Combined therapeutic benefit of mitochondria-targeted antioxidant, MitoQ_{10}, and angiotensin receptor blocker, losartan, on cardiovascular function

Objective: Mitochondria-derived reactive oxygen species (ROS) play important roles in the development of cardiovascular disease highlighting the need for novel targeted therapies. This study assessed the potential therapeutic benefit of combining the mitochondria-specific antioxidant, MitoQ_{10}, with the low-dose angiotensin receptor blocker (ARB), losartan, on attenuation of hypertension and left ventricular hypertrophy. In parallel, we investigated the impact of MitoQ_{10} on cardiac hypertrophy in a neonatal cardiomyocyte cell line.

Methods and results: Eight-week-old male stroke-prone spontaneously hypertensive rats (SHRSPs, n = 8–11) were treated with low-dose losartan (2.5 mg/kg per day); MitoQ_{10} (500 μmol/l); a combination of MitoQ_{10} and losartan (M+L); or vehicle for 8 weeks. Systolic pressure and pulse pressure were significantly lower in M+L rats (167.1 ± 2.9 mmHg, 50.2 ± 2.05 mmHg) than in untreated SHRSP (206.6 ± 9 mmHg, P < 0.001; 63.7 ± 2.7 mmHg, P = 0.001) and demonstrated greater improvement than MitoQ_{10} or low-dose losartan alone, as measured by radiotelemetry. Left ventricular mass index was significantly reduced from 22.8 ± 0.74 to 20.1 ± 0.61 mg/mm in the combination group (P < 0.05). Picrosirius red staining showed significantly reduced cardiac fibrosis in M+L rats (0.82 ± 0.22 A.U.) compared with control (5.94 ± 1.35 A.U., P < 0.01). In H9c2 neonatal rat cardiomyocytes, MitoQ_{10} significantly inhibited angiotensin II mediated hypertrophy in a dose-dependent manner (500 nmol/l MitoQ_{10} 153.7 ± 3.1 microns vs. angiotensin II 200.1 ± 3.6 microns, P < 0.001).

Conclusion: Combining MitoQ_{10} and low-dose losartan provides additive therapeutic benefit, significantly attenuating development of hypertension and reducing left ventricular hypertrophy. In addition, MitoQ_{10} mediates a direct antihypertrophic effect on rat cardiomyocytes in vitro. MitoQ_{10} has potential as a novel therapeutic intervention in conjunction with current antihypertensive drugs.

Keywords: cardiac fibrosis, cardiac hypertrophy, hypertension, mitochondria, reactive oxygen species

Abbreviations: A.U., arbitrary units; ACE, angiotensin-converting enzyme; AngII, angiotensin II; ARB, angiotensin receptor blocker; AUC, area under curve; CMI, cardiac mass index; De, external diameter; Di, internal diameter; dTPP, decyl triphenylphosphonium; eGFR, estimated glomerular filtration rate; KCl, potassium chloride; L-NAME, N\textsuperscript{-}nitro-l-arginine methyl ester; LVMI, left ventricular mass index; M+L, MitoQ_{10} and losartan; MRA, mesenteric resistance artery; NOS, nitric oxide synthase; ROS, reactive oxygen species; SHRSP, stroke prone spontaneously hypertensive rat

INTRODUCTION

Accumulating evidence demonstrates that mitochondria-derived reactive oxygen species (ROS) play an important role in the development of cardiovascular disease [1,2]. Conventional antioxidants cannot impact on mitochondria-derived ROS owing to their limited accumulation within mitochondria, highlighting the need for targeted therapies. Previously, we demonstrated that oral administration of the mitochondria-targeted antioxidant MitoQ_{10} attenuates the development of hypertension, improves endothelial nitric oxide bioavailability and reduces cardiac hypertrophy in the stroke-prone spontaneously hypertensive rat (SHRSP) [3]. Although the MitoQ_{10}-mediated reduction in blood pressure was modest, it may represent an important novel therapeutic agent for resistant hypertension and end-organ damage if combined with established antihypertensive drugs. Resistant hypertension remains a common problem with an estimated prevalence of 20–30%, despite a three-drug regimen [4].

Journal of Hypertension 2014, 32:555–564

*BHF Glasgow Cardiovascular Research Centre, University of Glasgow, Glasgow and bMRC Mitochondrial Biology Unit, Cambridge, UK

Correspondence to Dr Delyth Graham, Institute of Cardiovascular and Medical Sciences, BHF Glasgow Cardiovascular Research Centre, University of Glasgow, 126 University Place, Glasgow G12 8TA, UK. Tel: +44 141 300 2524; fax: +44 330 6997; e-mail: Delyth.Graham@glasgow.ac.uk

Received 21 December 2012 Accepted 15 October 2013

J Hypertens 32:555–564 © 2014 Wolters Kluwer Health | Lippincott Williams & Wilkins. This is an open-access article distributed under the terms of the Creative Commons Attribution-NonCommercial-NoDerivatives 3.0 License, where it is permissible to download and share the work provided it is properly cited. The work cannot be changed in any way or used commercially.

DOI:10.1097/HJH.0000000000000554
This suggests existence of blood pressure elevating mechanisms, which are not fully addressed by current antihypertensive therapies. Combining a mitochondria-targeted antioxidant such as MitoQ10 with commonly used antihypertensive agents may provide additive effects against the morbidity and mortality associated with resistant hypertension.

Studies by Dai et al. [5,6] indicate a critical role of mitochondrial ROS in cardiac hypertrophy, fibrosis and failure strengthening the rationale for mitochondria-targeted drugs. Our previous studies suggest a direct effect of MitoQ10 on cardiac hypertrophy, demonstrated by a significant reduction in cardiac mass index (CMI) despite relatively small blood pressure attenuation [3]. Similarly, direct blood pressure independent effects on cardiac hypertrophy have been demonstrated with low-dose angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARBs) [7–9]. Combining MitoQ10 with a low-dose commonly used antihypertensive agent may achieve additional risk reduction beyond blood pressure lowering by addressing both cytosolic and mitochondrial mechanisms of ROS generation. The aims of this study were to investigate the potential complementary effects of a combined therapeutic strategy on the development of hypertension and cardiac hypertrophy using the mitochondria-targeted antioxidant MitoQ10 and a low-doseARB, losartan. In parallel, we investigated the contribution of mitochondrial oxidative damage to cardiomyocyte hypertrophy in vitro using H9c2 neonatal cardiomyocytes. These studies suggest new therapeutic interventions for resistant hypertension and related organ damage in humans.

MATERIALS AND METHODS

An expanded Methods section is available in the Online Data Supplement, http://links.lww.com/HJH/A299.

In-vivo experimental procedures

An inbred colony of SHRSP has been maintained at the University of Glasgow since 1991. Animals were housed under 12 h light/dark cycles at ambient temperature and were maintained on normal rat chow (Rat and Mouse No. 1 maintenance diet, Special Diet Services). All studies were conducted in accordance with the Animals Scientific Procedures Act 1986. Eight-week-old male SHRSP (8–11 rats per group) were treated with low-dose losartan (2.5 mg/kg per day); MitoQ10 (500 μmol/l); a combination of MitoQ10 and losartan (M + L); or vehicle for 8 weeks. MitoQ10 was administered in drinking water, and losartan potassium (Sigma-Aldrich, Gillingham, Dorset, UK) administered daily and mixed with highly palatable baby food. SBP was measured by tail-cuff plethysmography [10,11] for the first 4 weeks of study. At 12 weeks of age, rats were implanted with radiotelemetry probes (Dataquest IV telemetry system; Data Sciences International, St Paul, Minnesota, USA) for haemodynamic measurement over the final 4 weeks of treatment [3,12]. Metabolic cages were used for the measurement of volume intake, urine output and collection of 24-h urine samples from control and treated rats at 16 weeks of age. At sacrifice, blood samples were taken by cardiac puncture, followed by measurement of heart weight, left ventricle as well as septum weight and kidney weight for the calculation of CMI, left ventricular mass index and renal mass index, respectively (CMI, LVMI, RMI corrected for tibia length). Heart apexes were fixed in 10% formalin for histological fibrosis assessment by picrosirius red staining. Aortae were taken for organ bath pharmacology and liver samples snap-frozen in liquid nitrogen.

Ex-vivo experimental procedures

Organ bath pharmacology

Organ bath pharmacology was used as described previously [3,11] to test contractile responses in aorta from control and drug-treated SHRSP. Vessels (approximately 4 mm in length) were pretreated with potassium chloride (KCl, 100 mmol/l), followed by construction of cumulative concentration–response curves to phenylephrine (10 nmol/l to 10 μmol/l in the absence and presence of N5-nitro- L-arginine methyl ester (L-NAME, 100 μmol/l) to inhibit nitric oxide species (NOS). Consecutive dose–response curves were carried out in the same vessel, measured first in the absence of L-NAME, followed by two washes with Krebs’ buffer and then in the presence of L-NAME. The difference in tension in the absence and presence of L-NAME provides a measure of basal NO bioavailability and was calculated over the full dose–response curve and expressed as area under the curve (AUC). In addition, the rings were preconstricted to the EC50 of phenylephrine and a concentration–response curve for relaxation to carbachol (10 nmol/l to 10 μmol/l) was obtained to measure stimulated NO release for which AUC was calculated. Responses to phenylephrine were standardized against the initial contractile response to KCl.

Histology

Five-micrometre paraffin-embedded sections of heart apex were cut and deparaffinized with two washes in Histoclear (Fisher Scientific, Loughborough, UK) followed by rehydration through an ethanol concentration gradient. Fibrosis was assessed using picrosirius red staining (Sigma-Aldrich) specific for collagen type I and III. Sections were incubated under dark conditions in 0.1% picrosirius red solution, washed, rehydrated and cover-slip mounted. A colour threshold application was used to measure the average intensity of picrosirius red stain in five sections per heart apex from 3–8 rats per treatment group (ImageProPlus 4.1; Media Cybernetics, Marlow, UK). This involved transformation of pixel values to optical density units using the Area of Interest selection tool and Macro from the ImageProPlus software [13].

Biochemical analysis

Urine sodium, potassium, urea and total protein levels were measured by routine biochemical analysis (Biochemistry Department, Gartnavel General Hospital, Glasgow, UK). Urinary and plasma creatinine were assessed for estimated glomerular filtration rate (eGFR) by the QuantiChrom Creatinine Assay Kit [Universal Biologicals (Cambridge) Ltd, Cambridge, UK] and urinary protein concentrations assessed by bicinchoninic acid (BCA) Protein Assay (Pierce,
Lipid peroxidation in liver samples was determined by malondialdehyde (MDA) assay kit (Bioxytech LPO-586 Assay Kit; OxisResearch, Portland, Oregon, USA) according to manufacturer's instructions and normalized to protein concentration.

**In-vitro treatment of cardiomyocytes with MitoQ**

In-vitro assays were conducted in the H9c2 immortalized cardiomyocyte cell line derived from rat neonatal cardiomyocytes [14,15]. Cells were pretreated with MitoQ10 or the

---

**FIGURE 1** Effect of MitoQ10 treatment alone and in combination with low-dose losartan on the haemodynamic profile of SHRSF. (a) SBP measured by tail-cuff plethysmography and radiotelemetry was reduced by MitoQ10 and losartan alone, and demonstrated greatest reduction with combined therapy (*P* = 0.004, **P** < 0.001 vs. control, *P* = 0.006 vs. MitoQ10 and *P* = 0.05 vs. losartan). (b) DBP was significantly reduced by individual and combination treatment (**P** = 0.032, ***P** = 0.005, **P** = 0.003 vs. control). (c) Pulse pressure shows small but significant reduction in losartan-only treated rats, and more pronounced reduction with combination therapy (**P** = 0.025, ***P** = 0.001 vs. control, *P* = 0.01 vs. MitoQ10, *P* = 0.057 vs. losartan only). (d) Heart rate measured by radiotelemetry was significantly reduced by MitoQ10 (**P** = 0.012 vs. control, *P* = 0.001 vs. losartan). (e) Night-time heart rate was significantly lowered by MitoQ10 alone and by combination treatment compared with control and losartan only (**P** = 0.02, ***P** = 0.001 vs. control, *P* = 0.006 **P** < 0.001 vs. losartan only; n = 8–11).

---

Combined benefit of MitoQ10 and losartan on cardiovascular function
control compound decyl triphenylphosphonium (dTPP) (10–500 nmol/l) for 18 h prior to angiotensin II (AngII) stimulation (100 nmol/l). After 96 h, cells were fixed with 2% paraformaldehyde, stained overnight with 2% crystal violet and cell size measured using ImageProPlus 4.1 software (Media Cybernetics). For each condition, 180 random cells were measured from 18 fields of view; experiments were repeated in triplicate on three independent occasions.

The MitoQ₁₀ analogue IBTP was colocalized to mitochondria using confocal microscopy as previously demonstrated (see supplementary methods, http://links.lww.com/HJH/A299) [16].

Cell Titre 96R Non-Radioactive Cell Proliferation Assay (Promega, Southampton, UK) was used according to manufacturer’s instructions to test the cytotoxicity of MitoQ₁₀ and dTPP. A spectrometer (Wallac Victor2 plate-reader, Perkin
Elmer, Turku, Finland) measured absorbance of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at 570 nm. Experiments were repeated in quadruplicate on three independent occasions.

**Statistical analysis**

Results are expressed as mean ± SEM. In-vivo experiments were performed with 8–11 rats per group. Repeated-measures analysis of variance (ANOVA) with Tukey pairwise comparison was used to compare radiotelemetry data. Ex-vivo comparisons were performed by one-way ANOVA with Tukey’s multiple comparison test. In-vitro comparisons between groups were performed by one-way ANOVA with Bonferroni’s multiple comparison test. Statistical significance was considered with P values less than 0.05.

**RESULTS**

Average day-time SBP of control SHRSP increased from 129.0 ± 4.9 mmHg to a maximum of 206.6 ± 9.0 mmHg over the 8-week study (Fig. 1a). MitoQ₁₀ or low-dose losartan alone resulted in significant attenuation of SBP (MitoQ₁₀ 185.5 ± 6.0 mmHg, P = 0.032, Los; 172.9 ± 1.7 mmHg, P = 0.004). When both drugs were used in combination, there was a highly significant reduction in SBP compared with control (167.1 ± 2.9 mmHg, P < 0.001), which was significantly lower than either treatment alone. DBP was significantly reduced by either drug alone (MitoQ₁₀ 126.3 ± 3.7 mmHg, P = 0.032, Los; 118.2 ± 0.77 mmHg, P = 0.003) or in combination (116.3 ± 2.1 mmHg, P = 0.005) or in combination (142.6 ± 6.5 mmHg, Fig. 1b) but suggest no additive benefit of combined therapy. Pulse pressure was significantly reduced by combination treatment (50.2 ± 2.1 mmHg, P = 0.001) compared with control (63.7 ± 2.7 mmHg) and indicates additional benefit compared with either drug alone (Fig. 1c). Heart rate was significantly lower in MitoQ₁₀-treated SHRSP (329.3 ± 3.2 bpm) than in control (351.6 ± 4.3 bpm, P = 0.012, Fig. 1d). Night-time heart rate, when the rats are most active, was significantly reduced in MitoQ₁₀-treated SHRSP (126.3 ± 3.7 mmHg, P = 0.032, Los; 118.2 ± 0.77 mmHg, P = 0.003) or in combination (116.3 ± 2.1 mmHg, P = 0.005) or in combination (142.6 ± 6.5 mmHg, Fig. 1b) but suggest no additive benefit of combined therapy. Pulse pressure was significantly reduced by combination treatment (50.2 ± 2.1 mmHg, P = 0.001) compared with control (63.7 ± 2.7 mmHg) and indicates additional benefit compared with either drug alone (Fig. 1c).

Basal nitric oxide bioavailability in aorta from control and drug-treated SHRSP is illustrated in Fig. 2a and expressed as AUC in Fig. 2b. Basal nitric oxide bioavailability was significantly improved in losartan-treated SHRSP compared with control (P < 0.05) and in combination treatment compared with control (**P < 0.05) and MitoQ₁₀ only (**P < 0.05). Relaxation to carbachol was not significantly different in aortic rings from control and drug-treated SHRSP (Fig. 2d). Treatment with losartan only resulted in a significant decrease in liver MDA levels (P < 0.05). The other treatment groups demonstrated similar trends but were not significantly different to untreated controls. No significant differences were observed for basal superoxide production in aortic rings or plasma lipid peroxidation (Supplementary Figure ii, http://links.lww.com/HJH/A299).

Kidney and urine characteristics for MitoQ₁₀, losartan and combination-treated SHRSP were assessed (Table 1). There were no significant differences in kidney mass index, eGFR or urinary levels of Na⁺, K⁺ or urea between control and treatment groups. However, total urinary protein excretion was significantly reduced in MitoQ₁₀-treated rats (P < 0.05) with a similar trend in the combination therapy group.

Combination treatment significantly reduced cardiac mass index (CMI) (M + L 27.3 ± 0.77 mg/mm, control 30.9 ± 0.98 mg/mm, P < 0.05) and LVMI (M + L 20.1 ± 0.61 mg/mm, control 22.8 ± 0.74 mg/mm, P < 0.05) compared with untreated controls (Fig. 3a, b). Cardiac fibrosis was assessed by picrosirius red staining (Fig. 3c) and collagen I and III immunohistochemistry (Supplementary Figure iii, http://links.lww.com/HJH/A299). Elevated levels of fibrillar collagen in perivascular and interstitial regions (Fig. 3c) in control hearts were significantly attenuated by combination treatment (P < 0.01) (Fig. 3d).

Exposure of H9c2 cardiomyocytes to AngII increased cell size from 159.2 ± 3.3 to 200.1 ± 3.6 μm (P < 0.001) (Fig. 4a), and this was prevented by MitoQ₁₀ but not by the control compound dTTP (Fig. 4b). MitoQ₁₀ had no effect on cell size in unstimulated H9c2 cells and cell viability was not affected by MitoQ₁₀ or dTTP (Supplementary Figure iv, http://links.lww.com/HJH/A299). Immunofluorescence to detect the MitoQ₁₀ analogue IBTP in cardiomyocytes demonstrated uptake and colocalization to mitochondria during hypertrophy (Fig. 4c).

**TABLE 1. Effect of MitoQ₁₀ alone and in combination with low-dose losartan on renal parameters**

|                      | Control              | MitoQ₁₀              | Losartan             | M + L     |
|----------------------|----------------------|----------------------|----------------------|-----------|
| Kidney/tibia ratio (mg/mm) | 27.1 ± 1.06          | 28.1 ± 0.90          | 27.6 ± 1.04          | 28.1 ± 0.91 |
| eGFR (ml/min per g kidney weight) | 0.25 ± 0.03          | 0.17 ± 0.02          | 0.22 ± 0.02          | 0.20 ± 0.01 |
| Urinary protein (mg/ml per 24 h) | 120.7 ± 15.4         | 75.8 ± 10.5*         | 110.5 ± 8.0          | 83.6 ± 7.7 |
| Urinary Na⁺ (mmol/24h) | 0.92 ± 0.21          | 0.84 ± 0.35          | 0.85 ± 0.08          | 1.07 ± 0.20 |
| Urinary K⁺ (mmol/24h) | 2.06 ± 0.28          | 2.00 ± 0.31          | 2.14 ± 0.20          | 2.18 ± 0.19 |
| Urea (mmol/24h)      | 6.99 ± 0.54          | 4.92 ± 1.36          | 5.74 ± 1.26          | 6.02 ± 0.41 |

Values are means ± SEM; n = 8–11 rats per group. eGFR, estimated glomerular filtration rate. *P < 0.05 compared with untreated control.
DISCUSSION

This study demonstrates that administration of MitoQ\textsubscript{10} provides complementary therapeutic benefit to an established antihypertensive agent. Our data suggest that MitoQ\textsubscript{10} targets elements of the hypertensive and hypertrophic processes involving mitochondrial oxidative damage that are not fully addressed by current antihypertensive drugs. Furthermore, our in-vitro studies demonstrate that MitoQ\textsubscript{10} has a direct antihypertrophic action in cardiomyocytes indicating that its in-vivo action may be partly independent of blood pressure lowering.

We have previously demonstrated that the mitochondria-specific antioxidant MitoQ\textsubscript{10} has important

---

**FIGURE 3** Effect of MitoQ\textsubscript{10} alone and in combination with low-dose losartan on cardiac and left ventricular hypertrophy and fibrosis. (a) CMI and (b) LVMI were significantly reduced in combination-treated SHRSP compared with control \((n = 8–11, P < 0.05\) vs. control). Single-drug therapy had no significant effect on CMI or LVMI. (c) Representative images of perivascular and interstitial cardiac fibrosis (magnification \(\times 10\), scale bar \(= 100\) mm) analysed with picrosirius red staining and quantified using ImageProPlus. (d) Perivascular and (e) interstitial fibrosis was significantly reduced in combination-treated SHRSP compared with controls \((P < 0.05\) vs. control, \(n = 3–8\)).
antihypertensive action in young SHRSP [3] emphasizing a key role for mitochondrial-oxidative stress in the development of hypertension. Other in-vivo studies [17–21] have shown that MitoQ<sub>10</sub> is effective against mitochondrial oxidative damage in rodent models of sepsis, cardiac reperfusion, metabolic syndrome and diabetic nephropathy. Together, these findings highlight the importance of targeted antioxidant therapy in a range of diseases affected by mitochondrial oxidative damage.

Several sources of ROS may contribute to the development of cardiovascular disease, including NADPH oxidase, xanthine oxidase, uncoupled nitric oxide synthase (NOS) and the mitochondrial electron transport chain [1,22–24]. There is a complex interplay between these different ROS sources in the cardiovascular system, and the role of MitoQ<sub>10</sub> in modulating this balance remains an area of active investigation.

**FIGURE 4** Effect of MitoQ<sub>10</sub> on AngII-induced cardiomyocyte hypertrophy. (a) Representative images of control and AngII-stimulated H9c2 cardiomyocytes after 96 h incubation and crystal violet staining (magnification ×10, scale bar = 100 μm). (b) MitoQ<sub>10</sub> prevented development of AngII-stimulated hypertrophy in a dose-dependent manner (*P < 0.001 vs. control, *P < 0.05 and $P < 0.001 vs. 10 nmol/l MitoQ<sub>10</sub>), whereas dTPP had no effect. (c) The MitoQ<sub>10</sub> analogue, IBTP, was detected in cells after 96 h, co-localized with MitoTracker Red. Nuclei were counterstained with DAPI (magnification ×63, scale bar = 50 μm).
and the precise contributions to the underlying disease mechanisms remain obscure. There is evidence for ROS-induced ROS production, with mitochondria being both stimulated by NADPH oxidases and also acting as the initiating stimulus for further ROS generation [2,5,24–26], creating a potential feed-forward cycle of ROS production. In the present study, MitoQ\textsubscript{10} was tested in combination with a low-dose commonly prescribed ARB. This combined therapy demonstrated significant additive haemodynamic benefit, anti-hypertrophic and anti-fibrotic action when compared with control or single therapies. Only DBP failed to show additive reduction during combined versus single therapy, suggesting that MitoQ\textsubscript{10} and losartan share a common anti-hypertensive mechanism for diastolic pressure. The lack of an additive effect on DBP is potentially beneficial, as evidence suggests that therapeutically induced diastolic hypertension can be a risk factor for increased coronary events [27–29]. In line with the non-additive effects on DBP, we also observe similar significant equivalent improvements in aortic nitric oxide bioavailability in the combination and losartan-only group. The significant reduction in heart rate by MitoQ\textsubscript{10} treatment, either alone or in combination with losartan, during the active (night-time) period, was not observed with single losartan therapy. MitoQ\textsubscript{10} may alter heart rate by reducing oxidative stress within the rostral ventrolateral medulla (RVLM), as mitochondrial-derived ROS has been shown to mediate sympathoexcitation within this brain stem cardiovascular control centre [30]. We also demonstrate reduced urinary protein levels in MitoQ\textsubscript{10} and combination-treated rats indicating that mitochondria-specific antioxidants may also play a role in reducing end-organ damage in the kidney.

Increased ROS production stimulates myocardial fibrosis and is associated with reduced cardiac function [31]. This may be due to the accumulation of perivascular collagen compressing coronary arterioles and the accumulation of fibrillar collagen in the myocardial interstitium increasing wall stiffness and impairing cardiac systolic function [32]. Significant cardiac fibrosis has previously been reported in male SHRSP by 16 weeks of age [33,34], and in the current study, we demonstrate prevention of cardiac fibrosis by combination treatment. The design of the current study does not allow direct mechanistic assessment of the anti-fibrotic action of MitoQ\textsubscript{10}. This would require investigation in alternative models of cardiac fibrosis such as the transverse aortic constriction (TAC) model or the renal hypertensive rat (RHR), permitting pre and post-surgery assessment of MitoQ\textsubscript{10} action. However, we suggest that the significant reduction in collagen deposition by combination therapy is likely to be due to decreased oxidative damage. Losartan lowers blood pressure and decreases left ventricular hypertrophy by blocking AT\textsubscript{1} receptors, and reducing AngII-dependent activation of NADPH oxidases, [35,36]. This, in turn, can reduce mitochondrial-derived ROS production [24,26] in addition to the direct mitochondrial antioxidant potential of MitoQ\textsubscript{10}. Our data suggest that combining a mitochondria-targeted antioxidant with losartan may provide superior anti-hypertensive, anti-hypertrophic and anti-fibrotic action by interrupting the vicious cycle of ROS production and oxidative damage.

Although this study was unable to demonstrate direct MitoQ\textsubscript{10}-induced improvement in ROS, this was not entirely unexpected considering that O$_2^-$, the primary ROS produced by the mitochondria, is not readily diffusible across mitochondrial membranes and is largely dismutated to H$_2$O$_2$ [37]. Moreover, it is well recognized that measurement of reactive molecules in biological environments is inherently challenging due to their short lifespan and the limited selectivity of detection systems [38–40].

Cardiac hypertrophy is an adaptive response to increased blood pressure, and as a result, practically all antihypertensive agents reduce LVMI, emphasizing the importance of haemodynamic load in the pathogenic process. However, convincing evidence from studies with low-dose ACE inhibitors and ARBs also demonstrate blood pressure independent attenuation of left ventricular hypertrophy [7–9]. The beneficial action of MitoQ\textsubscript{10} on cardiac hypertrophy despite modest blood pressure reduction suggests a direct role for mitochondrial oxidative stress in the hypertrophic process rather than simply a secondary effect of blood pressure reduction [3]. In support of this, H9c2 cardiomyocytes, a well established model of hypertrophy [14,15,41], showed dose-dependent inhibition of hypertrophy by MitoQ\textsubscript{10}. These data suggest a potentially important therapeutic role for MitoQ\textsubscript{10} in heart disease independent of its haemodynamic effects.

The current preclinical study has investigated the beneficial action of MitoQ\textsubscript{10} during the developmental of hypertension and left ventricular hypertrophy. The next stage will be to determine the effects of mitochondria-specific antioxidant efficacy in the more clinically relevant setting of established hypertension. These future studies will provide important information for potential clinical trials of human resistant hypertension, as MitoQ\textsubscript{10} was proven well tolerated in two phase II clinical trials [42,43].

Clinical perspectives
Oxidative stress and mitochondrial dysfunction are increasingly implicated in cardiovascular disease and yet this is a neglected aspect in current treatment strategies. We demonstrate that MitoQ\textsubscript{10} is a potential complementary therapeutic intervention to current antihypertensive agents, particularly in the treatment of haemodynamic changes and end-organ damage associated with resistant hypertension.

ACKNOWLEDGEMENTS
British Heart Foundation Chair (CH98001) and Programme grant funding (RG/07/005), EU Sixth Framework Programme Integrated Project (LSHG_CT 2005-019015 EURA-Tools), EU Community’s Seventh Framework Programme (FP7/2007–2013) under grant agreement (HEALTH-F4–2010–241504 EURATRANS) awarded to A.F.D. BHF PhD studentship (FS/07/029/23022) and project grant (PG/11/82/29136) awarded to D.G. and A.F.D.

Conflicts of interest
M.P.M. is on the scientific advisory board of, and holds shares in, Antipodean Pharmaceuticals Inc, which is commercializing MitoQ\textsubscript{10}.
REFERENCES

1. Gutierrez J, Bullinger SW, Darley-Usmar VM, Landar A. Free radicals, mitochondria, and oxidised lipids. The emerging role in signal transduction in vascular cells. Circ Res 2006; 99:924–932.

2. Dikalova AE, Biknevica AV, Badzyn K, Nazarewicz RR, McCann L, Lewis W, et al. Therapeutic targeting of mitochondrial superoxide in hypertension. Circ Res 2010; 107:106–116.

3. Graham D, Huynh NN, Hamilton CA, Beattie E, Smith RA, Cochemé HM, et al. Mitochondria-targeted antioxidant MitoQ10 improves endothelial function and attenuates cardiac hypertrophy. Hypertension 2009; 54:322–328.

4. Calhoun DA, Jones D, Textor S, Goff DC, Murphy TP, Toto RD, et al. Resistant hypertension: diagnosis, evaluation, and treatment. A scientific statement from the American Heart Association Professional Education Committee of the Council for High Blood Pressure Research. Hypertension 2008; 51:1403–1419.

5. Dai DF, Johnson SC, Villarin M, Kutyavin V, Santana LF, Rabinowitch PS Mitochondrial targeted antioxidant peptide ameliorates hypertensive cardiomyopathy. J Am Coll Cardiol 2011; 58:76–82.

6. Dai DF, Chen T, Szeto H, Nieves-Cintrón M, Kutyavin V, Santana LF, Rabinowitch PS Mitochondrial targeted antioxidant peptide ameliorates hypertensive cardiomyopathy. J Am Coll Cardiol 2011; 58:76–82.

7. Roman MJ, Alderman MH, Pickerling TG, Piri R, Keating JO, Sealey JE, Devereux RB. Differential effects of angiotensin converting enzyme inhibition and diuretic therapy on reductions in ambulatory blood pressure, left ventricular mass, and vascular hypertrophy. Am J Hypertens 1998; 11:387–396.

8. London GM, Pannier B, Guerin AP, Marchais SJ, Safar ME, Cuche JL. Prevention of diabetic nephropathy in stroke-prone spontaneously hypertensive rat: sex differences. Free Radic Biol Med 2003; 35:851–859.

9. Yamasaki K, Maeda K, Watanabe T, Nakamura M, Yoshikawa J, Asada A. Comparative effects of ACE inhibition and calcium channel blockade. Circulation 1994; 90:2786–2796.

10. Evans AL, Brown W, Kenyon CJ, Maxted KJ, Smith DC. Improved system for measuring systolic blood pressure in the conscious rat. Med Biol Eng Comput 1994; 32:101–102.

11. Graham D, Hamilton C, Beattie E, Spiers A, Dominiczak AF. Comparison of the effects of omapatrilat versus amloidipine on left ventricular mass and reactive oxygen species formation by monocytes in hypertensive patients with left ventricular hypertrophy. J Am Coll Cardiol 2004; 45:2116–2123.

12. Jeffs B, Negrin CD, Graham D, Clark JS, Anderson NH, Griendling KK, Hamilton CA, Beattie E, Spiers A, Dominiczak AF. Comparison of the effects of omapatrilat and irbesartan/hydrochlorothiazide on reductions in ambulatory blood pressure, left ventricular mass, and vascular hypertrophy. Am J Hypertens 2008; 21:329–337.

13. Yang G, Li L, Volk A, Emmell E, Petley T, Giles-Komar J, et al. Angiotensin-(1-9) attenuates cardiac fibrosis in the rostral ventrolateral medulla of spontaneusly hypertensive rats. Hypertension 2008; 52:841–849.

14. Qin F, Patel R, Yan C, Liu W. NADPH oxidase is involved in angiotensin I-induced cardiac hypertrophy and Galpahox overexpression-induced heart failure. Circ Res 2011; 108:857–846.

15. Dai DF, Chen T, Szeto H, Nieves-Cintrón M, Kutyavin V, Santana LF, Rabinowitch PS Mitochondrial targeted antioxidant peptide ameliorates hypertensive cardiomyopathy. J Am Coll Cardiol 2011; 58:76–82.

16. Ross MF, Prime TA, Abakumova I, James AM, Porteous CM, Smith RA, et al. Combined benefit of MitoQ10 and losartan on cardiovascular function.
Reviewers’ Summary Evaluations

Referee 1
The manuscript explores the efficacy of MitoQ10 as a combination therapy with an angiotensin receptor blocker in reducing cardiac fibrosis with hypertension. Although, there are several antihypertensives that can be used clinically to prolong survival in hypertension induced heart failure, there is still a need to find better therapeutic molecules. One of the possible targets is reducing the reactive oxygen species generated by mitochondria thus limiting cardiac fibrosis and the consequent heart failure. Among several antioxidants tested so far, none has been shown to be effective. Something like MitoQ10 may provide a benefit as combination therapy, provided the animal experiments are confirmed in humans.

Referee 2
There is convincing evidence that oxidative stress is involved in the pathogenesis of hypertension but data from clinical trials with antioxidants are inconsistent. Targeting the enzymes responsible for reactive oxygen species (ROS) generation could be an effective strategy. Mitochondria is an important source of ROS at cardiovascular level. This study shows that the combination of the mitochondria-targeted antioxidant MitoQ10 and losartan provides additive effects, attenuating hypertension and left ventricular hypertrophy of stroke-prone spontaneously hypertensive rats. Thus, mitochondria-targeted antioxidants would provide complementary therapeutic benefit to the inhibitors of renin-angiotensin-system in the treatment of hypertension. However, the clinically relevance of the observed effects in this work are unknown.