can cause further activation of PKCβ2. ROS activates ASK1 leading to apoptosis. Protective properties of PI3K(p110α) are highlighted in red. Further studies will be required to assess whether PI3K(p110α) can enhance mitochondrial function in a setting of diabetes *in vivo*. 
Figure 2

(a) Non-diabetic | Diabetic
Ntg | ![](image1)
dnP13K | ![](image2)
cP13K | ![](image3)
Bars = 40µm

(b) Non-diabetic | Diabetic
Ntg | ![](image4)
dnP13K | ![](image5)
cP13K | ![](image6)
Bars = 20µm

(c) Ntg | dnP13K | caP13K
BAX | ![](image7)
BCL2 | ![](image8)

(d) Ntg | dnP13K | caP13K
pAKT | ![](image9)
total AKT | ![](image10)

(e) Ntg | dnP | caP
Pim1 | ![](image11)
Gapdh | ![](image12)
Figure 5

a

\[ \text{Tfam} / \text{Gapdh} \]

\[ \text{Fold change} \]

b

\[ \text{Ucp3} / \text{Gapdh} \]

\[ \text{Fold change} \]

c

\[ \text{UCP3} / \text{GAPDH} \]

\[ \text{Fold change} \]

d

\[ \text{OCR} (\text{pmol/min/10^6 cells}) \]

\[ \text{Time (mins)} \]

H9c2 - High glucose

\[ \text{H9c2 - High glucose} \]
Figure 6

High Glucose

Cardiomyocyte

PKCβ2

p22phox

NADPH oxidase complex

ROS e.g. O₂⁻

mitochondrial dysfunction

mtDNA e.g. Tfam

mtDNA dysfunction e.g. ROS

mtDNA e.g. Tfam

ROS

Mitochondrion

H₂O₂

Enhanced mitochondrial function

PI3K (p110α)

pAKT

ASK1

APOPTOSIS

Growth factor receptors e.g. IGF1R

Enhanced mitochondrial dysfunction

Mitochondrion

H₂O₂

Enhanced mitochondrial function