BJP British Journal of Pharmacology

DOI:10.1111/bph.12847
www.brjpharmacol.org

RESEARCH PAPER

Glutathione administration reduces mitochondrial damage and shifts cell death from necrosis to apoptosis in ageing diabetic mice hearts during exercise

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Received
1 December 2013
Revised
30 June 2014
Accepted
9 July 2014

BACKGROUND AND PURPOSE

The effect of antioxidants on ageing type 2 diabetic (T2D) hearts during exercise is unclear. We hypothesized that GSH therapy during exercise reduces mitochondrial oxidative stress (mOXS) and cell death in ageing *db/db* mice hearts.

EXPERIMENTAL APPROACH

The effect of GSH on cardiac mOXS and cell death was evaluated both *in vivo* and *in vitro*.

KEY RESULTS

During exercise, GSH treatment protected *db/db* hearts from exaggerated mOXS without reducing total cell death. Despite similar cell death, investigations on apoptosis-specific single-stranded DNA breaks and necrosis-specific damage provided the first *in vivo* evidence of a shift from necrosis to apoptosis, with reduced fibrosis following GSH administration in exercised *db/db* hearts. Further support for a GSH-regulated 'switch' in death phenotypes came from NIH-3T3 fibroblasts and H9c2 cardiomyocytes treated with H_2O_2, a reactive oxygen species (ROS). Similar to *in vivo* findings, augmenting GSH by overexpressing glutamyl cysteine ligase (GCLc) protected fibroblasts and cardiomyocytes from necrosis induced by H_2O_2, but elevated caspase-3 and apoptosis instead. Similar to *in vivo* findings, where GSH therapy in normoglycaemic mice suppressed endogenous antioxidants and augmented caspase-3 activity, GCLc overexpression during staurosporine-induced death, which was not characterized by ROS, increased GSH efflux and aggravated death in fibroblasts and cardiomyocytes, confirming that oxidative stress is required for GSH-mediated cytoprotection.

CONCLUSIONS AND IMPLICATIONS

While GSH treatment is useful for reducing mOXS and attenuating necrosis and fibrosis in ageing T2D hearts during exercise, such antioxidant treatment could be counterproductive in the healthy heart during exercise.

Abbreviations

8-OHG, 8-hydroxyguanosine; AFC, 7-amino-4-trifluoromethylcoumarin; AMPK, AMP kinase; cTnT, cardiac troponin T; DTNB, 5,5-dithiobis-2-nitrobenzoic acid; ETC, electron transport chain; Exe, exercised group of mice; GCLc, catalytic subunit of gamma glutamyl ligase; GSH, active glutathione or 'reduced' form of glutathione; GSSG, 'oxidized' glutathione; H2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide; mOXS, mitochondrial oxidative stress; PARP1, polyADPribose polymerase 1; ROS, reactive oxygen species; SOD, superoxide dismutase; ssDNA, single-stranded DNA; T2D, type 2 diabetes/diabetic; WT, wild-type mice; $\Delta\psi$, mitochondrial membrane potential

Table of Links

TARGETS	LIGANDS
AMPK	ATP
Caspase 3	Glutathione (GSH)
Caspase 7	H_2O_2
Caspase 8	Nitroglycerin
Caspase 9	NO
	Staurosporine

This Table lists key protein targets and ligands in this document, which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013).

Introduction

The International Diabetes Federation (IDF) predicts that nearly 196 million people worldwide between the ages of 60 and 79 years will develop diabetes by 2030, the vast majority of whom will be type 2 diabetic (T2D) patients. Cardiovascular disease is the leading cause of mortality in elderly T2D patients and regular exercise is routinely recommended for improving cardiovascular function and various metabolic, physical and psychological benefits for this age group (Tessier *et al.*, 2000). However, the direct effect of exercise on the ageing T2D heart remains unclear. It is becoming increasingly apparent that the effects of exercise on elderly patients with long-term diabetes are different from the effects observed in younger people and animals with more recent diabetes (Short *et al.*, 2003). Cardiac heat shock protein 70, involved in adaptation to stress, is only up-regulated by exercise in younger animals (Lawler *et al.*, 2009). In earlier studies, moderate exercise increased cardiac antioxidants in 3-month-old *db/db* mice with diabetes for 5 weeks (Moien-Afshari *et al.*, 2008) but not in 8-month-old *db/db* mice with diabetes for 22 weeks (Botta *et al.*, 2013; Laher *et al.*, 2013).

The use of antioxidant therapy to combat increased reactive oxygen species (ROS) generation during exercise remains controversial. Although earlier studies demonstrated benefits of antioxidants in the diabetic state (Golbidi *et al.*, 2011), recent studies have observed that the introduction of exogenous antioxidants may block exercise-induced up-regulation of endogenous antioxidants in healthy individuals (Ristow *et al.*, 2009; Gliemann *et al.*, 2013). As during long-standing, pro-oxidative conditions such as diabetes or ageing, exercise may fail to up-regulate endogenous antioxidants such as glutathione (GSH; Rousseau *et al.*, 2006; Lappalainen *et al.*, 2009; Laher *et al.*, 2013); whether antioxidant therapy in ageing diabetic heart promotes benefit remains unclear.

Cardiac GSH is crucial for preventing cell death during diabetes and diet-induced obesity (Ghosh *et al.*, 2005; 2011). In fact, a loss of GSH, increased cell death and elevated mitochondrial oxidative stress (mOXS) are all characteristics of elderly human diabetic hearts (Frustaci *et al.*, 2000; Anderson *et al.*, 2009). We hypothesized that increasing GSH during exercise would alleviate mOXS and reduce cardiac cell death in ageing *db/db* mice.

We report that increasing GSH during exercise prevents mOXS and changes cell death from necrosis to apoptosis in ageing *db/db* hearts *in vivo*. In addition, when GSH is genetically increased in cardiac cells such as cardiomyocytes and fibroblasts *in vitro*, cell death is also switched to classical apoptotic pathways under oxidative insults. Our results also provide evidence that increasing GSH in the absence of oxidative stress is counterproductive and precipitates mitochondrial toxicities both *in vivo* and *in vitro*. We conclude that while antioxidant support may be useful in reducing mOXS and necrosis following exercise in ageing diabetic hearts, antioxidant therapy as prophylaxis could be detrimental in healthy hearts during exercise.

Methods

Animals, exercise and GSH administration

All protocols were approved by the University of British Columbia Animal Care Committee. Male *db/db* (BKS.cg-m +/+ Leprdb/J strain) and control [wild type (WT), C57BLKS/J] mice were purchased and maintained under a 12 h light/dark cycle at 26°C with free access to food and water until they were at least 32 weeks of age. BKS.cg-m +/+ Leprdb/J mice are obese by 1 month, become diabetic by 2 months and die by 10 months due to beta-cell depletion (Ghosh *et al.*, 2010). Therefore, our experiments were conducted when mice reached 80% of their projected life span. *db/db* and WT mice were randomly placed into sedentary and exercise (Exe) groups ($n = 6$ in each group). Exercising mice were run on a motorized exercise wheel system at a speed of 5.2 m min^{-1} for 1 h for 5 days a week for 3 weeks. Sedentary *db/db* or WT mice ($n = 6$ in each group) were placed in non-rotating wheels for the same duration. For GSH treatment studies, a group of exercised WT and *db/db* mice were given daily i.p. injections of freshly prepared GSH in sterile saline (1.3 mmol·kg^{-1}, pH 7.4) for the duration of the exercise. After completion of the study, animals were anaesthetized

with 65 mg kg^{-1} pentobarbital, blood collected and the hearts removed for biochemical, analytical and histological studies. Blood plasma was separated and stored at –80°C. Commercial kits were used to measure free fatty acids, total cholesterol (Wako, Richmond, VA, USA) or LDH (Sigma-Aldrich, St. Louis, MO, USA) from plasma. Mouse cardiac troponin T (cTnT) was determined by an ELISA kit (USCN Life Science Inc., Houston, TX, USA). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Oxidative stress assays

Oxidative stress-induced lipid hydroperoxides and protein carbonyls in the mitochondrial and cytosolic fractions were estimated as described previously (Ghosh *et al.*, 2005). Total protein amounts in all assays were determined according to the Bradford method.

Glutathione and other antioxidants

Mitochondrial and cytosolic glutathione (both GSH and GSSG) were measured in fresh deproteinated heart samples using a kit (Trevigen, Gaithersburg, MD, USA). In brief, sulphydryl groups in reduced GSH in deproteinated samples were treated with 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) to produce the yellow 5-thio-2-nitrobenzoic acid (TNB). GSSG that is produced is recycled to GSH by glutathione reductase and NADPH to produce more TNB (Griffith, 1980). In this kinetic reaction, the rate of TNB production is directly proportional to this recycling reaction, which, in turn, is directly proportional to the total concentration of GSH in the sample. In a separate reaction, GSSG was estimated by first conjugating all GSH using 2-vinylpyridine, followed by the recycling reaction. The difference between total GSH and GSSG indicated the levels of reduced GSH. Protein assays were performed according to the Bradford method. Superoxide dismutase (SOD) and catalase activities were assessed in flash-frozen hearts using assay kits (Cayman Chemicals, Ann Arbor, MI, USA).

In situ *localization of oxidative DNA damage*

8-Hydroxyguanosine (8-OHG) is a DNA oxidation by-product that exists in the nuclei or mitochondria. Positive extranuclear 8-OHG staining in the myocardium predominantly reflects mitochondrial oxidative stress (Ghosh *et al.*, 2006). Deparaffinized sections were incubated with 1:50 goat anti-8-OHG (Enzo Life Sciences, Farmingdale, NY, USA) for 1 h followed by incubation with 0.3% H_2O_2 and HRP-conjugated secondary anti-goat peroxidase. Sections were visualized under bright view at 200× using Olympus IX81 microscope (Shinjuku, Tokyo, Japan).

Mitochondrial enzyme and ATP assays

Commercial kits were used to measure cytochrome *c* oxidase, citrate synthase, succinate dehydrogenase (Biovision Inc., Milpitas, CA, USA) and ATP (Sigma-Aldrich).

Total cell death

Total cardiac cell death was evaluated using TUNEL (EMD Biosciences, Gibbstown, NJ, USA). To identify cardiomyocytes, sections were stained with cardiac troponin I and Dylight 594 anti-rabbit secondary antibody (ABMGood, Richmond, CA, USA). Sections were visualized using the Olympus IX81 microscope at 400× magnification. TUNEL-positive nuclei are expressed as the mean of TUNEL-positive nuclei counted in 6 random sections per heart sample (n = 6 per group).

Single-stranded DNA (ssDNA) quantification

A specific way of identifying apoptosis-related DNA damage is to identify ssDNA breaks initiated by caspase-3/7 activity (Frankfurt, 2004). Intact apoptotic DNA is more susceptible to denaturation at lower temperatures than that of non-apoptotic cells (Frankfurt, 2004). In brief, formalin-fixed sections were deparaffinized, treated with 5 mM $MgCl_2$ and boiled for 5 min. The sections were then cooled and treated with anti-ssDNA, clone F7-26 (Millipore, Billerica, MA, USA) using the M.O.M (mouse-on-mouse) kit (Vector Laboratories, Burlingame, CA, USA). Sections were incubated with M.O.M. biotinylated anti-mouse IgG and FITC-streptavidin. Sections were counterstained with 4′,6-diamidino-2-phenylindole and examined under Olympus IX81 microscope using 200× or 400× magnification. The number of ssDNA-positive nuclei for each group was the mean of ssDNA-positive nuclei counted in 6 random sections per heart sample per group (n = 6 hearts per group).

Fibrosis and necrosis

To estimate the relationship between necrosis and fibrosis, formalin-fixed sections were stained with Masson's trichrome stain (Laher *et al.*, 2013). Interstitial fibrosis was estimated by Picrosirius Red staining as per standard protocols (Malkusch *et al.*, 1995). NIH Image J software (Bethesda, MD, USA) was used to calculate the ratio of the fibrosis area (stained in blue) to total surface area for the heart for 6 random sections per heart (n = 6 mice per group) by a blinded observer. Commercial kits measured LDH (Sigma-Aldrich) and cTnT (ELISA; USCN Life Science Inc.) in the plasma.

Caspase activities

Activities of caspase-3, -8 and -9 were determined in heart homogenates using caspase-specific fluorescent substrates DEVD-7-amino-4-trifluoromethylcoumarin (AFC; Anaspec, San Jose, CA, USA), IETD-AFC and LEHD-AFC (EMD Biosciences) respectively. Enzyme-catalysed release of AFC was quantified in a Glomax (Promega, Madison, WI, USA) fluorimeter at 380/500 nm wavelengths.

Western blotting

Western blotting was performed as previously described (Laher *et al.*, 2013). Primary antibodies (Supporting Information Table S1) were used to probe targets on nitrocellulose membranes followed by quantification with Image J and expressed as a ratio to GAPDH signal.

Cell studies

NIH/3T3 (CRL-1658) mouse fibroblasts and H9c2 (CRL-1446) rat myoblasts were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were maintained in DMEM with 10% FBS at 37°C under 5% CO_2. H9c2

was differentiated into mature cardiomyocyte with daily addition of 0.1 μM *all-trans* retinoic acid for 4 days as previously described (Botta *et al.*, 2013). Augmentation of GSH in fibroblasts and cardiomyocytes was achieved by overexpressing the catalytic subunit of glutamate cysteine ligase (GCLc), the rate-limiting enzyme for GSH biosynthesis (Griffith, 1999). Overexpression of the catalytic subunit of glutamyl cysteine ligase (mouse GCLc, Origene, Rockville, MD, USA; rat GCLc; Transomic Technologies, Huntsville, AL, USA) in both NIH-3T3 and H9c2 were achieved using Attractene (Qiagen, Toronto, ON, Canada). A 14 h incubation was chosen after a time-course study, demonstrating that this was appropriate for separation of apoptotic (caspase-3 activity) and necrotic (LDH release) events in both cell types *in vitro* (data not shown). Cells transfected with empty vectors (pTCN; also called CON) did not change any parameters tested in both cardiomyocytes and fibroblasts compared with untreated cells (data not shown). After a 48 h recovery, cells were treated with varying concentrations of H_2O_2 (hydrogen peroxide) or staurosporine (Sigma-Aldrich) as indicated, following which LDH (Affymetrix, Santa Clara, CA, USA) and caspase-3 (Anaspec) assays were preformed using kits according to manufacturer's instructions. Cellular GSH was measured using the *ortho*-phthalaldehyde assay (Senft *et al.*, 2000). Briefly, cells were lysed, treated with *N*-ethylmaleimide and *ortho*-phthalaldehyde for 30 min. Fluorescence was measured at 365/460 nm. Nuclear condensation as a marker for apoptosis-specific nuclear damage was evaluated after treating cells with Hoescht 33342, a live cell nuclear dye. Images of Hoechst 33342 stained nuclei were acquired on Olympus IX81 microscope under 200× magnification. Cells with condensed nuclei out of 100 nuclei from each treatment were counted in triplicate and expressed as percentage of total nuclei as an indicator of *in vitro* apoptosis.

Mitochondrial membrane potential and ROS

Mitochondrial membrane potential was estimated using the ratiometric dye, JC-1 (Anaspec), as described previously (Ghosh *et al.*, 2005). Mitochondrial depolarization is indicated by a decrease in the red/green fluorescence ratio. Red fluorescence was measured at 525/630 nm and green fluorescence at 490/530 using GloMax plate reader (Promega). Images of JC-1-treated cells were acquired on an Olympus IX81 epifluorescent microscope under 200× magnification. For the estimation of ROS, H9c2 and NIH-3T3 fibroblasts were treated with 1 μM staurosporine for 14 h and incubated with H2DCFDA (2′,7′-dichlorodihydrofluorescein diacetate), a redox-sensitive dye, and fluorescence measured at 490/530 using GloMax reader (Promega).

Statistical analysis

Results are expressed as mean ± SEM. Two-way ANOVA tests were performed to evaluate the interactions of exercise and glutathione supplementation between groups with multiple comparisons using the Bonferroni tests to determine differences between group mean values. The level of statistical significance was set at $P < 0.05$. GraphPad Prism 4.0 was used for plotting and analysis (GraphPad Software Inc., San Diego, CA, USA).

Results

GSH treatment raises mitochondrial GSH but not other non-thiol antioxidants in db/db *hearts*

Moderate exercise for 3 weeks with or without GSH did not induce whole-body metabolic improvements as elevated circulating free fatty acids and total cholesterol or insulin sensitivity in *db/db* mice remained unchanged following GSH treatment (Supporting Information Fig. S1). Compared with sedentary WT hearts, mitochondrial GSH was lower in sedentary *db/db*, which was lowered further by exercise (Figure 1A). GSH treatment augmented mitochondrial GSH in exercising *db/db* hearts (Figure 1A). Mitochondrial GSSG was increased in sedentary *db/db* compared with WT hearts (Figure 1B). Exercise, irrespective of GSH therapy, increased mitochondrial GSSG in *db/db* hearts, indicating that exercise augments GSH utilization within the mitochondria (Figure 1B). Overall, mitochondrial 2GSH/GSSG ratio specifically increased in *db/db* mice hearts receiving GSH therapy (Figure 1C), whereas in the hearts of exercised WT mice receiving GSH, it was cytosolic GSH that was increased (Figure 1D). Selective GSH accretion in the ageing diabetic mitochondria, but not the cytosol during GSH treatment in *db/db* mice hearts, could be viewed as the result of an evolutionarily conserved mechanism of mitochondrial self-preservation from mOXS under stress (Fernandez-Checa *et al.*, 1998).

Cytosolic GSSG or cytosolic 2GSH/GSSG ratios were not different in any experimental groups (Figure 1E and F). Exercise increased the endogenous antioxidant catalase (Figure 1G) and SOD (Figure 1H) only in WT but not in *db/db* mice hearts. Such increases in antioxidants with exercise were lowered with exogenous GSH in WT hearts (Figure 1G and H). These results are in agreement with recent studies demonstrating blunting of endogenous antioxidant response to exercise-induced oxidative stress with exogenous antioxidants (Ristow *et al.*, 2009; Gliemann *et al.*, 2013).

GSH reduces mOXS in db/db *hearts*

Evaluation of oxidative stress markers of cardiac mitochondrial lipid (lipid peroxides; Figure 2A) and proteins (protein carbonyls; Figure 2B) suggested increased mOXS in sedentary *db/db* hearts compared with WT hearts, which was exacerbated by exercise. GSH treatment attenuated mOXS in exercising *db/db* mice (Figure 2A and B). Levels of mOXS were unaffected in hearts from any of the WT experimental groups (Figure 2A and B). Staining for 8-OHG, a DNA oxidation by-product revealed immunopositivity across Z-lines of the myofibrils from sedentary *db/db* mouse hearts in comparison to WT mice hearts from the various experimental groups (Figure 2C). Exercise-induced increase in 8-OHG staining was associated with complete dissolution of Z-line architecture along some areas of ageing *db/db* hearts, indicating severe mitochondrial and myofibrillar damage (Figure 2C; *db/db* + Exe). GSH treatment attenuated 8-OHG immunopositivity in ageing *db/db* hearts (Figure 2C; *db/db* + Exe + GSH). There were no significant differences in staining observed in any WT heart sections (Figure 2C).

Next, we hypothesized that increased mOXS in *db/db* hearts could down-regulate mitochondrial enzyme activities.

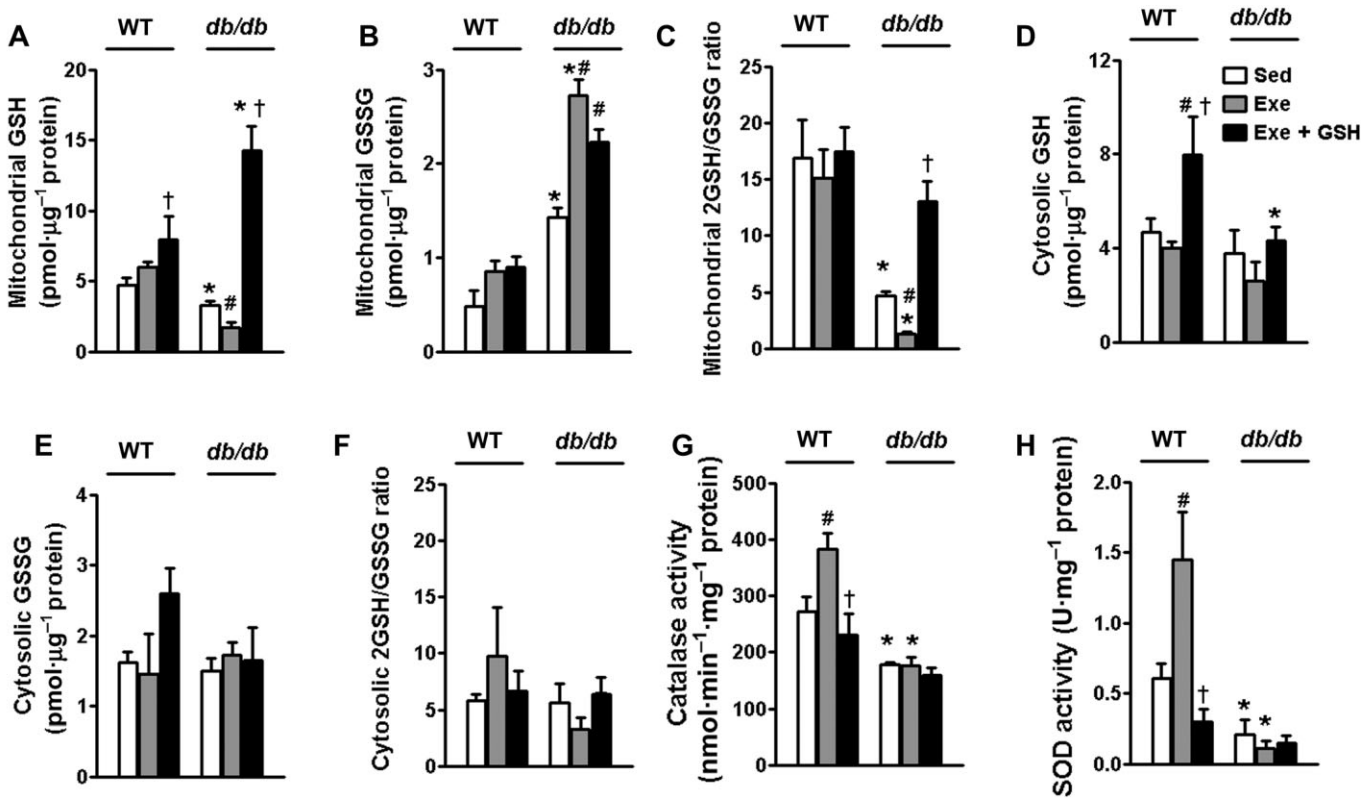

Figure 1

Cardiac mitochondrial GSH is restored with GSH therapy in mature *db/db* hearts. (A) Cardiac GSH in mitochondria; (B) cardiac 'oxidized' glutathione (GSSG) in mitochondria; (C) cardiac mitochondrial 2GSH/GSSG ratios; (D) cardiac GSH in cytosol; (E) cardiac 'oxidized' glutathione (GSSG) in cytosol; and (F) cardiac cytosolic 2GSH/GSSG ratios as determined by recycling assays from WT and *db/db* mice in mitochondrial and cytosolic fractions. Other non-thiol antioxidants such as (G) catalase and (H) superoxide dismutase activities were also assessed in the whole heart using commercial kits. GSH administration increased mitochondrial GSH specifically in *db/db* mice hearts. Data were analysed using two-way ANOVA with the Bonferroni tests, $P < 0.05$ ($n = 5$–6). $^{*}P < 0.05$ versus similar treatments WT group; $^{\#}P < 0.05$ versus sedentary group of the same genotype; $^{\dagger}P < 0.05$ versus Exe group of the same genotype. Abbreviations: Exe, exercised; Sed, sedentary; WT, wild type.

Accordingly, activities of the following mitochondrial enzymes were measured: succinate dehydrogenase [from complex II of the electron transport chain (ETC)], cytochrome *c* oxidase (complex IV of ETC) and citrate synthase (from Krebs cycle) (Figure 2D–F). Exercise did not influence succinate dehydrogenase (Figure 2D) but reduced citrate synthase (Figure 2E) and cytochrome *c* oxidase (Figure 2F) activities in hearts from *db/db* mice. GSH therapy combined with exercise significantly prevented such loss of enzyme activities in *db/db* hearts (Figure 2E and F). Unlike *db/db* mice, exercise did not influence mitochondrial enzyme activities in WT mice. However, in contrast to *db/db* mice, GSH treatment reduced succinate dehydrogenase (Figure 2D) and cytochrome *c* oxidase (Figure 2F) activities in WT hearts, suggesting that GSH treatment in the normoglycaemic mice could impair mitochondrial enzyme function in the myocardium.

Exercise without GSH treatment augments energy stress, necrosis and fibrosis in db/db *hearts*

We next hypothesized that a direct result of augmented mOXS and loss of enzyme activities from the ETC or the Krebs cycle (Figure 2) would result in the loss of ATP and enhancement of necrosis, an energy-independent form of cell death in *db/db* hearts (Proskuryakov *et al.*, 2003). Energy stress was indicated by a specific increase in 'activated' threonine 172 phosphorylated adenosine monophosphate kinase (AMPK) in exercised *db/db* hearts, which was attenuated by GSH administration (Figure 3A). AMPK phosphorylation status did not change with exercise either with or without GSH in WT hearts (Figure 3A). Activation of AMPK was accompanied with a decrease in total ATP in sedentary *db/db* hearts, which was further reduced by exercise alone (Figure 3B). GSH treatment prevented the loss of cardiac ATP in exercised *db/db* mice (Figure 3B) but could not increase ATP levels to that observed in WT mice. It is likely that short-term exogenous GSH could only prevent further deterioration of mitochondrial function by protecting against oxidative insults induced by exercise and not stimulate mitochondrial biogenesis in a mature post-translational organ such as the heart.

Masson's trichrome staining of the myocardium revealed necrotic foci and reparative fibrosis in the hearts of sedentary *db/db* compared with WT mice (Figure 3C; *db/db*). In agreement with our hypothesis, exercise increased necrotic foci in hearts from *db/db* mice (Figure 3C; *db/db* + Exe). Interestingly,

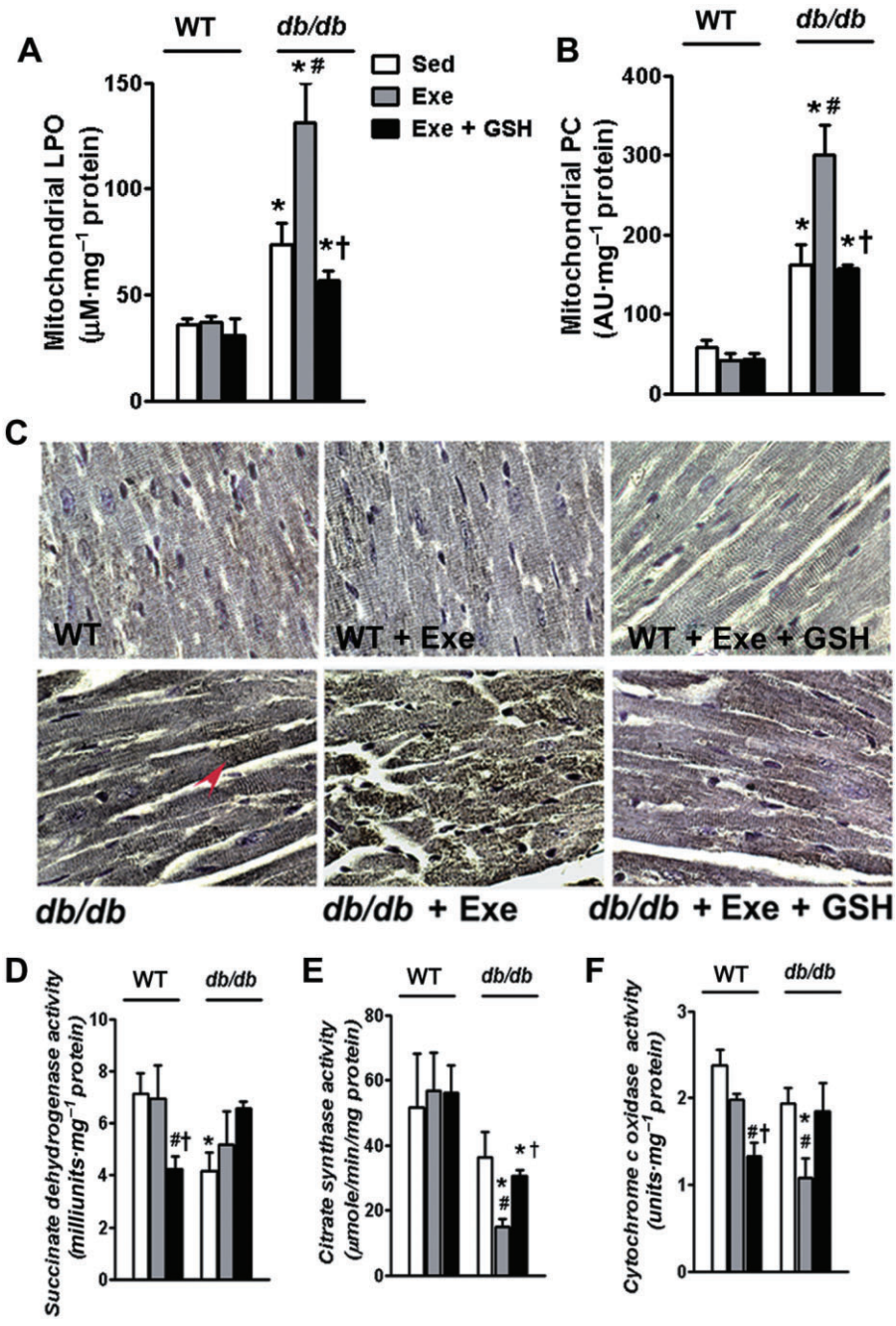

Figure 2

GSH treatment prevents mitochondrial oxidative stress following exercise in *db/db* hearts. (A) Mitochondrial lipid peroxides and (B) mitochondrial protein carbonyls as estimated using enzymatic assays in WT and *db/db* hearts following exercise with or without GSH supplementation. (C) *In situ* localization of 8-hydroxyguanosine (8-OHG) in WT and *db/db* hearts following exercise with or without GSH supplementation. 8-OHG staining was more pronounced in *db/db* mice after at least 22 weeks of diabetes. Exercise without GSH supplementation increased 8-OHG staining intensity within damaged myofibrils (with disrupted Z bands) in the *db/db* + Exe group but this was reduced with GSH supplementation (*db/db* + Exe + GSH group). Magnification 400×. Activities of mitochondrial metabolic enzymes like (D) succinate dehydrogenase; (E) citrate synthase; and (F) cytochrome *c* oxidase were measured using commercial kits. Data were analysed using two-way ANOVA with Bonferroni tests ($n = 5–6$). $^{*}P < 0.05$ versus similar treatments WT group; $^{\#}P < 0.05$ versus sedentary group of the same genotype; $^{\dagger}P < 0.05$ versus Exe group of the same genotype. Abbreviations: AU, arbitrary units; Exe, exercised; LPO, lipid peroxides; PC, protein carbonyls; Sed, sedentary; WT, wild type.

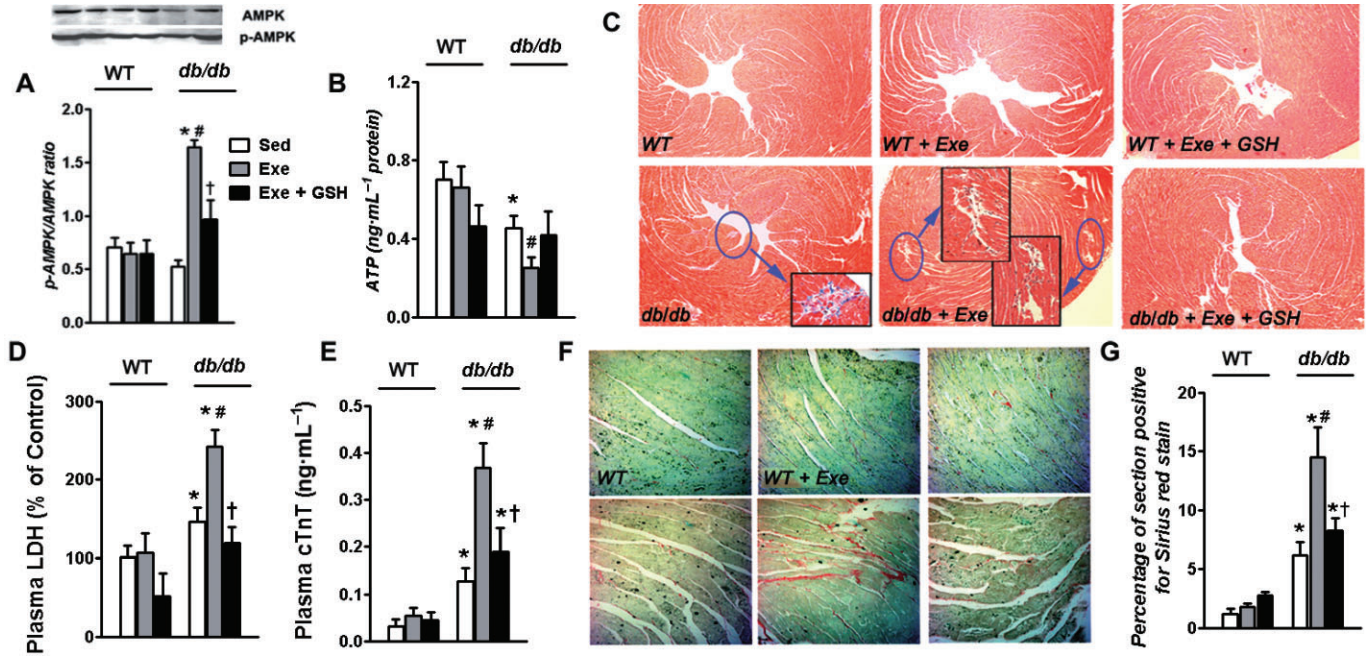

Figure 3

GSH treatment attenuates exercise-induced energy stress, necrosis and fibrosis in ageing *db/db* hearts. (A) Ratio of protein expressions for phosphorylated AMPKα (thr172) to total AMPKα as estimated by Western blot. Inset depicts representative gels. (B) Total cardiac ATP as estimated by luciferase assay on flash-frozen samples. (C) Formalin-fixed heart sections were treated with Masson's trichrome stain and the entire cross section of hearts depicted at 40×. Highlighted areas (blue box) depicts magnified (200×) areas of the myocardium exhibiting necrotic foci. Blue stains in magnified *db/db* mice sections represent collagen deposition. Darker nuclei in *db/db* + Exe sections represent polymorphonuclear (PMN) cell infiltration, which were attenuated with GSH treatment. (D) Serum LDH values as measured using an enzymatic assay kit and (E) plasma cTnT assay as measured by an ELISA. (F) Interstitial fibrosis by Sirius Red stain in heart sections; magnification 200×. (G) Quantification of fibrotic area in the myocardium. Exercise alone increased energy stress and cardiac necrosis whereas GSH therapy prevented such an outcome in *db/db* mice hearts. Data were analysed using two-way ANOVA with Bonferroni tests (n = 5–6). $^{*}P < 0.05$ versus similar treatments WT group; $^{\#}P < 0.05$ versus sedentary group of the same genotype; $^{\dagger}P < 0.05$ versus Exe group of the same genotype. Abbreviations: AMPK, adenosine monophosphate kinase; cTnT, cardiac troponin T; Exe, exercised; Sed, sedentary; WT, wild type.

some of these cardiac necrotic foci from exercised *db/db* mice did not demonstrate fibrosis (unlike sedentary *db/db* hearts) and instead were characterized by leukocyte infiltration, indicating more recent damage, probably due to the short exercise period prior to being killed (Figure 3C; *db/db* + Exe). Ongoing necrosis was confirmed by elevated plasma LDH following exercise in *db/db* hearts (Figure 3D). As plasma LDH can also result from non-cardiac necrosis, we measured cTnT by ELISA (Figure 3E). cTnT is the most sensitive and specific biochemical marker of myocardial injury/necrosis available (Thygesen *et al.*, 2010). Both plasma LDH and cTnT levels were attenuated by GSH in *db/db* mice (Figure 3D and E). Exercise also induced an acute rise in fibrosis in *db/db* hearts, which was attenuated by concurrent GSH therapy (Figure 3F and G).

GSH treatment fails to attenuate total cardiac cell death in exercised db/db *hearts*

Cardiac cell death was estimated by TUNEL in troponin I positive areas for cardiomyocytes (Figure 4A). Exercise with or without GSH had no effect on TUNEL in WT hearts. Sedentary *db/db* hearts demonstrated augmented TUNEL in both non-cardiomyocytes (Figure 4B) and cardiomyocytes (Figure 4C) compared with WT hearts. Exercise for 3 weeks increased TUNEL positivity in both of these cell types in *db/db* mice hearts (Figure 4A–C). Surprisingly, despite antioxidant benefits and lower necrosis, GSH-treated *db/db* mice hearts did not demonstrate any change in TUNEL in any cardiac cell (Figure 4A–C).

GSH treatment increases ssDNA breaks and caspase-3 in exercising db/db *mice hearts*

TUNEL is an indicator of total cardiac cell death and is unable to differentiate between apoptotic and necrotic death (Grasl-Kraupp *et al.*, 1995). Therefore, with lower necrosis and fibrosis, we speculated an increase in apoptosis, which could be responsible for sustained TUNEL positivity in GSH-treated *db/db* mice hearts. To verify apoptosis-specific nuclear DNA damage, monoclonal antibody-based ssDNA staining was performed. Cardiac ssDNA was augmented in hearts from sedentary *db/db* mice compared with sedentary WT mice, but was abolished with exercise alone (Figure 5A and B). GSH treatment reversed this trend and augmented ssDNA in hearts from exercising *db/db* mice, thus confirming increased apoptosis in GSH-treated exercising *db/db* hearts (Figure 5A and B).

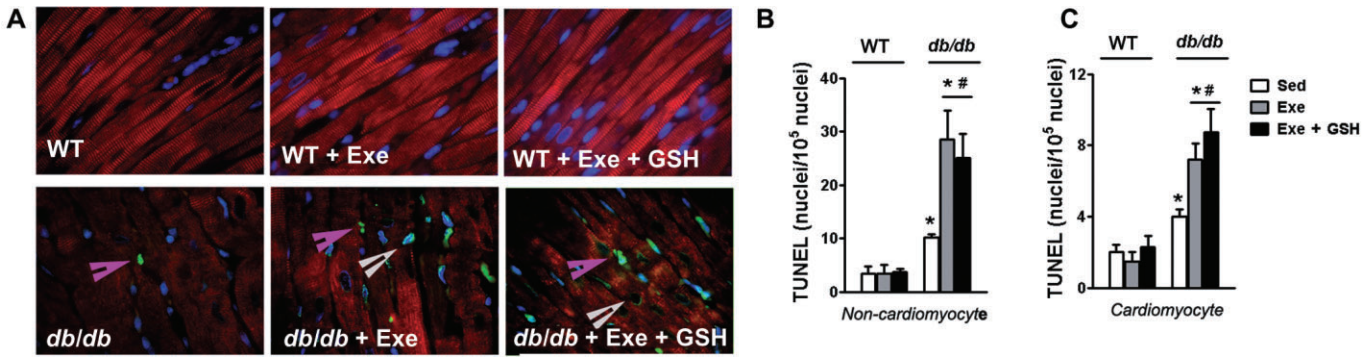

Figure 4

GSH treatment fails to attenuate total cardiac cell death in exercised ageing *db/db* hearts. TUNEL assay was performed on formalin-fixed heart sections using Olympus IX81 at 400× magnification. (A) Representative micrographs of heart sections demonstrating red fluorescence correspond to cardiac troponin I specific staining and green fluorescence corresponds to TUNEL-positive nuclei. Blue stains correspond to normal nuclei stained with DAPI. (B) Quantification of TUNEL-positive nuclei from non-cardiomyocytes (troponin I negative; pink arrows). (C) Quantification of TUNEL-positive nuclei from cardiomyocytes (troponin I positive; white arrows). Quantification was performed using the Metamorph software as described in the Methods section. GSH therapy was unable to reduce TUNEL positivity in exercised *db/db* hearts. Data were analysed using two-way ANOVA with the Bonferroni tests (n = 5–6). *P < 0.05 versus similar treatments WT group; $^{\#}P$ < 0.05 versus sedentary group of the same genotype. Abbreviations: Exe, exercised; Sed, sedentary; WT, wild type.

As ssDNA breaks are mediated by caspases, we verified caspase-9, -8 and -3 activities. Activities of caspase-9 and caspase-8, principal initiator caspases from the 'intrinsic' and 'extrinsic' pathways, were both increased with diabetes but were down-regulated following exercise in *db/db* mice (Figure 5C and D). GSH therapy specifically increased cardiac caspase-9 (Figure 5C) activity in *db/db* mice. Caspase-3 activity, which orchestrates nuclear DNA damage, was increased in the hearts from sedentary, ageing *db/db* mice (Figure 5E). Similar to caspase-9, caspase-3 activity was also reduced in exercised *db/db* hearts, while GSH treatment restored cardiac caspase-3 activity in *db/db* mice (Figure 5E). In addition to artificial fluorescent substrates, we confirmed endogenous caspase-3 activity by analysing cleavage products of its cellular substrates. Caspase-3 cleaves actin, giving rise to 'fractin', a 32 kDa fraction (Rossiter *et al.*, 2002). Additionally, caspase-3 also cleaves whole 116 kDa polyADP ribose polymerase 1 (PARP1) to produce an 89 kDa fragment (Los *et al.*, 2002). Both cardiac fractin (Figure 5F) and cleaved PARP-1 (Figure 5G) were increased GSH-treated *db/db* mice.

Overall, these results indicate that while total cardiac cell death was not affected (TUNEL; Figure 4), exogenous GSH caused a shift from necrosis (Figure 3) to increased caspase-3 activity and apoptosis in exercised *db/db* mice hearts.

Increasing GSH promotes apoptosis and inhibits necrosis in fibroblasts under elevated oxidative stress

To better understand the relationship between GSH and modes of cardiac cell death, we simulated *in vivo* interactions *in vitro* by incubating NIH3T3 fibroblasts and differentiated H9c2 cardiomyocytes with hydrogen peroxide (H_2O_2) to represent exercise-induced increases in oxidative stress. In fibroblasts, GCLc overexpression increased GSH (Figure 6A). Mitochondrial membrane potential (delta psi, $\Delta\psi$), an indicator of mitochondrial health, was assessed with the potentiometric dye JC-1 in fibroblasts (Figure 6B). All concentrations of H_2O_2 (with or without GCLc) reduced $\Delta\psi$ in fibroblasts (Figure 6B and F). Loss of $\Delta\psi$ in fibroblasts was partially rescued by GCLc overexpression at 1000 and 2000 μM H_2O_2 (Figure 6B and F). Despite such protection, both forms of cell death, namely caspase-3 activity, condensed nuclei (characteristic of apoptosis) and LDH release (characteristic of necrosis) increased in fibroblasts with 1000 μM H_2O_2, irrespective of GCLc overexpression (Figure 6C–E), demonstrating that moderate oxidative stress can induce both apoptotic and necrotic forms of death in the same cell population (Zuliani *et al.*, 2003). However, at 2000 μM H_2O_2, caspase-3 activity (Figure 6C) and condensed nuclei (Figure 6D) decreased with a corresponding increase in LDH release (Figure 6E) with an appearance of diffuse, swollen nuclei (Figure 6F), characteristic of predominantly necrotic phenotype (Teramoto *et al.*, 1999). These data are in agreement with published reports of fibroblasts demonstrating a co-existence of apoptosis and necrosis at moderate oxidative insults induced with 1000 μM H_2O_2 but necrosis only at higher oxidative insults between above 1 mM (Teramoto *et al.*, 1999). At this H_2O_2 concentration (2 mM), overexpression of GCLc increased apoptotic characteristics [caspase-3 activity (Figure 6C) and condensed nuclei (Figure 6D and F)] while attenuating necrotic features like LDH release, thus promoting an apoptotic phenotype over necrosis under levels of high oxidative insults.

Increasing GSH promotes apoptosis and inhibits necrosis in cardiomyocytes under elevated oxidative stress

In cardiomyocytes, GCLc overexpression augmented cellular GSH in the absence or presence of H_2O_2 (Figure 7A). Similar to the findings with fibroblasts, all concentrations of H_2O_2 reduced $\Delta\psi$ in fibroblasts (Figure 7B). However, unlike fibroblasts, increasing concentrations of H_2O_2 induced a weaker induction of caspase-3 or formation of condensed nuclei,

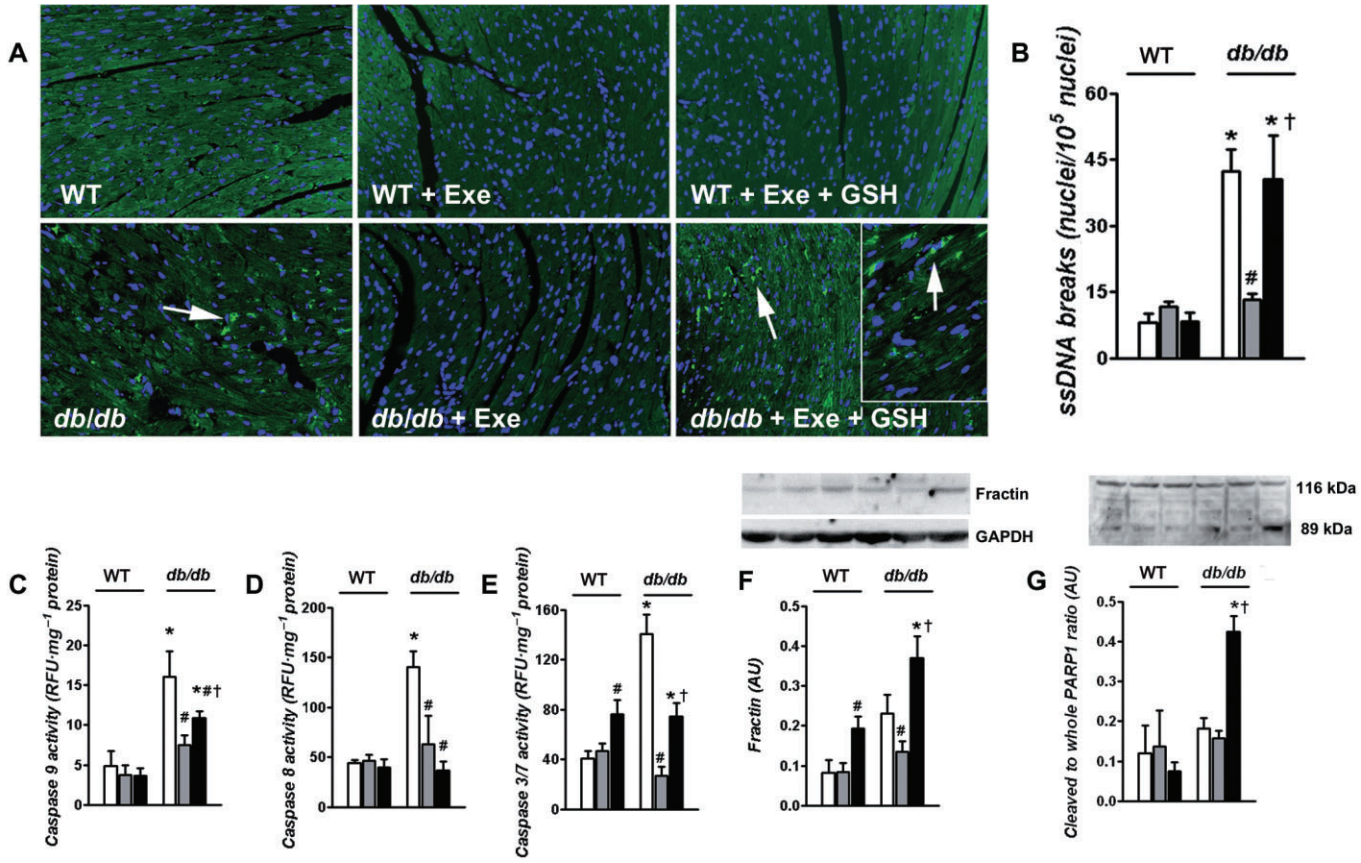

Figure 5

GSH treatment increases ssDNA breaks and caspase-3 in exercising *db/db* hearts. (A) ssDNA breaks in formalin-fixed heart sections as identified using a monoclonal antibody specifically directed against a ssDNA fragment of 25 bp or longer. Green fluorescence depicts positive staining (white arrows), which was co-localized with DAPI stains (inset, magnified image in *db/db* + Exe + GSH). (B) Quantification of ssDNA breaks across heart sections using Metamorph software. (C) Caspase-9, (D) caspase-8 and (E) caspase-3/7 activities were quantified using aminofluorocoumarin (AFC)-conjugated substrates in flash-frozen samples. Validation of caspase-3 activity was achieved by studying (F) fractin and (G) whole to cleaved PARP1 ratio. Inset depicts representative gels depicting fractin and cleavage of whole PARP1 (116 kDa) to its cleavage product (89 kDa). GSH therapy increased ssDNA breaks and caspase-3 activity in exercised *db/db* hearts. Data were analysed using two-way ANOVA with the Bonferroni tests (n = 5–6). $^{*}P < 0.05$ versus similar treatments WT group; $^{\#}P < 0.05$ versus sedentary group of the same genotype; $^{\dagger}P < 0.05$ versus Exe group of the same genotype. Abbreviations: AU, arbitrary units; Exe, exercised; PARP-1, polyADP ribose polymerase 1; RFU, relative fluorescence units; Sed, sedentary; ssDNA, single-stranded DNA; WT, wild type.

indicating resistance to apoptosis in differentiated H9c2 cardiomyocytes (Figure 7C and D). Others have reported that differentiated cardiomyocytes are relatively resistant to caspase-3-mediated nuclear damage (Bahi *et al.*, 2006), and unlike fibroblasts, differentiated H9c2 cells have more mitochondria, greater respiratory activity and hence possess increased tolerance to low concentrations of H_2O_2 (Comelli *et al.*, 2011). Indeed, 1000 μM H_2O_2 induced extensive loss of viability in CON cardiomyocytes, predominantly through LDH release, characterized by swollen nuclei (Figure 7D and F), which is indicative of necrosis. More interestingly, at baseline (i.e. no H_2O_2), GCLc overexpression reduced $\Delta\psi$ in cardiomyocytes, indicating toxic effects of GSH without oxidative stress (Figure 7B and F).

These data suggest that similar to GSH treatment of *db/db* mice undergoing exercise, augmenting GSH by GCLc overexpression *in vitro* also sustains caspase-3 activity in both fibroblasts and cardiomyocytes exposed to oxidative stress. Taken together, these studies imply that increased cellular GSH has an important role in preventing necrosis and activation of caspase-3-mediated classical apoptotic pathways induced by increased oxidative stress in both fibroblasts and cardiomyocytes.

Increasing GSH without oxidative stress impairs mitochondrial membrane potential and potentiates cell death

GSH treatment of exercised mice produced several detrimental effects in WT hearts, such as reducing antioxidant (Figure 1G and H) and ETC enzyme (Figure 2D and F) activities in addition to increasing caspase-3 and fractin formation (Figure 5E and F). As WT hearts experience lower oxidative stress (Figure 2A–C), we hypothesized that GSH up-regulation would have detrimental effects in both cardiomyocytes and fibroblasts without oxidative stress.

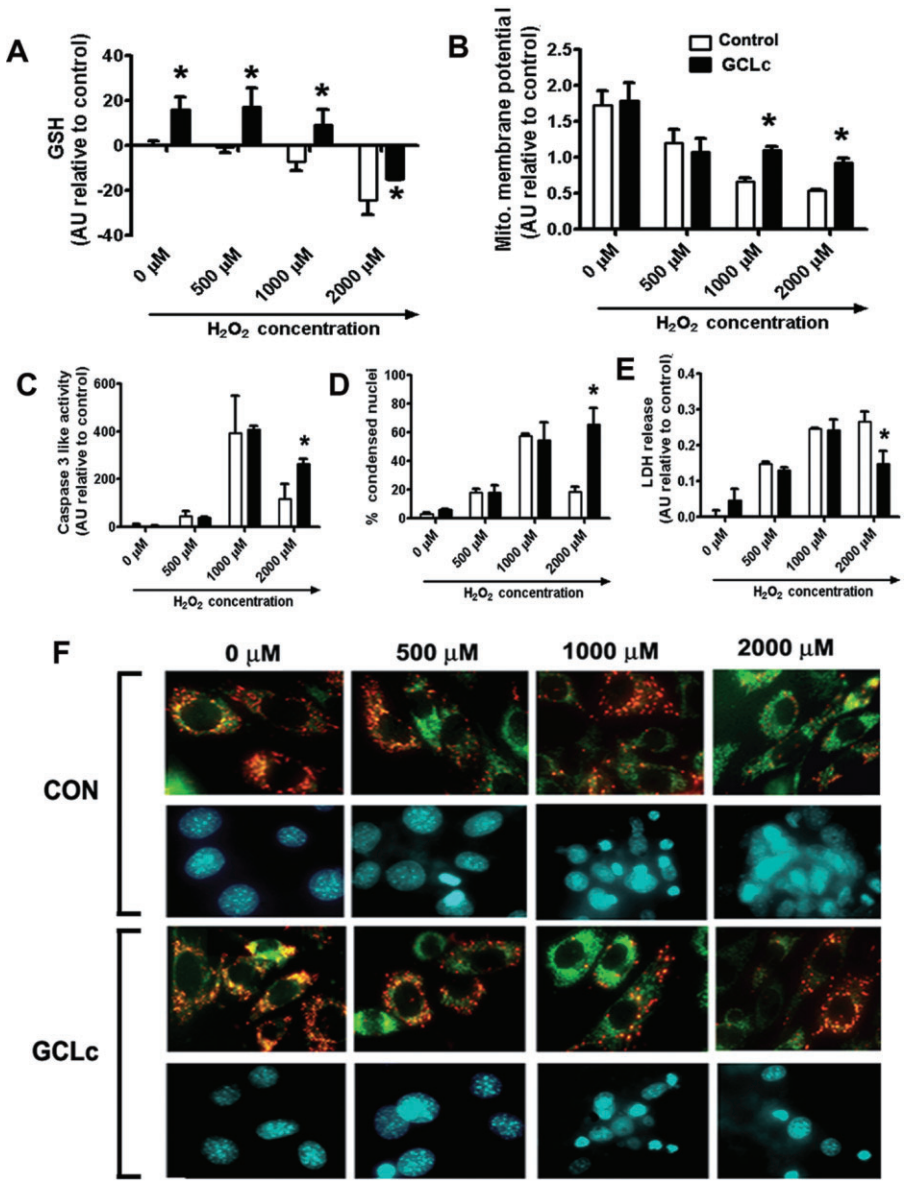

Figure 6

Increasing GSH promotes apoptosis and inhibits necrosis in fibroblasts under elevated oxidative stress. Effect of 14 h of H_2O_2 treatment on fibroblasts overexpressing GCLc or empty vectors (CON). Fibroblasts were analysed for (A) GSH content using *o*-phthalaldehyde assay; (B) mitochondrial membrane potential ($\Delta\psi$) with the ratiometric JC-1 dye using a fluorescent plate reader; (C) caspase-3 activity using fluorescent substrates; (D) percentage of nuclear condensation as estimated through microscopy after counting condensed nuclei out of 100 total nuclei from each treatment as an indicator of apoptotic nuclear damage; (E) LDH release in the media using enzymatic substrates; and (F) representative micrographs of JC-1 and Hoechst fluorescence using Texas Red, FITC and DAPI filters, respectively, at 400× magnification. Higher ratio of red : green fluorescence indicates healthy mitochondria with elevated $\Delta\psi$. Data were analysed using one-way ANOVA with Bonferroni tests; $^*P < 0.05$ versus corresponding control under the same treatment condition. GCLc overexpression increases GSH and protects against loss of mitochondrial membrane potential. Experiments were carried out at least twice in triplicate. Abbreviations: AU, arbitrary units; CON; control, with overexpression of empty vectors; GCLc; with overexpression of glutamate cysteine ligase catalytic subunit; Mito., mitochondrial.

To investigate this hypothesis, we selected staurosporine, a classic inducer of cell death that induces both caspase-dependent and caspase-independent cell death not involving the generation of ROS (Belmokhtar *et al.*, 2001; Ramiro-Cortes and Moran, 2009). In this study, staurosporine lowered baseline ROS after 14 h of incubation in both cardiomyocytes and fibroblasts (Figure 8A). Interestingly, GCLc overexpression caused a greater loss of GSH from both fibroblasts and cardiomyocytes following staurosporine treatment (Figure 8B). Further, unlike H_2O_2-treated cells, GCLc overexpression decreased mitochondrial membrane potential in fibroblasts (Figure 8C) or had no effect in preventing loss of

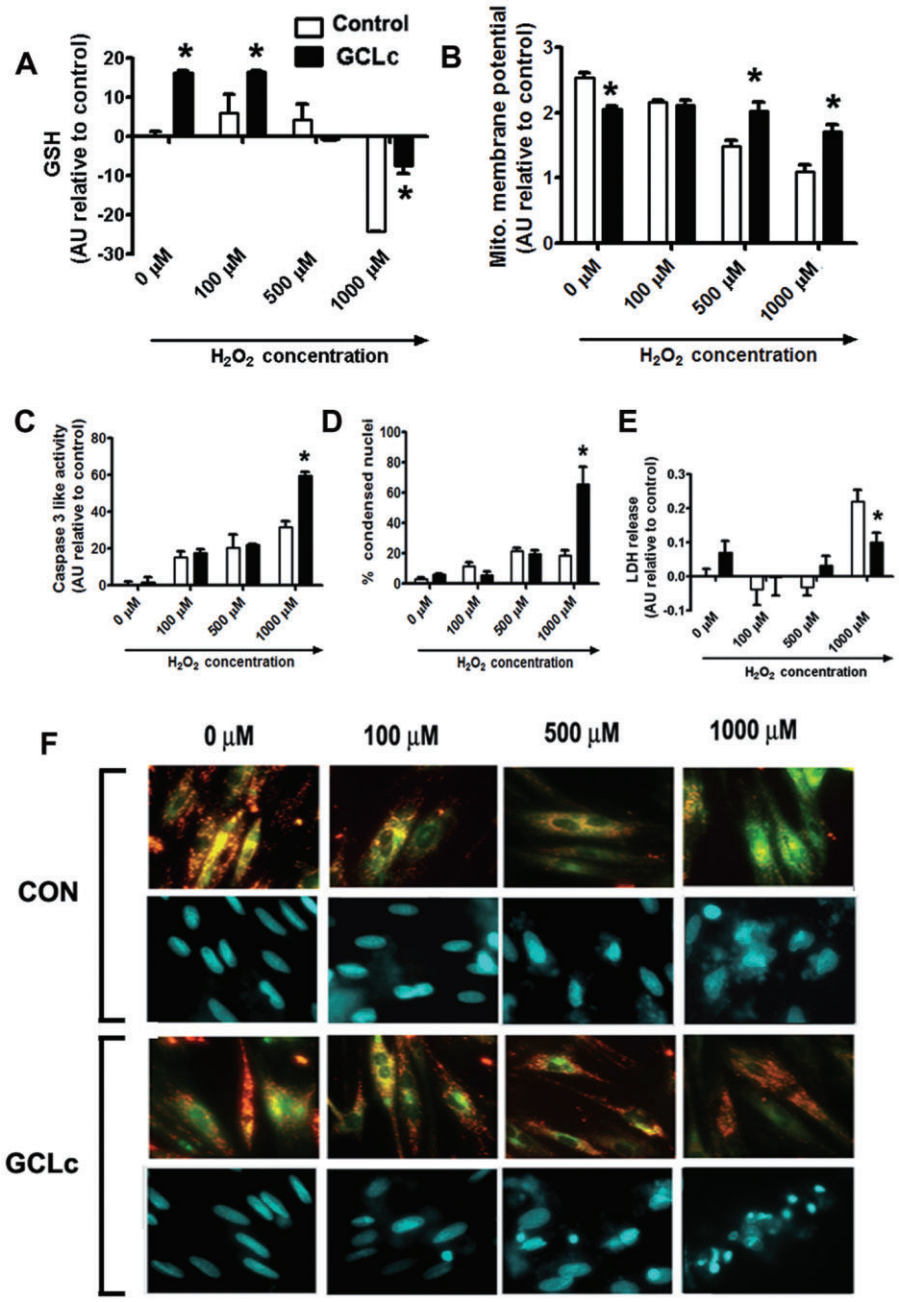

Figure 7

Increasing GSH promotes apoptosis and inhibits necrosis in cardiomyocytes under elevated oxidative stress. GCLc or empty vectors (CON) were overexpressed in cardiomyocytes, which were then treated with increasing concentrations of H_2O_2 as indicated for 14 h. Cardiomyocytes were analysed for (A) GSH content using *o*-phthalaldehyde assay; (B) mitochondrial membrane potential ($\Delta\psi$) with the ratiometric JC-1 dye using a fluorescent plate reader; (C) caspase-3 activity using fluorescent substrates; (D) percentage of nuclear condensation as estimated through microscopy after counting condensed nuclei out of 100 total nuclei from each treatment as an indicator of apoptotic nuclear damage; (E) LDH release in the media using enzymatic substrates. (F) representative micrographs of JC-1 and Hoechst fluorescence using Texas Red/ FITC and DAPI filters, respectively, at 400× magnification. Higher ratio of red: green fluorescence indicates healthy mitochondria with elevated $\Delta\psi$. Data were analysed using one-way ANOVA with Bonferroni tests. *$P < 0.05$ versus corresponding control under the same treatment condition. GCLc overexpression increases GSH and protects against loss of mitochondrial membrane potential. Experiments were carried out at least twice in triplicate. Abbreviations: AU, arbitrary units; CON; control, with overexpression of empty vectors; GCLc; with overexpression of glutamate cysteine ligase catalytic subunit; Mito., mitochondrial.

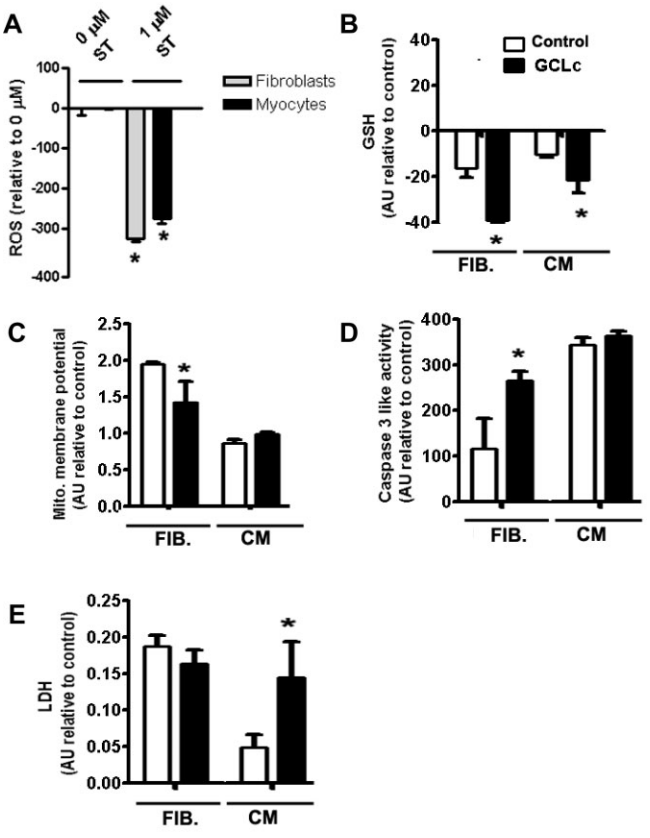

Figure 8

Increasing GSH without oxidative stress impairs mitochondria and increases cell death. Effects of GCLc overexpression on fibroblasts and cardiomyocytes undergoing staurosporine-induced death was evaluated at 14 h by measuring (A) intracellular ROS using H2DCFDA dye; (B) cellular GSH using *o*-phthalaldehyde assay; (C) mitochondrial membrane potential using JC-1 fluorescence ratio; (D) caspase-3 activity using fluorescent substrates as described in the Methods section; and (E) LDH release using enzymatic assays. GCLc overexpression in the absence of incumbent oxidative stress cannot sustain cellular GSH, lowers mitochondrial membrane potential and potentiates cell death in both fibroblasts and cardiomyocytes. Experiments were carried out at least twice in triplicate. Data were analysed using one-way ANOVA with Bonferroni tests. $*P < 0.05$ versus corresponding control cells under the same treatment condition. Abbreviations: AU, arbitrary units; CM, cardiomyocytes; FIB, fibroblasts; GCLc, glutamate cysteine ligase catalytic subunit; Mito., mitochondrial.

membrane potential in cardiomyocytes following staurosporine treatment (Figure 8C). Caspase-3 was preferentially increased in fibroblasts with GCLc overexpression with staurosporine (Figure 8D). In contrast, GCLc-treated cardiomyocytes led to higher LDH release than fibroblasts in response to staurosporine (Figure 8E).

Overall, these data suggest that in the absence of augmented ROS generation, GCLc overexpression induced a greater GSH efflux from dying cells. GCLc could not protect against mitochondrial impairment during ROS-independent cell death and instead potentiated either LDH release or caspase-3 activities in both fibroblasts and cardiomyocytes.

Discussion

GSH is an endogenous thiol that accumulates spontaneously within the mitochondria as a means of self-protection during increased mOXS (Mari *et al.*, 2009). The clinical importance of GSH is highlighted by the fact that elevation of this thiol underlies the benefit of most therapeutically useful antioxidants (Suh *et al.*, 2004; McManus *et al.*, 2011; Hwang *et al.*, 2012). A seminal prospective study demonstrated that low levels of glutathione peroxidase (but not superoxide dismutase) was an independent risk factor for cardiovascular events in 636 patients over 4.7 years (Blankenberg *et al.*, 2003). As glutathione peroxidase depends upon GSH as its co-factor, this effect may have been related to GSH deficiency. A recent study by Damy *et al.* confirmed that a lack of atrial GSH was proportional to structural abnormalities and severity of heart failure in patients undergoing cardiac surgery for coronary artery disease, aortic stenosis or cardiomyopathy (Damy *et al.*, 2009). As a large proportion of patients enrolled in these studies were geriatric patients with T2D, a correlation between a lack of GSH and cardiovascular complications is conceivable in aged diabetic hearts.

An important physiological consequence of exercise is the generation of ROS, which then up-regulates endogenous antioxidant expression as an adaptive response. We reported earlier that moderate exercise increased cardiac antioxidant levels in 3-month-old *db/db* mice with diabetes for 5 weeks (Moien-Afshari *et al.*, 2008). In the current study, such effects were absent in 8-month-old *db/db* hearts with diabetes for 24 weeks. Such a discrepancy could be due to 'tolerance' of older diabetic tissues to ROS. Both ageing and hyperglycaemia independently induce ROS release, and in combination, can desensitize the heart to additional ROS released by exercise. A parallel can be drawn between exercise and the effects of ischaemic preconditioning, which also involves ROS release, as a major mechanism to protect against future ischaemic events in the heart (Otani, 2004). Both diabetes and ageing blunts the cardioprotective effects of preconditioning in humans (Ishihara *et al.*, 2001; van den Munckhof *et al.*, 2013). Like ROS, 'tolerance' to other signalling molecules such as NO have also been reported, where repeated dosing of nitroglycerin leads to endothelial dysfunction and tolerance to vasodilator effects of nitroglycerin (Gori *et al.*, 2010).

A dose-dependent effect of oxidative stress on cell death phenotype or caspases has long been recognized (Sen and Roy, 2001). One study demonstrated a gradual rise in caspase-3 activity with lower concentrations of H_2O_2 in Jurkat T lymphocytes, followed by a rapid drop at higher concentrations of H_2O_2 where apoptosis was replaced by necrosis (Hampton and Orrenius, 1997). Interestingly, H_2O_2 can directly inactivate caspases (Borutaite and Brown, 2001). As ATP concentrations are critical in determining cardiac cell death phenotype, a critical determinant of cellular ATP under stress is the duration and extent of loss of $\Delta\psi$ (otherwise known as mitochondrial membrane potential). Loss of $\Delta\psi$ for short and long periods can induce apoptosis and necrosis respectively (Skulachev, 2006). This is due to persistent loss of $\Delta\psi$, which inhibits oxidative phosphorylation and depletes ATP, leading to the failure of membrane ion pumps to maintain osmolarity, thus causing cell swelling, membrane leakage and LDH release. In contrast, a moderate and reversible loss

of $\Delta\psi$ leads to the release of mitochondrial cytochrome *c* and initiation of apoptotic process but maintains sufficient oxidative phosphorylation levels to permit ATP-dependent steps for the execution of apoptosis. As an example, active nuclear transport, activation of stress kinases, phosphatidyl serine externalization and apoptosome formation are ATP-dependent processes necessary for apoptosis, which are initiated by transient loss of $\Delta\psi$ (Nicotera and Melino, 2004).

However, caution is warranted while trying to predict the occurrence of apoptosis or necrosis based solely upon the degree of oxidative stress. Other factors such as the state of differentiation or cell type may also dictate such outcomes. As an example, in this study using 1000 μM H_2O_2, CON fibroblasts demonstrated greater caspase-3 activity and higher condensed nuclei than in CON cardiomyocytes (Figure 6C and D vs. Figure 7C and D). This is in agreement with our *in vivo* data where the number of TUNEL-positive nuclei was significantly higher among fibroblasts in the interstitial space (labelled as non-cardiomyocytes; Figure 4B) and not in cardiomyocytes. Differentiated cells such as epithelial cells and cardiomyocytes are more resistant to caspase-3 activity-induced nuclear damage under oxidative stress. Activation of caspase-3 in cardiomyocytes often involves cleavage of contractile proteins such as actin to fractin and not nuclear damage *per se* (Communal *et al.*, 2002; Rossiter *et al.*, 2002). Alternatively, it may take up to 5000 μM H_2O_2 to initiate apoptosis in differentiated cells such as human airway epithelial cells (Kazzaz *et al.*, 1996).

Despite such assertions *in vitro*, factors controlling cell death phenotypes in the heart *in vivo* were uncertain (Anathy *et al.*, 2012). Using novel apoptosis-specific ssDNA probes in the intact heart *in vivo* as well as various doses of H_2O_2 in both cardiomyocytes and fibroblasts *in vitro*, we present the first evidence suggesting that GSH is a key in controlling the mode of death in heart cells both *in vivo* and *in vitro* under oxidative challenges. Our study indicates that short-term exercise greatly augments oxidative stress in *db/db* hearts (Figure 2). This results in a 'switch' of cell death from predominantly apoptotic DNA damage (represented by ssDNA breaks in sedentary *db/db* hearts; Figure 5) to non-specific DNA damage (indicated by TUNEL; Figure 4) with elevated necrosis and fibrosis (Figure 3) in exercised *db/db* hearts. Thus, GSH supplementation during exercise alters the balance between cell death phenotypes to augment apoptosis in *db/db* hearts. Indeed, GSH provision during cardiac oxidative stress related to doxorubicin treatment promotes greater cardiac caspase-3 activity (Asakura *et al.*, 1999). The failure of GSH therapy to completely prevent cell death in our study could be due to the fact that circulating glucose levels remaining unchanged with GSH therapy in exercised *db/db* mice, as hyperglycaemia *per se* is an independent predictor of cardiac cell death (Cai *et al.*, 2002).

In this study, GSH therapy during exercise demonstrated several detrimental effects in WT hearts. Exogenous GSH reduced exercise-induced up-regulation of antioxidants such as catalase and SOD, lowered ETC enzyme activities, as well as increased caspase-3 and fractin formation in WT hearts during exercise. As WT hearts had low oxidative stress, increasing GSH concentrations could induce 'reductive stress' in these hearts *in vivo* as suggested recently under *in vitro* studies (Zhang *et al.*, 2012). Consistent with such results, without incident ROS, staurosporine induced a much greater GSH efflux, in GCLc overexpressing fibroblasts and cardiomyocytes at 14 h. Others have shown that in colonic epithelial cell lines not experiencing oxidative stress, GSH efflux potentiates staurosporine-induced cell death (Circu *et al.*, 2009). Loss of GSH from dying cells that may not need them (i.e. due to a lack of oxidative stress) is a conserved mechanism to redistribute this important antioxidant to cells that could require GSH to combat oxidative stress (Coppola and Ghibelli, 2000). Thus, overproduction of GSH without oxidative stress could be detrimental as excessive GSH extrusion can magnify damage by committing cells to the death process (Coppola and Ghibelli, 2000).

We summarize our results in Figure 9. Exercise and antioxidant supplementation has different effects depending upon whether they are diabetic or normoglycaemic. GSH (or other antioxidants that increase GSH) in ageing diabetic hearts ameliorates mOXS, prevents necrosis, while activating caspase-3 and apoptosis during exercise. In contrast, in the *absence* of chronic oxidative stress (i.e. normoglycaemic hearts), efforts to increase GSH blocks physiological up-regulation of ROS and antioxidants, lowers mitochondrial function and induces 'stress'.

Older human T2D hearts demonstrate both apoptosis and necrosis (Frustaci *et al.*, 2000). Although any form of cell death can induce functional deficit, cardiac necrosis is the primary mediator of heart failure in humans (Nakayama *et al.*, 2007). Necrosis (and not apoptosis) involves permeation of cardiac cell membrane and extruded cell debris can provoke inflammation in the failing heart, at the site of

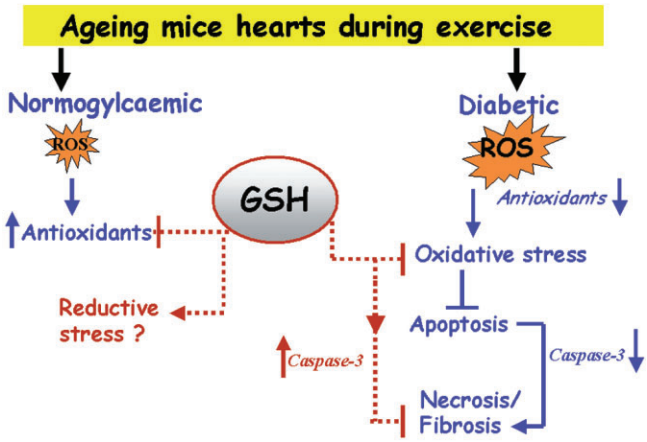

Figure 9

Graphical abstract explaining effects of GSH on cardiac cell death in ageing hearts during exercise. Exercise and antioxidant supplementation has different effects depending on whether they are diabetic or normoglycaemic. GSH (or other antioxidants that increase GSH) in ageing diabetic hearts ameliorates mOXS, prevents necrosis, while activating caspase-3 and apoptosis during exercise. In contrast, in the *absence* of chronic oxidative stress (i.e. normoglycaemic hearts), efforts to increase GSH block physiological up-regulation of ROS and antioxidants, and induces 'stress'. Blue fonts and lines represent events occurring without antioxidant intervention in both ageing normoglycaemic and diabetic hearts during exercise. Red lines and fonts represent events in the same following GSH therapy.

injury (Frangogiannis *et al.*, 2002). Moreover, calcium overload and hyperactivation of β-adrenergic receptors also accelerate deterioration of cardiac function during necrosis (Nakayama *et al.*, 2007). In this study, exercised *db/db* hearts also demonstrated extensive fibrosis, which was attenuated with exogenous GSH. Therefore, by inducing a 'switch' from necrosis to apoptosis, GSH therapy could provide anti-inflammatory and anti-fibrotic effects in the older diabetic myocardium during exercise. We conclude that while GSH therapy [or GSH enhancing agents such as lipoic acid (Ziegler *et al.*, 2011)] during exercise may benefit in ageing diabetic hearts by attenuating mOXS, necrosis or fibrosis; antioxidant therapy during exercise could damage the mitochondria and activate death signalling in the healthy heart.

Acknowledgements

This work was supported by the Scholar award from Canadian Diabetes Association, and Grant-In-Aids from the internal UBC Research Office and CDA to S. G., Heart and Stroke Foundation of British Columbia and Yukon to I. L. CIHR Doctoral award is also gratefully acknowledged for A. B. Editorial help of Brieanne Matier during finalization of this manuscript is also much appreciated.

Author contributions

S. G. performed all animal work and experiments and helped analyse the data. A. B. performed cardiomyocyte experiments, analysed all *in vitro* data and wrote the first draft of the manuscript. S. G. performed fibroblast experiments and Western blots. A. N. performed all immunohistochemistry studies. I. L. assessed the results and wrote the manuscript. S. G. conceived, guided and supported experiments, assessed the results and wrote the manuscript.

Conflict of interest

None.

References

Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M *et al.* (2013). The Concise Guide to PHARMACOLOGY 2013/14: Enzymes. Br J Pharmacol 170: 1797–1867.

Anathy V, Roberson EC, Guala AS, Godburn KE, Budd RC, Janssen-Heininger YM (2012). Redox-based regulation of apoptosis: S-glutathionylation as a regulatory mechanism to control cell death. Antioxid Redox Signal 16: 496–505.

Anderson EJ, Kypson AP, Rodriguez E, Anderson CA, Lehr EJ, Neufer PD (2009). Substrate-specific derangements in mitochondrial metabolism and redox balance in the atrium of the type 2 diabetic human heart. J Am Coll Cardiol 54: 1891–1898.

Asakura T, Sawai T, Hashidume Y, Ohkawa Y, Yokoyama S, Ohkawa K (1999). Caspase-3 activation during apoptosis caused by glutathione-doxorubicin conjugate. Br J Cancer 80: 711–715.

Bahi N, Zhang J, Llovera M, Ballester M, Comella JX, Sanchis D (2006). Switch from caspase-dependent to caspase-independent death during heart development: essential role of endonuclease G in ischemia-induced DNA processing of differentiated cardiomyocytes. J Biol Chem 281: 22943–22952.

Belmokhtar CA, Hillion J, Segal-Bendirdjian E (2001). Staurosporine induces apoptosis through both caspase-dependent and caspase-independent mechanisms. Oncogene 20: 3354–3362.

Blankenberg S, Rupprecht HJ, Bickel C, Torzewski M, Hafner G, Tiret L *et al.* (2003). Glutathione peroxidase 1 activity and cardiovascular events in patients with coronary artery disease. N Engl J Med 349: 1605–1613.

Borutaite V, Brown GC (2001). Caspases are reversibly inactivated by hydrogen peroxide. FEBS Lett 500: 114–118.

Botta A, Laher I, Beam J, Decoffe D, Brown K, Halder S *et al.* (2013). Short term exercise induces PGC-1alpha, ameliorates inflammation and increases mitochondrial membrane proteins but fails to increase respiratory enzymes in aging diabetic hearts. PLoS ONE 8: e70248.

Cai L, Li W, Wang G, Guo L, Jiang Y, Kang YJ (2002). Hyperglycemia-induced apoptosis in mouse myocardium: mitochondrial cytochrome C-mediated caspase-3 activation pathway. Diabetes 51: 1938–1948.

Circu ML, Stringer S, Rhoads CA, Moyer MP, Aw TY (2009). The role of GSH efflux in staurosporine-induced apoptosis in colonic epithelial cells. Biochem Pharmacol 77: 76–85.

Comelli M, Domenis R, Bisetto E, Contin M, Marchini M, Ortolani F *et al.* (2011). Cardiac differentiation promotes mitochondria development and ameliorates oxidative capacity in H9c2 cardiomyoblasts. Mitochondrion 11: 315–326.

Communal C, Sumandea M, De Tombe P, Narula J, Solaro RJ, Hajjar RJ (2002). Functional consequences of caspase activation in cardiac myocytes. Proc Natl Acad Sci U S A 99: 6252–6256.

Coppola S, Ghibelli L (2000). GSH extrusion and the mitochondrial pathway of apoptotic signalling. Biochem Soc Trans 28: 56–61.

Damy T, Kirsch M, Khouzami L, Caramelle P, Le Corvoisier P, Roudot-Thoraval F *et al.* (2009). Glutathione deficiency in cardiac patients is related to the functional status and structural cardiac abnormalities. PLoS ONE 4: e4871.

Fernandez-Checa JC, Kaplowitz N, Garcia-Ruiz C, Colell A (1998). Mitochondrial glutathione: importance and transport. Semin Liver Dis 18: 389–401.

Frangogiannis NG, Smith CW, Entman ML (2002). The inflammatory response in myocardial infarction. Cardiovasc Res 53: 31–47.

Frankfurt OS (2004). Immunoassay for single-stranded DNA in apoptotic cells. Methods Mol Biol 282: 85–101.

Frustaci A, Kajstura J, Chimenti C, Jakoniuk I, Leri A, Maseri A *et al.* (2000). Myocardial cell death in human diabetes. Circ Res 87: 1123–1132.

Ghosh S, Pulinilkunnil T, Yuen G, Kewalramani G, An D, Qi D *et al.* (2005). Cardiomyocyte apoptosis induced by short-term diabetes requires mitochondrial GSH depletion. Am J Physiol Heart Circ Physiol 289: H768–H776.

Ghosh S, Kewalramani G, Yuen G, Pulinilkunnil T, An D, Innis SM *et al.* (2006). Induction of mitochondrial nitrative damage and

cardiac dysfunction by chronic provision of dietary omega-6 polyunsaturated fatty acids. Free Radic Biol Med 41: 1413–1424.

Ghosh S, Golbidi S, Werner I, Verchere BC, Laher I (2010). Selecting exercise regimens and strains to modify obesity and diabetes in rodents: an overview. Clin Sci (Lond) 119: 57–74.

Ghosh S, Sulistyoningrum DC, Glier MB, Verchere CB, Devlin AM (2011). Altered glutathione homeostasis in heart augments cardiac lipotoxicity associated with diet-induced obesity in mice. J Biol Chem 286: 42483–42493.

Gliemann L, Schmidt JF, Olesen J, Bienso RS, Peronard SL, Grandjean SU *et al.* (2013). Resveratrol blunts the positive effects of exercise training on cardiovascular health in aged men. J Physiol 591: 5047–5059.

Golbidi S, Ebadi SA, Laher I (2011). Antioxidants in the treatment of diabetes. Curr Diabetes Rev 7: 106–125.

Gori T, Dragoni S, Di Stolfo G, Sicuro S, Liuni A, Luca MC *et al.* (2010). Tolerance to nitroglycerin-induced preconditioning of the endothelium: a human *in vivo* study. Am J Physiol Heart Circ Physiol 298: H340–H345.

Grasl-Kraupp B, Ruttkay-Nedecky B, Koudelka H, Bukowska K, Bursch W, Schulte-Hermann R (1995). In situ detection of fragmented DNA (TUNEL assay) fails to discriminate among apoptosis, necrosis, and autolytic cell death: a cautionary note. Hepatology 21: 1465–1468.

Griffith OW (1980). Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. Anal Biochem 106: 207–212.

Griffith OW (1999). Biologic and pharmacologic regulation of mammalian glutathione synthesis. Free Radic Biol Med 27: 922–935.

Hampton MB, Orrenius S (1997). Dual regulation of caspase activity by hydrogen peroxide: implications for apoptosis. FEBS Lett 414: 552–556.

Hwang TL, Tsai CJ, Chen JL, Changchien TT, Wang CC, Wu CM (2012). Magnesium ascorbyl phosphate and coenzyme Q10 protect keratinocytes against UVA irradiation by suppressing glutathione depletion. Mol Med Rep 6: 375–378.

Ishihara M, Inoue I, Kawagoe T, Shimatani Y, Kurisu S, Nishioka K *et al.* (2001). Diabetes mellitus prevents ischemic preconditioning in patients with a first acute anterior wall myocardial infarction. J Am Coll Cardiol 38: 1007–1011.

Kazzaz JA, Xu J, Palaia TA, Mantell L, Fein AM, Horowitz S (1996). Cellular oxygen toxicity. Oxidant injury without apoptosis. J Biol Chem 271: 15182–15186.

Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG (2010). Animal research: reporting *in vivo* experiments: the ARRIVE guidelines. Br J Pharmacol 160: 1577–1579.

Laher I, Beam J, Botta A, Barendregt R, Sulistyoningrum D, Devlin A *et al.* (2013). Short-term exercise worsens cardiac oxidative stress and fibrosis in 8-month-old db/db mice by depleting cardiac glutathione. Free Radic Res 47: 44–54.

Lappalainen Z, Lappalainen J, Oksala NK, Laaksonen DE, Khanna S, Sen CK *et al.* (2009). Diabetes impairs exercise training-associated thioredoxin response and glutathione status in rat brain. J Appl Physiol 106: 461–467.

Lawler JM, Kwak HB, Kim JH, Suk MH (2009). Exercise training inducibility of MnSOD protein expression and activity is retained while reducing prooxidant signaling in the heart of senescent rats. Am J Physiol Regul Integr Comp Physiol 296: R1496–R1502.

Los M, Mozoluk M, Ferrari D, Stepczynska A, Stroh C, Renz A *et al.* (2002). Activation and caspase-mediated inhibition of PARP: a molecular switch between fibroblast necrosis and apoptosis in death receptor signaling. Mol Biol Cell 13: 978–988.

Malkusch W, Rehn B, Bruch J (1995). Advantages of Sirius Red staining for quantitative morphometric collagen measurements in lungs. Exp Lung Res 21: 67–77.

Mari M, Morales A, Colell A, Garcia-Ruiz C, Fernandez-Checa JC (2009). Mitochondrial glutathione, a key survival antioxidant. Antioxid Redox Signal 11: 2685–2700.

McGrath JC, Drummond GB, McLachlan EM, Kilkenny C, Wainwright CL (2010). Guidelines for reporting experiments involving animals: the ARRIVE guidelines. Br J Pharmacol 160: 1573–1576.

McManus MJ, Murphy MP, Franklin JL (2011). The mitochondria-targeted antioxidant MitoQ prevents loss of spatial memory retention and early neuropathology in a transgenic mouse model of Alzheimer's disease. J Neurosci 31: 15703–15715.

Moien-Afshari F, Ghosh S, Elmi S, Khazaei M, Rahman MM, Sallam N *et al.* (2008). Exercise restores coronary vascular function independent of myogenic tone or hyperglycemic status in db/db mice. Am J Physiol Heart Circ Physiol 295: H1470–H1480.

van den Munckhof I, Riksen N, Seeger JP, Schreuder TH, Borm GF, Eijsvogels TM *et al.* (2013). Aging attenuates the protective effect of ischemic preconditioning against endothelial ischemia-reperfusion injury in humans. Am J Physiol Heart Circ Physiol 304: H1727–H1732.

Nakayama H, Chen X, Baines CP, Klevitsky R, Zhang X, Zhang H *et al.* (2007). Ca^{2+}- and mitochondrial-dependent cardiomyocyte necrosis as a primary mediator of heart failure. J Clin Invest 117: 2431–2444.

Nicotera P, Melino G (2004). Regulation of the apoptosis-necrosis switch. Oncogene 23: 2757–2765.

Otani H (2004). Reactive oxygen species as mediators of signal transduction in ischemic preconditioning. Antioxid Redox Signal 6: 449–469.

Pawson AJ, Sharman JL, Benson HE, Faccenda E, Alexander SP, Buneman OP *et al.*; NC-IUPHAR (2014). The IUPHAR/BPS Guide to PHARMACOLOGY: an expert-driven knowledgebase of drug targets and their ligands. Nucl. Acids Res 42 (Database Issue): D1098–106.

Proskuryakov SY, Konoplyannikov AG, Gabai VL (2003). Necrosis: a specific form of programmed cell death? Exp Cell Res 283: 1–16.

Ramiro-Cortes Y, Moran J (2009). Role of oxidative stress and JNK pathway in apoptotic death induced by potassium deprivation and staurosporine in cerebellar granule neurons. Neurochem Int 55: 581–592.

Ristow M, Zarse K, Oberbach A, Kloting N, Birringer M, Kiehntopf M *et al.* (2009). Antioxidants prevent health-promoting effects of physical exercise in humans. Proc Natl Acad Sci U S A 106: 8665–8670.

Rossiter JP, Anderson LL, Yang F, Cole GM (2002). Caspase-3 activation and caspase-like proteolytic activity in human perinatal hypoxic-ischemic brain injury. Acta Neuropathol 103: 66–73.

Rousseau AS, Margaritis I, Arnaud J, Faure H, Roussel AM (2006). Physical activity alters antioxidant status in exercising elderly subjects. J Nutr Biochem 17: 463–470.

Sen CK, Roy S (2001). Redox control of caspases. Environ Toxicol Pharmacol 10: 215–220.

Senft AP, Dalton TP, Shertzer HG (2000). Determining glutathione and glutathione disulfide using the fluorescence probe o-phthalaldehyde. Anal Biochem 280: 80–86.

Short KR, Vittone JL, Bigelow ML, Proctor DN, Rizza RA, Coenen-Schimke JM *et al*. (2003). Impact of aerobic exercise training on age-related changes in insulin sensitivity and muscle oxidative capacity. Diabetes 52: 1888–1896.

Skulachev VP (2006). Bioenergetic aspects of apoptosis, necrosis and mitoptosis. Apoptosis 11: 473–485.

Suh JH, Wang H, Liu RM, Liu J, Hagen TM (2004). (R)-Alpha-lipoic acid reverses the age-related loss in GSH redox status in post-mitotic tissues: evidence for increased cysteine requirement for GSH synthesis. Arch Biochem Biophys 423: 126–135.

Teramoto S, Tomita T, Matsui H, Ohga E, Matsuse T, Ouchi Y (1999). Hydrogen peroxide-induced apoptosis and necrosis in human lung fibroblasts: protective roles of glutathione. Jpn J Pharmacol 79: 33–40.

Tessier D, Menard J, Fulop T, Ardilouze J, Roy M, Dubuc N *et al*. (2000). Effects of aerobic physical exercise in the elderly with type 2 diabetes mellitus. Arch Gerontol Geriatr 31: 121–132.

Thygesen K, Mair J, Katus H, Plebani M, Venge P, Collinson P *et al*. (2010). Recommendations for the use of cardiac troponin measurement in acute cardiac care. Eur Heart J 31: 2197–2204.

Zhang H, Limphong P, Pieper J, Liu Q, Rodesch CK, Christians E *et al*. (2012). Glutathione-dependent reductive stress triggers mitochondrial oxidation and cytotoxicity. FASEB J 26: 1442–1451.

Ziegler D, Low PA, Litchy WJ, Boulton AJ, Vinik AI, Freeman R *et al*. (2011). Efficacy and safety of antioxidant treatment with alpha-lipoic acid over 4 years in diabetic polyneuropathy: the NATHAN 1 trial. Diabetes Care 34: 2054–2060.

Zuliani T, Duval R, Jayat C, Schnebert S, Andre P, Dumas M *et al*. (2003). Sensitive and reliable JC-1 and TOTO-3 double staining to assess mitochondrial transmembrane potential and plasma membrane integrity: interest for cell death investigations. Cytometry A 54: 100–108.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

http://dx.doi.org/10.1111/bph.12847

Figure S1 GSH does not improve metabolic profile or cardiac antioxidants in exercised db/db mice.
Table S1 Sources of antibodies used in study.