# Targeting the upregulation of reactive oxygen species subsequent to hyperglycemia prevents type 1 diabetic cardiomyopathy in mice

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## ABSTRACT

Cardiac oxidative stress is an early event associated with diabetic cardiomyopathy, triggered by hyperglycemia. We tested the hypothesis that targeting left-ventricular (LV) reactive oxygen species (ROS) upregulation subsequent to hyperglycemia attenuates type 1 diabetes-induced LV remodeling and dysfunction, accompanied by attenuated proinflammatory markers and cardiomyocyte apoptosis. Male 6-week-old mice received either streptozotocin (55 mg/kg/day for 5 days), to induce type 1 diabetes, or citrate buffer vehicle. After 4 weeks of hyperglycemia, the mice were allocated to coenzyme Q$_{10}$ supplementation (10 mg/kg/day), treatment with the angiotensin-converting-enzyme inhibitor (ACE-I) ramipril (3 mg/kg/day), treatment with olive oil vehicle, or no treatment for 8 weeks. Type 1 diabetes upregulated LV NADPH oxidase (Nox2, p22$^{phox}$, p47$^{phox}$ and superoxide production), LV uncoupling protein UCP3 expression, and both LV and systemic oxidative stress (LV 3-nitrotyrosine and plasma lipid peroxidation). All of these were significantly attenuated by coenzyme Q$_{10}$. Coenzyme Q$_{10}$ substantially limited type 1 diabetes-induced impairments in LV diastolic function (E:A ratio and deceleration time by echocardiography, LV end-diastolic pressure, and LV $-dP/dt$ by micromanometry), LV remodeling (cardiomyocyte hypertrophy, cardiac fibrosis, apoptosis), and LV expression of proinflammatory mediators (tumor necrosis factor-$\alpha$, with a similar trend for interleukin IL-1$\beta$). Coenzyme Q$_{10}$'s actions were independent of glycemic control, body mass, and blood pressure. Coenzyme Q$_{10}$ compared favorably to improvements observed with ramipril. In summary, these data suggest that coenzyme Q$_{10}$ effectively targets LV ROS upregulation to limit type 1 diabetic cardiomyopathy. Coenzyme Q$_{10}$ supplementation may thus represent an effective alternative to ACE-Is for the treatment of cardiac complications in type 1 diabetic patients.

## Introduction

The alarming escalation in the global incidence and prevalence of diabetes mellitus over the past 20 years, to its current estimates

of almost 300 million, is projected to further increase to 440 million individuals by the year 2030. Of these, up to 70% will succumb to cardiovascular disease secondary to their diabetes [1,2]. Hyperglycemia-induced impairments in redox balance are considered a key trigger of diabetic complications, through upregulated generation of reactive oxygen species (ROS) [3–5], together with an impaired ability of the endogenous antioxidant defense system to remove them [6–8]. The heart is particularly susceptible to oxidative damage, as it possesses lower levels of endogenous antioxidants in comparison to other organs [9]. The predominant sources of ROS in the heart include NADPH oxidase (particularly Nox2 oxidase) and mitochondrial oxidative phosphorylation [10–12]. Recent evidence now suggests that NADPH oxidase-derived superoxide is a key trigger of mitochondrial dysfunction and upregulated mitochondrial superoxide generation [12,13]. Moreover, ROS upregulation is an important driver of cardiovascular inflammation, remodeling, and dysfunction [10,13–16]. Targeting this ROS upregulation is thus an

attractive therapeutic approach for the cardiac complications of diabetes.

One of the potential candidate drugs targeting ROS is coenzyme $Q_{10}$. Coenzyme $Q_{10}$ is an important component of the mitochondrial respiratory chain, in which it functions primarily as an electron transfer intermediate [17]. In its reduced state, it acts as a potent antioxidant by preventing the reaction of hydroxyl and superoxide radicals with the lipid membrane (lipid peroxidation) [18]. Clinical evidence suggests coenzyme $Q_{10}$ supplementation attenuates cardiac dysfunction in congestive heart failure and may also ameliorate cardiovascular risk [19,20]. Our own evidence specifically in the diabetes context demonstrates that coenzyme $Q_{10}$ supplementation ameliorates renal remodeling and dysfunction, accompanied by improvements in renal mitochondrial function and renal mitochondrial coenzyme $Q_{10}$ content [4] in *db/db* mice. Coenzyme $Q_{10}$ also effectively attenuated cardiomyocyte hypertrophy, cardiac remodeling, and diastolic dysfunction in *db/db* mice [21]. These beneficial effects were accompanied by a reduction in blood pressure and improved glycemic control, which may have indirectly contributed to the improvements observed in this experimental model of type 2 diabetes.

Diabetic patients are more susceptible to coronary heart disease and peripheral vascular disease; independent of (but often coexistent with) these macrovascular abnormalities, diabetic patients are also at considerable risk of developing distinct impairments directly at the level of the myocardium, termed "diabetic cardiomyopathy" [22–24]. Diabetic cardiomyopathy is characterized by early impairments in cardiac relaxation (diastolic dysfunction, identifiable on echocardiography and magnetic resonance imaging) [24,25], accompanied by structural abnormalities such as cardiomyocyte hypertrophy, myocardial fibrosis, and increased cardiomyocyte apoptosis [26–28]. Patients with type 1 diabetes suffer the disease for several decades (more than those with type 2 diabetes) [29], probably further increasing their likelihood of developing diabetes-induced heart failure, even when adjusted for age and coronary artery disease [30,31]. There remains a lack of choice of therapies for managing the changes in cardiac structure and function specifically in the diabetic heart, and the "gold standard" therapy for treating this diabetic cardiomyopathy, angiotensin-converting-enzyme inhibitors [32], are not well tolerated by a significant number of patients [32,33].

The objective of this study was to test the hypothesis that targeting cardiac ROS upregulation *after* hyperglycemia is established attenuates diabetes-induced remodeling and dysfunction, in a mouse model of type 1 diabetes, and this cardioprotection is associated with attenuation of proinflammatory markers and cardiomyocyte apoptosis. Chronic supplementation with the endogenous antioxidant coenzyme $Q_{10}$ was selected to target ROS in this study, as diabetic individuals are often coenzyme $Q_{10}$-deficient [34]. We now demonstrate in a male, nonobese mouse model of type 1 diabetes that chronic supplementation with coenzyme $Q_{10}$, commenced 4 weeks after the onset of hyperglycemia, effectively targets both the structural and the functional aspects of diabetic cardiomyopathy, independent of glycemic control and blood pressure. Moreover we demonstrate for the first time that these cardioprotective effects of coenzyme $Q_{10}$ are associated with restoration of NADPH oxidase expression (Nox2, p22[phox], and p47[phox]), oxidative stress, and proinflammatory signaling in the diabetic myocardium.

## Material and methods

### Animal model

All animal research was conducted in accordance with the National Health and Medical Research Council of Australia guidelines, and approval was obtained from the Alfred Medical Research and Education Precinct (AMREP) Animal Ethics Committee. Age-matched male FVB/N mice were bred and housed in the AMREP Precinct Animal Centre and maintained under a 12-h light/dark cycle with up to four littermates per cage. At 6 weeks of age, mice were assigned to either nondiabetic (sham) or diabetic groups. Type 1 diabetes was induced in mice by five consecutive daily intraperitoneal injections of streptozotocin (STZ; 55 mg/kg body wt, in 0.1 mol/L citrate buffer, pH 4.5; Sigma–Aldrich, St. Louis, MO, USA) [35–37]. An equivalent volume of citrate buffer vehicle was administered to the sham group. Four weeks after the initial STZ/citrate buffer injection, the mice were further allocated into the untreated group, the coenzyme $Q_{10}$-treated group (10 mg/kg/day, dissolved in olive oil vehicle, administered daily via oral gavage; Sigma), the vehicle-treated group (olive oil alone, oral gavage), and the ramipril-treated group (3 mg/kg/day in drinking water; Sigma). Doses of both coenzyme $Q_{10}$ and ramipril were based on those previously effective in a mouse model of type 2 diabetes [21]. Mice were followed for a further 8 weeks before euthanasia and tissue collection. Blood glucose levels were monitored fortnightly using a handheld glucometer (Accu-Check Advantage; Roche, Basel, Switzerland). Animals with blood glucose levels exceeding 28 mmol/L were considered diabetic. Blood was also retained for measurement of glycated hemoglobin (GHb) by HPLC (CLC330 GHb Analyzer; Primus, Kansas City, MO, USA) [38].

### Analysis of cardiac function in vivo

Two-dimensional M-mode echocardiography and Doppler echocardiography were employed to noninvasively assess endpoint LV function. At 18 weeks of age, mice were anesthetized with a cocktail of ketamine, xylazine, and atropine (100, 10, and 1.2 mg/kg ip). Echocardiographic images were obtained using a Philips iE33 ultrasound machine with a 15-MHz linear array transducer as previously described [21,35,36]. Parameters derived from M-mode echocardiography included LV end-systolic dimension (LVESD), LV end-diastolic dimension (LVEDD), and fractional shortening (calculated as $((LVEDD − LVESD)/LVEDD) \times 100\%$). LV filling (LV *E:A* ratio, the ratio of peak early, *E,* to late, *A,* transmitral blood flow velocities across the mitral valve) and deceleration time (on *E* velocity) were assessed using Doppler echocardiography. As previously described [21,36], we measured by micromanometry ventricular and aortic blood pressure parameters including LV end-diastolic pressure (LVEDP), aortic systolic (AoSBP) and diastolic blood pressures, and LV $\pm dP/dt$ (maximal rate of LV pressure rise or fall during contraction or relaxation), in addition to tau.

### Tissue collection and histology

The wet weight of the heart and lungs and the length of the tibia bone were recorded and used to normalize the heart weight (HW: TL). A portion of the ventricle was fixed and stained with hematoxylin and eosin (H&E) or 0.1% picrosirius red for determination of cardiomyocyte width and LV interstitial collagen deposition, as previously described [21,36]. Cardiomyocyte cross-sectional area was determined as a second measure of cardiomyocyte hypertrophy, from > 60 individual cardiomyocytes per mouse, calculated from cell outlines using Image-Pro Plus (Media Cybernetics, Bethesda, MD, USA). Apoptosis was examined in NBF-fixed ventricular sections using the CardioTACS In Situ Apoptosis Detection Kit (Trevigen, Gaithersburg, MD, USA). Fifty fields per ventricle section were randomly captured and used for the quantitation of apoptotic cells, which were expressed as a percentage of nonapoptotic cells [21,36]. For immunohistochemical assessment of the p22[phox] subunit of NADPH oxidase, ventricular sections were deparaffinized in xylene, rehydrated, and rinsed in phosphate-buffered saline (PBS), before proteolytic-induced epitope retrieval using proteinase K [36].