Therapeutic Targeting of Oxidative Stress with Coenzyme Q$_{10}$ Counteracts Exaggerated Diabetic Cardiomyopathy in a Mouse Model of Diabetes with Diminished PI3K(p110$\alpha$) Signalling

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Abstract

Diabetes-induced cardiac complications include left ventricular (LV) dysfunction and heart failure. We previously demonstrated that LV phosphoinositide 3-kinase p110α (PI3K) protects the heart against diabetic cardiomyopathy, associated with reduced NADPH oxidase expression and activity. Conversely, in dominant negative PI3K(p110α) transgenic mice (dnPI3K), reduced cardiac PI3K signalling exaggerated diabetes-induced cardiomyopathy; associated with upregulated NADPH oxidase.

Aim: To examine whether chronic supplementation with the antioxidant coenzyme Q₁₀ (CoQ₁₀) could attenuate LV superoxide and diabetic cardiomyopathy in a setting of impaired PI3K signalling.

Methods: Diabetes was induced in 6-week-old non-transgenic and dnPI3K male mice via streptozotocin. After 4 weeks of diabetes, CoQ₁₀ supplementation commenced (10mg/kg i.p., 3 times/week, 8 weeks). At study end (12 weeks of diabetes), markers of LV function, cardiomyocyte hypertrophy, collagen deposition, NADPH oxidase, oxidative stress (3-nitrotyrosine, 4-hydroxynonenal) and concentrations of CoQ₉ and CoQ₁₀ were determined.

Results: LV NADPH oxidase (Nox2 gene expression and activity, on lucigenin-enhanced chemiluminescence), as well as oxidative stress, were increased by diabetes, exaggerated in diabetic dnPI3K mice, and attenuated by CoQ₁₀. Diabetes-induced LV diastolic dysfunction (prolonged deceleration time, elevated end-diastolic pressure, impaired E/A ratio), cardiomyocyte hypertrophy and fibrosis, expression of atrial natriuretic peptide, connective tissue growth factor and β-myosin heavy chain, were all attenuated by CoQ₁₀.

Conclusions: Chronic CoQ₁₀ supplementation attenuates aspects of diabetic cardiomyopathy, even in a setting of reduced cardiac PI3K protective signalling. Given CoQ₁₀ supplementation has been suggested to have positive outcomes in heart failure patients, chronic CoQ₁₀ supplementation may be an attractive adjunct therapy for diabetic heart failure.
Introduction

Diabetes imposes a significant burden on society, and with its incidence predicted to affect up to 439 million adults globally by 2030, it represents a major threat to human health [1]. Increased morbidity and mortality are evident, in part due to a greater prevalence of heart failure [2, 3]. Diabetes-induced cardiac complications include coronary heart disease, atherosclerosis and distinct diabetic cardiomyopathy [4]; their progression is closely linked to increased generation of reactive oxygen species (ROS) such as superoxide (O$_2^-$) [4, 5]. Hyperglycaemia-induced upregulation of NADPH oxidase is a key trigger of diabetes-induced ROS production in the heart [6, 7]. The heart is particularly susceptible to oxidative damage due to low levels of endogenous cardiac antioxidants, which are further impaired by hyperglycaemia [8, 9]. In the diabetic mouse heart, LV NADPH oxidase is upregulated, in terms of gene expression, protein levels and activity [6, 10].

The p110α isoform of phosphoinositide 3-kinase [PI3K(p110α)] is a critical regulator of beneficial physiological heart growth and a negative regulator of cardiac pathology [11-15]. Mice expressing constitutively active PI3K(p110α) (caPI3K) specifically in the heart are protected against a broad spectrum of cardiac disorders (including pressure-overload induced-hypertrophy, myocardial infarction and dilated cardiomyopathy) [13-15]. Conversely, dnPI3K transgenic mice, which exhibit a 77% reduction in cardiac PI3K(p110α) activity, exhibit exaggerated responses to these cardiac pathologies, including impaired LV contractile function and increased fibrosis [11, 13, 15]. We have recently shown that dnPI3K mice also exhibit an augmented diabetic cardiomyopathy [10], in which diabetes-induced cardiomyocyte hypertrophy, cardiac fibrosis and elevated LV end-diastolic pressure (LVEDP) were exaggerated compared with non-transgenic (Ntg) control mice. Reduced cardiac PI3K signalling was also associated with upregulation of LV NADPH oxidase activity (as assessed by lucigenin-enhanced chemiluminescence) and gene expression. In contrast, caPI3K transgenic mice were completely protected from these diabetes-induced increases in
LV NADPH oxidase, LV remodelling and diastolic dysfunction [10]. The combination of diabetes and diminished PI3K(p110α) signalling thus represents a more severe setting of diabetic cardiomyopathy, likely a consequence of increased oxidative stress.

The antioxidant, coenzyme Q_{10} (CoQ_{10}), an endogenous component of all cellular membranes and mitochondrial respiration, limits cardiac oxidative stress by inhibiting lipid peroxidation and O_{2}• levels [16]. Furthermore, CoQ_{10} supplementation preserves both mitochondrial and endothelial function in patients subjected to LV ischaemic insults [17, 18]. Heart failure is associated with a reduction in myocardial CoQ_{10} content, with similar findings of reduced plasma levels of CoQ_{10} in diabetic patients. This CoQ_{10} deficiency is an independent predictor of mortality in affected patients [19-21]. Seventeen years ago, meta-analysis of CoQ_{10} supplementation in patients with congestive heart failure revealed improvements in LV ejection fraction and cardiac output [22]. A concomitant study of 7 patients with hypertrophic cardiomyopathy revealed trends for improved LV wall thickness by echocardiography [23]. Appropriately-powered, prospective, double-blind randomised clinical trials investigating whether CoQ_{10} supplementation is beneficial in heart failure are limited. A recent editorial has highlighted the potential benefits of CoQ_{10} supplementation in the management of chronic heart failure but additional studies to confirm this are clearly warranted [24]. The recent Q-SYMBIO study suggested that long-term CoQ_{10} supplementation in this context improved cardiac hemodynamic parameters and heart failure symptoms and tended to improve incidences of hospitalisations and mortality, however the study was limited in terms of slow recruitment and protocol changes such as alterations to the CoQ_{10} dosage [25]. Given that diabetes not only escalates risk of heart failure and its incidence (>2.5-fold) independent of other concomitant co-morbidities, together with the identification of diabetes as an independent predictor of poor outcome [4], new adjunct therapies specifically for the diabetic heart are urgently required. Previous studies from our laboratory have demonstrated that CoQ_{10} supplementation attenuates diabetic
cardiomyopathy in two conventional pre-clinical models of diabetic cardiomyopathy [6, 7], but its efficacy in a more severe context of diabetic cardiomyopathy, in which endogenous protective mechanisms are impaired, remained unresolved. This is important to assess because obesity and diabetes have been associated with depressed or defective PI3K signaling [26-28]. The goal of the current study was to examine whether chronic supplementation with CoQ₁₀ could attenuate LV superoxide generation in a setting of severe diabetic cardiomyopathy due to impaired PI3K signalling.
Methods

Animals

All animal research was conducted in accordance with the ‘National Health and Medical Research Council of Australia’ guidelines, and by the Alfred Medical Research and Education Precinct (AMREP) Animal Ethics committee. All mice were bred and housed in the AMREP Animal Centre and maintained under a 12-hr light/dark cycle with up to 4 littermates per cage. Heterozygous dnPI3K transgenic mice (FVB/N background) were originally produced by cloning a truncated p110 mutant lacking the active kinase domain into the α-MHC promoter construct [29]. The catalytically inactive p110 molecule competes with endogenous p110 for binding with the p85 regulatory subunit, therefore inhibiting the function of endogenous p110 in vivo, as previously described [29]. Mice were genotyped for the presence of the p110Δkinase transgene to identify dnPI3K mice and their non-transgenic (Ntg) littermate controls (Supplementary Figure 1). Briefly, DNA was extracted from mouse tail clips followed by PCR with α-MHC (forward primer) and p110Δkinase (reverse primer) [29, 30]. Samples were then run on a 2% agarose gel and imaged using an UV Illuminator.

Treatments

Age-matched Ntg and dnPI3K transgenic male mice (n=20/group) were assigned to either the diabetic or nondiabetic group. At 6 weeks of age, mice were administered with daily i.p. injections of either streptozotocin (STZ, 55mg/kg, Sigma-Aldrich, USA) to induce type 1 diabetes (T1DM) or with a citrate buffer vehicle (Citric Acid, Sigma-Aldrich, USA) for 5 consecutive days. Four weeks following administration of STZ or citrate buffer, diabetic and nondiabetic animals were assigned to receive either CoQ10 (i.p., 10mg/kg/day; LiQsorb, Tishcon Corp, NY, USA) three times per week for 8 weeks, or remain untreated. Eight weeks
later, mice were killed by exsanguination under anaesthesia and tissues were collected for analysis. Endpoint parameters were thus measured at 18 weeks of age.

**Glucose measurements**

Fortnightly and endpoint collection of blood from diabetic mice was used for evaluation of hyperglycaemia (≥26 mM), and hence onset of T1DM. Saphenous vein bleeds were conducted fortnightly following STZ or citrate buffer administration and glucose was measured using a glucometer (Accu-check Advantage; Roche, Basel, Switzerland).

**LV function (M-mode and Doppler echocardiography)**

M-mode and Doppler echocardiography were performed at baseline before STZ or citrate buffer administration, and at study endpoint. Mice were anaesthetised (cocktail of i.p. ketamine, xylazine and atropine, KXA: 60:6:0.7 mg/kg, respectively). Echocardiography was performed using a Philips iE53 ultrasound system with 15MHz linear (M-mode) and 12MHz sector (Doppler) transducers. Parameters measured from M-mode echocardiography included external LV dimension, anterior and posterior wall thickness, LV end-diastolic dimension (LVEDD) and LV end-systolic dimension (LVESD). Fractional shortening (FS), a measure of systolic function, was calculated (% FS = (LVEDD – LVESD) / LVEDD x 100). Diastolic transmitral LV inflow images were obtained from apical 4-chamber views using colour flow mapping-guided pulsed-wave Doppler and were used to calculate early (E) and late (atrial, A) peak filling blood flow velocities and E-wave deceleration time.
**Micromanometry**

LV function was examined via cardiac catheterisation in anaesthetised mice (KXA, 85:8.5:1.0 mg/kg, i.p.) immediately prior to cull and collection of tissues. A micro-manometer-tipped catheter (1.4F, Millar Instrument Co, Texas, USA) was inserted through the right carotid artery and into the LV. Pressure recordings were obtained and calculated for LV +dP/dt (indicator of systolic function), LV -dP/dt (indicator of diastolic function) and LVEDP [31]. Aortic systolic (AoSBP) and diastolic (AoDBP) blood pressure was recorded with the catheter positioned in the ascending aorta [31].

**Tissue collection**

Following cardiac catheterisation, blood was collected and heart and lungs were removed and weighed. The top third portion of LV was fixed in 10% neutral-buffered formalin (NBF, Australian Biostain, Melbourne, Australia), followed by paraffin-embedding, and subsequent histology and immunohistochemistry. The apex of the LV (approximately 5mg) was used for assessment of $\text{O}_2^-$ by lucigenin-enhanced chemiluminescence. The remainder was snap frozen for determination of gene expression, protein content and CoQ tissue determination. A single hind leg was removed for determination of tibia bone length to normalise heart and lung weight.

**Histology**

LV sections were cut (4μm) using a microtome (Leica Microsystems, Wetzlar, Germany). Slides were stained with haematoxylin and eosin (H&E) and cardiomyocyte width was determined by measuring across the shortest cross-sectional axis, while cardiomyocyte area was measured as the cross-sectional area within the perimeter, of individual cardiomyocytes (400X magnification, 5-6 images, minimum 100 individual myocytes per heart).
Cardiomyocyte width and area were analysed with Olympus Image Pro-Plus (Version 6.0, Media Cybergenetics, Bethesda, USA) [10, 32].

Picrosirius red staining was used to measure LV collagen deposition. Slides were dewaxed and stained with 0.1% picrosirius red solution (Picric acid, Fluka, Buchs, Switzerland; pH 2.0). Collagen stained an intense red colour and was analysed using Olympus Image Pro-Plus as a ratio of area of collagen to total image area (200X magnification, 10 fields per image), as described previously [10, 32].

**Immunohistochemistry**

LV 3-nitrotyrosine (3-NT) was assessed in deparaffinised sections from all mice prior to enzymatic antigen retrieval using Proteinase K (30 µg/ml in 10 mM Tris-EDTA-CaCl₂ buffer, pH 8.0). Endogenous peroxidases were quenched in 3% H₂O₂, and sections were blocked with normal goat serum (NGS, Sigma Aldrich) followed by avidin-biotin block (Vector Laboratories, Burlingame, CA, USA). Sections were incubated overnight with Anti-Nitrotyrosine Antibody (Merck-Millipore, Darmstadt, Germany) followed by biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA). In addition, 4-hydroxynonenal (4-HNE) was assessed in a limited number of deparaffinised LV sections (in one mouse heart per experimental group) prior to heat-activated antigen retrieval with 10 mM sodium citrate buffer (pH 6.0). Endogenous peroxidases were quenched in 3% H₂O₂, and then blocked in Avidin-Biotin Block (Vector Laboratories, Burlingame, CA, USA). Sections were incubated with M.O.M.™ Mouse Ig Blocking Solution followed by M.O.M.™ diluent solution (Vector Laboratories, Burlingame, CA, USA). Sections were incubated overnight with the Mouse Anti-4-HNE Monoclonal Antibody (HNEJ-2, JaICA, Shizuoka, Japan) followed by M.O.M.™ Biotinylated Anti-Mouse IgG solution. Sections for both 3-NT and 4-
HNE were then incubated with Vectastain® Universal Elite® ABC Avidin–Biotinylated Horseradish Peroxidase Complex, developed using DAB peroxidase substrate solution (Vector Laboratories), then counterstained with haematoxylin. Both 3-NT and 4-HNE content were semi-quantitatively assessed for intensity of brown stain and were graded by a blinded observer as follows: score 0, negative stain; score 1, weak; score 2, moderate; score 3, strong; score 4, intense.

Assessment of LV NADPH oxidase activity and plasma MDA levels

LV O$_2^-$ levels were estimated in fresh LV tissue using NADPH-driven lucigenin (5µmol/L)-enhanced chemiluminescence, as previously described [6, 7, 32]. Results were normalised against tissue dry weight and expressed as relative light units (RLU) per second per milligram of tissue. Plasma levels of MDA were measured using a commercially available kit (Bioxytech MDA-586, Foster City, CA, USA), as per manufacturer instructions.

Assessment of coenzyme Q$_9$ and Q$_{10}$ levels

Levels of CoQ$_9$ and CoQ$_{10}$ in plasma were determined as described previously [33]. Briefly, mouse plasma (100 µL) was mixed with 25 µL freshly prepared 1,4 benzoquinone (2 mg/mL MilliQ water) and incubated for 30 min at room temperature (RT). Following incubation, 2 mL methanol containing 0.02% acetic acid and 10 mL of water-washed hexane were added, and vigorously mixed. Samples were centrifuged (1,430 xg, 5 min, 4°C) and 9 mL of the top hexane layer collected. In addition, LV CoQ$_9$ and CoQ$_{10}$ were measured in Ntg LV tissues (the ~30% smaller heart size in dnPI3K mice precluded sufficient remaining heart tissue for also determining this in dnPI3K mice). LV CoQ$_9$ and CoQ$_{10}$ were determined by homogenising 10-20 mg wet tissue in 200 µL PBS; a 10 µL aliquot was then removed for protein determination. The remaining homogenate (100 µg protein) was diluted with PBS,
mixed with 25 µL freshly prepared 1,4 benzoquinone (2 mg/mL MilliQ water) and incubated for 30 min at RT. PBS (1 mL) containing 1% SDS was added and incubated for a further 10 min at RT. Following incubation, 2 mL of ethanol:isopropanol alcohol (95:5; vol/vol) was added and mixed vigorously. Water-washed hexane (4 mL) was added, mixed and centrifuged (1,430 xg, 5 min, 4°C). The hexane (3.5 mL) was collected, the extraction repeated two more times, and the collected hexane combined (10.5 mL total). The hexane containing plasma or cardiac lipids was dried using a rotary evaporator. The resulting dried lipids were then redissolved in 150 µL of ice-cold mobile phase (ethanol:methanol:isopropanol:ammonium acetate pH 4.4, 65:30:3:2, vol/vol/vol/vol) and transferred into HPLC vials. CoQ9 and CoQ10 were determined by HPLC using a Supelcosil LC-C18 column (5 µm, 250x4.6 mm) eluted at 1 mL/min and connected to UV and electrochemical (ESA CoulArray 5600A) detectors. Peaks were identified by comparison with known standards. CoQ9 and CoQ10 were quantified at -700 and +500 mV, using CoQ8 as the internal standard. For plasma samples, UV210nm absorbance was used to quantify cholesterol by area comparison, using authentic cholesterol as a standard [33].

Analysis of LV gene expression and protein content

RNA was extracted from frozen LV and reverse transcribed as previously described [32]. Cardiac gene expression of pro-hypertrophic markers β-myosin heavy chain and ANP (atrial natriuretic peptide), the pro-fibrotic marker CTGF (connective tissue growth factor), the Nox2 subunit of NADPH oxidase, and the housekeeper 18S, were determined via real-time PCR using SYBR® Green (Applied Biosystems, Scoresby, Victoria, Australia). Primers were generated from murine sequences published on GenBank (Table 1). Quantitative real-time analysis was performed using ABI Prism® 7700 Sequence Detection System software, and the ΔΔCt method was employed to detect relative fold differences. In addition, frozen LV
samples (~30 mg) were homogenised and the protein concentration of the lysates were measured as previously described [10]. For assessment of protein levels of the Nox2 subunit of NADPH oxidase, blots were probed with anti-gp91[phox] (BD Transduction Laboratories, USA) with a band detected at 58 kDa, followed by β-tubulin antibody (#2146, Cell Signaling Technology, USA; 1:1,000), with a band detected at 55 kDa. The ratio of Nox2 to β-tubulin was calculated. Results of each western blot were analysed using Quantity One software (version 4.5.2, BIO-RAD, USA).

Statistical analysis

Results are presented as mean ± SEM. Two-way ANOVA was used to identify differences between groups followed by Tukey’s post-hoc test and correction for multiple comparisons. Specific comparisons (unpaired t-tests) were performed to test the a priori hypotheses that diabetes would increase, and dnPI3K would further increase, markers of oxidative stress, diastolic dysfunction, and cardiomyocyte hypertrophy and fibrosis, and these would be attenuated by CoQ10 supplementation. Data were considered significant where *P <0.05 (ANOVA) or †P <0.05 (unpaired t-test), unless otherwise stated.
Results

Blood glucose and body composition

Blood glucose levels were elevated in diabetic compared to nondiabetic mice, and neither genotype nor CoQ\textsubscript{10} treatment altered blood glucose concentrations (Table 2). Body weight was reduced in diabetic mice, regardless of genotype or treatment (Table 2). Total heart weight (HW) and HW to tibia length ratio (HW/TL) were reduced in dnPI3K mice compared to Ntg controls (Table 2). Total lung weight and lung weight to tibia length ratio (LW/TL) did not differ between groups, irrespective of genotype, diabetes or treatment (Table 2).

Coenzyme Q\textsubscript{10} limits diabetes-induced cardiac oxidative stress

Lucigenin-enhanced chemiluminescence, indicative of LV O\textsubscript{2}\textsuperscript{−} levels, was increased in both untreated Ntg diabetic mice and dnPI3K diabetic mice compared to their respective nondiabetic controls (Figure 1A). LV O\textsubscript{2}\textsuperscript{−} chemiluminescence was further increased in dnPI3K diabetic mice compared to Ntg diabetic mice (Figure 1A). CoQ\textsubscript{10} supplementation effectively decreased LV chemiluminescence O\textsubscript{2}\textsuperscript{−} in both Ntg and dnPI3K diabetic mice (Figure 1A). Nox2 gene expression was increased in both Ntg and dnPI3K diabetic mice, compared to Ntg citrate controls (Figure 1B). CoQ\textsubscript{10} supplementation effectively attenuated this increase in each of Ntg and dnPI3K, citrate and diabetic mice (Figure 1B). Nox2 protein was also increased in dnPI3K citrate and diabetic mice, compared to their respective Ntg mice (Figure 1C). CoQ\textsubscript{10} supplementation effectively attenuated the increased Nox2 protein levels in dnPI3K diabetic mice (Figure 1C).

LV 3-NT content was increased with diabetes in both Ntg and dnPI3K mice, and was further increased in the dnPI3K mice with diabetes compared to Ntg diabetic mice (Figure 2A, B). CoQ\textsubscript{10} supplementation effectively attenuated the increase in 3-NT content in both Ntg and
dnPI3K diabetic mice (Figure 2A, B). A similar trend was observed on LV 4-HNE content, which was determined in one mouse heart from each experimental group; representative images are shown (Figure 2D). Plasma MDA levels were increased in Ntg and dnPI3K diabetic mice, compared to their respective controls, and were further increased in dnPI3K diabetic mice (~27%) compared to Ntg diabetic mice (Figure 2C). CoQ$_{10}$ supplementation effectively attenuated the increase in MDA levels in both Ntg and dnPI3K diabetic mice (Figure 2C).

Coenzyme Q$_{10}$ limits diabetes-induced diastolic dysfunction

LV E/A ratio was reduced in Ntg diabetic mice compared to Ntg nondiabetic controls, which was attenuated with CoQ$_{10}$ supplementation (Figure 3A). Similarly, dnPI3K diabetic mice exhibited a reduced E/A ratio compared to dnPI3K citrate mice, which was also attenuated by CoQ$_{10}$ (Figure 3A). Deceleration time was prolonged in both Ntg and dnPI3K diabetic mice compared to their respective nondiabetic controls, with deceleration time further prolonged in dnPI3K diabetic mice (Figure 3B). CoQ$_{10}$ supplementation effectively attenuated this increased deceleration time in both Ntg and dnPI3K diabetic animals (Figure 3B). LVEDP was elevated in both Ntg and dnPI3K diabetic mice compared to their respective nondiabetic controls; this was attenuated by CoQ$_{10}$ (Figure 3C). Additionally, LVEDP was further increased in dnPI3K diabetic mice compared to Ntg diabetic mice (Figure 3C). LV -dP/dt was reduced in diabetic animals of both genotypes, and was further reduced in dnPI3K diabetic mice (Figure 3D). CoQ$_{10}$ treatment prevented the fall in LV -dP/dt in both Ntg and dnPI3K diabetic animals (Figure 3D). Heart rate, SBP and markers of systolic function including fractional shortening and LV +dP/dt, remained unaltered regardless of genotype, diabetes or treatment (Table 3).
**Coenzyme Q₁₀ limits diabetes-induced cardiomyocyte hypertrophy**

External LV dimension was unaltered in diabetes, however was reduced in dnPI3K mice compared to their respective Ntg controls (Table 3). Cardiomyocyte widths and areas were larger in both Ntg and dnPI3K diabetic mice compared with their citrate controls (Figure 4A-C). CoQ₁₀ supplementation effectively attenuated this cardiomyocyte hypertrophy in both Ntg and dnPI3K diabetic mice (Figure 4A-C). LV ANP expression was also increased by diabetes in both Ntg and dnPI3K mice, and was further increased in dnPI3K diabetic mice (Figure 5A). CoQ₁₀ effectively attenuated this increase, in both diabetic Ntg and dnPI3K mice (Figure 5A). Similarly, LV β-myosin heavy chain expression was increased in Ntg diabetic and dnPI3K diabetic mice compared to their respective controls (Figure 5B). CoQ₁₀ again effectively attenuated this hypertrophic gene expression, in both the Ntg and dnPI3K diabetic mice (Figure 5B).

**Coenzyme Q₁₀ limits diabetes-induced cardiac fibrosis**

LV CTGF expression was increased in diabetic Ntg mice compared to Ntg citrate controls, which was attenuated by CoQ₁₀ supplementation (Figure 6A). CTGF was further increased in dnPI3K citrate mice compared to Ntg citrate mice (Figure 6A), and showed a trend (P=0.07) for a further increase in dnPI3K diabetic mice compared to the upregulation already evident in dnPI3K citrate mice (Figure 6A). These increases in CTGF expression were effectively attenuated by CoQ₁₀ supplementation (Figure 6A). LV collagen deposition was increased in both Ntg and dnPI3K diabetic mice compared to their nondiabetic controls; these increases were effectively attenuated by CoQ₁₀ supplementation (Figure 6B-C). Additionally, there was
a trend for further increases in collagen deposition in the dnPI3K diabetic mice compared to Ntg diabetic mice ($P=0.08$, Figure 6B-C).

*Coenzyme Q<sub>9</sub> and Q<sub>10</sub> levels*

Diabetes was associated with increased plasma CoQ<sub>9</sub> levels, irrespective of whether the concentration was normalised to cholesterol, in both genotypes (Figure 7A, 7B). Supplementation with CoQ<sub>10</sub> effectively increased plasma CoQ<sub>10</sub> levels (both absolute concentration and normalised to cholesterol), except the Ntg citrate groups (Figure 7C, 7D). Neither diabetes nor CoQ<sub>10</sub> treatment altered LV concentrations of CoQ<sub>9</sub> (data not shown). Although there were no differences in LV concentrations of CoQ<sub>10</sub> between non-diabetic and diabetic mice, supplementation with CoQ<sub>10</sub> increased LV concentrations of CoQ<sub>10</sub> in diabetic mouse hearts (STZ Ntg untreated: $0.99 \pm 1.23$ pmol/µg protein; STZ Ntg CoQ<sub>10</sub>-treated: $1.24 \pm 0.06$ pmol/µg protein; $P = 0.048$).
Discussion

Heart failure, obesity and diabetes are often associated with defective or suboptimal PI3K(p110α) signalling. This is the first study to examine whether antioxidant supplementation can overcome the detrimental effects of the combined insults of diabetes and concomitant diminished cardiac PI3K(p110α) signalling, and its relationship to LV O$_2^-$ levels. We utilised a dnPI3K transgenic mouse model which exhibits a reduction in cardiac PI3K(p110α) signalling and then supplemented with CoQ$_{10}$ to demonstrate effective reductions in both NADPH oxidase and oxidative stress and multiple aspects of diabetic cardiomyopathy. Specifically, we have shown that exogenous CoQ$_{10}$ supplementation effectively reduced cardiac O$_2^-$ levels, plasma MDA levels and LV Nox2 expression. We also confirm that a reduction in cardiac PI3K(p110α) signalling leads to a more severe cardiomyopathy phenotype with concomitant type 1 diabetes, which is associated with increased oxidative stress. We show that CoQ$_{10}$ attenuated markers of LV diastolic dysfunction and effectively reduced the diabetic-induced increase in markers of LV cardiomyocyte hypertrophy.

*Coenzyme Q$_{10}$ supplementation reduces oxidative stress in diabetic dnPI3K animals*

Oxidative stress has been implicated in the development and progression of diabetic cardiomyopathy [4, 8, 34, 35]. Several studies have highlighted hyperglycaemia-induced changes in redox state as a critical stimulator of cardiac dysfunction and remodelling. The heart is particularly susceptible to oxidative damage as it possesses a low endogenous antioxidant content relative to other organs, and itself generates a large amount of ROS due to high mitochondrial concentration [36]. An additional source of endogenous cardiac O$_2^-$ is from NADPH oxidase [37]. We have examined several markers of oxidative stress in the
present study, both systemic (plasma MDA levels) and cardiac (LV lucigenin-enhanced chemiluminescence, Nox2 gene and protein expression, as well as cardiac 3-NT; a similar trend was evident for 4-HNE content). NADPH-driven lucigenin chemiluminescent detection of \( \text{O}_2^- \) was elevated in the Ntg diabetic heart compared with nondiabetic controls, accompanied by increases in plasma MDA levels, LV Nox2 gene expression and LV 3-NT content. Interestingly, dnPI3K diabetic mice exhibited even higher levels of plasma MDA levels, LV lucigenin chemiluminescence, LV Nox2 protein and LV 3-NT content (again with a similar trend for 4-HNE), in comparison to Ntg diabetic mice, consistent with previous observations in dnPI3K diabetic mice in vivo [10]. We have demonstrated that cardiac markers of oxidative stress, which are increased with diabetes, were effectively attenuated with CoQ\(_{10}\) supplementation, consistent with previous studies using less severe models of diabetic cardiomyopathy in vivo [6, 10].

*Coenzyme Q\(_{10}\) supplementation attenuates diastolic dysfunction in diabetic dnPI3K animals*

The mechanisms responsible for the distinct cardiomyopathy observed in diabetic patients, characterised by LV diastolic dysfunction and detrimental LV remodelling, are complex and multifaceted. Diastolic dysfunction results in impaired cardiac relaxation and is one of the earliest indicators of diabetic cardiomyopathy [4]. Activation of insulin-like growth factor-1 receptor (IGF1R)-PI3K(p110\(\alpha\)) signalling is protective against cardiac dysfunction under various pathological settings, [10, 15, 32, 38]. We have previously reported that diabetes is associated with a depressed E/A ratio, prolonged deceleration time, reduced LV -dP/dt and elevated LVEDP, all markers of impaired LV diastolic function [6, 32]. Additionally, we have shown that diabetic mice with increased cardiac PI3K activity due to expression of the caPI3K(p110\(\alpha\)) transgene were protected from diabetes-induced abnormal LV relaxation and
filling, whilst diabetic mice with reduced PI3K(p110α) signalling exhibited worsened LV diastolic function [10]. The present study provides further evidence that diabetes induces LV diastolic dysfunction, supporting the hypothesis that diminished PI3K(p110α) signalling further exacerbates cardiac dysfunction under a diabetic setting. In the current study, supplementation of CoQ₁₀ provided protection in our control Ntg T1DM model with diabetic cardiomyopathy, an observation previously reported in our type 2 diabetic mouse model (dbdb; [7]). The novel finding that CoQ₁₀ administration was also effective in protecting dnPI3K diabetic mice from diabetic cardiomyopathy is of particular significance given the increasing rates of obesity and our ageing population, settings associated with defective PI3K signalling [26-28].

*Coenzyme Q₁₀ administration attenuates diabetes-induced cardiomyocyte hypertrophy*

Early diastolic impairment in the diabetic heart is intricately linked to cardiac histopathology. Cardiomyocyte hypertrophy and cardiac fibrosis are frequently observed in both experimental models and clinical settings of diabetes [32, 39, 40]. Consistent with our previous findings [32], Ntg diabetic mice exhibited enlarged cardiomyocytes in terms of width and area compared to nondiabetic controls. This was accompanied by upregulation of hypertrophic gene expression in the absence of changes in heart weight, as previously observed [10, 32]. This may represent the net effect of increased LV apoptosis and the compensatory pathological hypertrophy of viable cardiomyocytes. As PI3K(p110α) signalling is a critical regulator of heart size [29], nondiabetic dnPI3K mice exhibited a lower HW/TL ratio and external LV diameter. Diabetic dnPI3K mice also displayed a lower HW/TL ratio despite elevated hypertrophic gene expression which was further elevated in dnPI3K diabetic mice compared to Ntg diabetic mice, consistent with diminished PI3K(p110α) signalling further
exacerbating pathological hypertrophic responses under cardiac pathology [12]. CoQ\textsubscript{10} supplementation effectively reduced cardiomyocyte width and area, and hypertrophic gene expression, in diabetic Ntg and dnPI3K mice. Similarly, diabetic mice (Ntg and dnPI3K) presented with increased cardiac collagen deposition and CTGF expression in comparison to their citrate controls, both effectively attenuated by CoQ\textsubscript{10} supplementation, consistent with CTGF as a key driver of diabetes-driven cardiac fibrosis.

Coenzyme Q\textsubscript{10} supplementation – potential mechanisms of protection

Antioxidant administration, in particular CoQ\textsubscript{10}, may protect the heart against diabetes-induced increased levels of oxidative stress and mitochondrial dysfunction which contribute to both cardiac dysfunction and adverse morphological changes. The main site of endogenous CoQ\textsubscript{10} action is considered to be at the mitochondria, where it functions primarily as an electron transporter and a O\textsubscript{2}– scavenger [41]. Whether exogenously administered CoQ\textsubscript{10} can be taken up by the cell and incorporated into the mitochondria remains to be elucidated. Interestingly, in the present study we did not detect a decrease in CoQ\textsubscript{9} or CoQ\textsubscript{10} concentrations with diabetes, as previously suggested in patients with diabetes [21] or heart failure [19, 20]. Indeed, the plasma concentration of CoQ\textsubscript{9}, the major form of CoQ in mice, was increased with diabetes. This increase was also observed for cholesterol-standardised CoQ\textsubscript{9}, indicating that it was independent of changes in the mevalonate pathway that is shared in the biosynthesis of cholesterol and CoQ. Importantly, we demonstrate that concentrations of plasma CoQ\textsubscript{10} and CoQ\textsubscript{9}, as well as LV CoQ\textsubscript{10}, were elevated with CoQ\textsubscript{10} administration. This is consistent with our previous findings that CoQ\textsubscript{10} levels increase in plasma and renal tissue by approximately 60% after chronic CoQ\textsubscript{10} supplementation in db/db diabetic mice [42], and the notion that increased tissue CoQ\textsubscript{10} attenuates oxidative stress, as assessed in the
present study. We administered CoQ\textsubscript{10} supplements via i.p. injection, whereas in humans CoQ\textsubscript{10} supplements are usually administered orally. Therefore, it will be important to establish whether in the mouse models used here, similar increases in cardiac CoQ\textsubscript{10} are observed with oral CoQ\textsubscript{10} supplements.

We have also previously shown that cardioprotection induced by increased LV IGF1R-PI3K(p110\textalpha) signalling may partially be attributed to improvements in mitochondrial function. Although not specifically examined in the present study, CoQ\textsubscript{10} treatment in dnPI3K diabetic animals may have reduced mitochondrial O\textsubscript{2}•\textsuperscript{-} levels whilst simultaneously improving mitochondrial bioenergetics; further studies are however required to interrogate this possibility. Additionally, studies are required to assess the impact of CoQ\textsubscript{10} supplementation on other markers of diabetic cardiomyopathy in this more severe setting, including apoptosis, and other NADPH oxidase subunits.

In conclusion, the outcomes of the present study demonstrate that exogenous CoQ\textsubscript{10} supplementation is effective in reducing the detrimental effects of diabetic cardiomyopathy in mouse models, including oxidative stress, diastolic dysfunction and cardiac hypertrophy and fibrosis, even in the exaggerated context of diminished LV PI3K activity. In settings of obesity, diabetes and ageing, PI3K signalling is often defective. Since PI3K signalling represents a key protective pathway in the heart, any therapy with the ability to overcome reduced cardioprotective signalling due to defective PI3K\textalpha, could be particularly valuable in a clinical setting. Furthermore, given that CoQ\textsubscript{10} supplementation may be beneficial in heart
failure patients, as recently reviewed [43], chronic CoQ_{10} supplementation may be a particularly attractive adjunct therapy for diabetic heart failure in light of our findings.
Funding sources

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Disclosure statement

The authors have nothing to disclose.

Author contributions

M.J.D., K.H., J.R.M., and R.H.R. contributed to the design of the study, data acquisition, data analysis, interpretation of results, and drafting of the manuscript. H. K., C.Q., X.J.D., A.A., N.C., R.S., and S.R. contributed to the design of some experiments, data acquisition and interpretation, and manuscript revision. All authors approved the final version of the manuscript.
References


phosphoinositide 3-kinase (p110alpha) activation increases the susceptibility to atrial fibrillation. *Am J Pathol* **175**:998-1009; 2009.


[38] Yang, J.; Pollock, J. S.; Carmines, P. K. NADPH oxidase and PKC contribute to increased Na transport by the thick ascending limb during type 1 diabetes. *Hypertension* **59**:431-436; 2012.


Figure legends:

**Figure 1.** CoQ10 treatment attenuates the diabetes-induced increase in cardiac Nox2.

Diabetes increased (A) LV lucigenin-enhanced chemiluminescence (a measure of $O_2^-\cdot$; n=5/group); (B) Nox2 gene expression (n=5/group); and (C) Nox2 protein (n=3-5/group). CoQ10 treatment attenuated all markers of oxidative stress. Data are presented as mean ± SEM; *$P<0.05$ (Two-way ANOVA); †$P<0.05$ (unpaired t-test).

**Figure 2.** Diabetes-induced cardiac and systemic oxidative stress was attenuated by CoQ10 treatment

LV 3-NT content and plasma MDA increased with diabetes in both genotypes, were further increased in diabetic dnPI3K mice, and were attenuated with CoQ10 (A, B, C). Data are presented as mean ± SEM; n=5/group; *$P<0.05$ (Two-way ANOVA); †$P<0.05$ (unpaired t-test). A similar trend was evident on 4-HNE content (images from one mouse heart per group are shown, D).

**Figure 3.** CoQ10 treatment attenuates diabetes-induced LV diastolic dysfunction.

(A) Diabetes reduced E/A ratio which was restored with CoQ10 treatment. (B) Diabetes prolonged deceleration time which was reduced with CoQ10 treatment. (C) Diabetes elevated LVEDP which was restored with CoQ10 treatment. (D) Diabetes reduced LV -dP/dt which was restored with CoQ10 treatment. Data are presented as mean ± SEM; n=5/group; *$P<0.05$ (Two-way ANOVA); †$P<0.05$ (unpaired t-test).

**Figure 4.** CoQ10 treatment attenuates diabetes-induced LV cardiomyocyte hypertrophy.

Cardiomyocyte width and area increased with diabetes in both genotypes and was attenuated with CoQ10 treatment (A, B). Representative images from each group are shown (C). Scale
bar = 20 µm. Data are presented as mean ± SEM; n=5/group; *P<0.05 (Two-way ANOVA); †P<0.05 (unpaired t-test).

**Figure 5.** CoQ₁₀ treatment attenuates markers of diabetes-induced LV hypertrophy.

Gene expression of LV (A) ANP and (B) β-myosin heavy chain were increased with diabetes in both genotypes and was attenuated with CoQ₁₀ treatment. Data are presented as mean ± SEM, fold change relative to Ntg citrate controls; n=5/group; *P<0.05 (Two-way ANOVA); †P<0.05 (unpaired t-test).

**Figure 6.** CoQ₁₀ treatment attenuates markers of diabetes-induced cardiac fibrosis.

LV expression of CTGF (connective tissue growth factor) (A) and LV collagen deposition (B) increased with diabetes and was attenuated by CoQ₁₀. Representative images of the presence of sirius-red stained LV collagen from each group are shown (C). Scale bar = 40 µm. Data are presented as mean ± SEM; n=5/group; *P<0.05 (Two-way ANOVA); †P<0.05 (unpaired t-test).

**Figure 7.** Plasma concentrations of coenzyme Q₉ and Q₁₀.

Plasma CoQ₉ and CoQ₉:cholesterol ratio increased with diabetes in both genotypes (A, B). Plasma CoQ₁₀ and CoQ₁₀:cholesterol ratio were increased in all CoQ₁₀ treatment groups except the Ntg citrate groups (C, D). Data are presented as mean ± SEM; n=5/group; *P<0.05 (Two-way ANOVA); †P<0.05 (unpaired t-test).
**Supplementary Figure 1. Genotyping of Ntg and dnPI3K mice.**

Mice were genotyped for the presence of the p110 transgene to identify dnPI3K mice and their non-transgenic (Ntg) littermate controls. DNA was extracted from mouse tail clips followed by PCR. DnPI3K mice display a visible band at 350bp.
<table>
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<td>18S</td>
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**Table 1. Oligonucleotide mouse sequences (5’-3’).**

Primers were generated from murine-specific sequences (GenBank) for SYBR® Green chemistry. ANP, atrial natriuretic peptide; CTGF, connective tissue growth factor; subunit of NADPH oxidase, Nox2; housekeeper, 18S.
### Table 2. Body composition of Ntg and dnPI3K mice treated with CoQ₁₀.

Data are presented as mean ± SEM and analysed by two-way ANOVA; n=5/group. *P<0.05 vs. same-genotype nondiabetic untreated mice, ^P<0.05 vs. same-genotype nondiabetic CoQ₁₀-treated mice, ‡P<0.05 vs. Ntg nondiabetic same-treatment mice, ‡P<0.05 vs. Ntg STZ same-treatment mice; CoQ₁₀; HW, heart weight; LW, lung weight; TL, tibia length.

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<td>93 ± 2^</td>
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<td>97 ± 2^</td>
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Table 3. Echocardiography and catheterisation analysis of heart size and function with CoQ_{10} treatment.

Data are presented as mean ± SEM and analysed by two-way ANOVA; n=5/group. *P<0.05 vs. Ntg nondiabetic same-treatment mice; ‡P<0.05 Ntg diabetic same-treatment mice. LVPW, left ventricular posterior wall thickness; LVEDD, left ventricular diastolic dimension; LVESD, left ventricular systolic dimension; SBP, systolic blood pressure; LVSP, left ventricular systolic pressure; +dP/dt, maximum rate of LVP change.

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Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Figure S1