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Cytosolic, but not mitochondrial, oxidative stress likely contributes to cardiac hypertrophy resulting from cardiac specific GLUT4 deletion in mice

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SUMMARY

We hypothesized that oxidative stress may contribute to the development of hypertrophy observed in mice with cardiac specific ablation of the insulin sensitive glucose transporter 4 gene (GLUT4, $G4H^{-/-}$). Measurements of oxidized glutathione (GSSG) in isolated mitochondria and whole heart homogenates were increased resulting in a lower ratio of reduced glutathione (GSH) to GSSG. Membrane translocation of the $p67^{phox}$ subunit of cardiac NOX2 was markedly increased in $G4H^{-/-}$ mice, suggesting elevated activity. To determine if oxidative stress was contributing to cardiac hypertrophy, 4-week old Con and $G4H^{-/-}$ mice were treated with either tempol (T, 1mM, drinking water), a whole cell antioxidant, or Mn(III)tetrakis(4-Benzoic acid) porphyrin chloride (MnTBAP, 10 mg/kg, ip), a mitochondrial targeted antioxidant, for 28d. Tempol attenuated cardiac hypertrophy in $G4H^{-/-}$ mice (heart:tibia, Con 6.82±0.35, $G4H^{-/-}$ 8.83±0.34, Con+T 6.82±0.46, $G4H^{-/-}$+T 7.57±0.3), without changing GSH:GSSG, GPX4, or membrane translocation of the $p67^{phox}$. Tempol did not modify phosphorylation of glycogen synthase kinase (GSK) 3β or thioredoxin-2. In contrast, MnTBAP lowered mitochondrial GSSG, improved GSH:GSSG, but did not prevent hypertrophy; indicating that mitochondrial oxidative stress may not be critical for hypertrophy in this model. The ability of tempol to attenuate cardiac hypertrophy suggests that a cytosolic source of reactive oxygen species, likely NOX2, may contribute to the hypertrophic phenotype in $G4H^{-/-}$ mice.

Keywords

ROS; mitochondria; cardiac hypertrophy; antioxidant; p67phox; GLUT4

INTRODUCTION

Epidemiological and clinical studies report that diabetes is associated with cardiac hypertrophy after adjusting for underlying coronary artery disease, ischemia or hypertension [1-3]. Cardiac hypertrophy alone is an independent risk factor for cardiovascular morbidity and mortality [4]. One commonly reported feature of the diabetic heart is an increase in reactive oxidative species (ROS) [5-7]. Major sources of ROS in the heart are NADPH oxidase and/or mitochondrial oxidative phosphorylation [8]. ROS are routinely removed by cellular antioxidants including superoxide dismutase (SOD, consisting of two isoforms; a mitochondrial Mn-SOD and cytosolic Cu-Zn-SOD), catalase, and glutathione peroxidase [8]. The characteristic increase in fatty acid β-oxidation (FAO) in diabetes coupled with increased cellular oxygen demand and mitochondrial dysfunction [9] increases the potential

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for ROS generation. Oxidative stress has been reported to play a critical role in cardiac hypertrophy [10, 11]. Similarly, impairment of endogenous antioxidant defenses through inhibition of thioredoxin results in cardiac hypertrophy [12], while the loss of superoxide dismutase (SOD) exacerbates hypertrophy in response to pressure overload [13].

We previously established a mouse model with cardiac specific deletion of the glucose transporter 4 gene (GLUT4, G4H$^{-/-}$) to study the effect of impaired cardiac glucose uptake on the ensuing cardiac phenotype [14]. Initial characterization revealed that cardiomyocyte loss of both GLUT4 mRNA and protein resulted in moderate cardiac hypertrophy without fibrosis [14]. This change in individual cardiomyocyte size develops by eight weeks of age in the absence of hyperinsulinemia, hyperglycemia or changes in serum concentrations of fatty acid, lactate, amino acids or ketones [14].

A recent study using an independently-generated murine model of GLUT4 deletion in the heart, superimposed upon reduced GLUT4 expression in skeletal muscle and fat, suggested that oxidative stress plays a role in cardiac hypertrophy, as treating adult mice (~150 d old) with tempol (a membrane permeable antioxidant) reversed cardiac hypertrophy [15]. Although tempol treatment in this study was associated with reduced mRNAs of NOX1 and gp91^{phox}, measured NADPH Oxidase activities were not altered in GLUT4 deficient hearts prior to and after tempol treatment. Thus the precise source of ROS in GLUT4 deficient hearts is not well characterized and the potential role that mitochondrial oxidative stress may play in stimulating cardiac hypertrophy in these hearts is incompletely understood. Moreover, the role of oxidative stress in the hypertrophy of GLUT4 deficient hearts remains to be clarified in a system that is not confounded by systemic insulin resistance or reduced expression of GLUT4 in other tissues. The contribution of mitochondria to ROS generation may be important in G4H$^{-/-}$ mice given the alterations in substrate utilization and FAO previously observed in insulin resistant hearts. Therefore, we hypothesized that reducing mitochondrial oxidative stress would prevent the development of cardiac hypertrophy in G4H$^{-/-}$ mice. To test this hypothesis we first determined the degree to which young and adult G4H$^{-/-}$ mice have cardiac oxidative stress, and then compared the effects of treating mice with 2 unique antioxidants; tempol and Mn(III) tetrakis (4-benzoic acid) porphyrin chloride (MnTBAP) on development of cardiac hypertrophy. Tempol is a whole cell antioxidant that has been shown to reduce markers of oxidative stress in membrane, cytosol, and mitochondrial compartments [16, 17], while MnTBAP is a mitochondrial-targeted superoxide 2 mimetic. We report here that G4H$^{-/-}$ mice show signs of mild oxidative stress, which likely stems from NOX2, a cytosolic source of ROS. Furthermore, while treatment with the whole cell antioxidant tempol attenuates cardiac hypertrophy, the mitochondrial-targeted antioxidant, MnTBAP, does not.

RESULTS

Cardiac hypertrophy and markers of oxidative stress

Mixed gender cohorts were used for all studies. Body mass (g) and tibia lengths were similar between groups (Table 1). In agreement with previous studies that have characterized the cardiac phenotype of these G4H$^{-/-}$ mice [14, 18], heart mass was increased and cardiac hypertrophy was present in G4H$^{-/-}$ mice compared to Con at 25 weeks of age but was not present at 5 weeks of age (Table 1). Multiple markers of oxidative stress were examined in hearts of 25-week old mice. Total ROS as determined by DCFDA assay in whole cardiac homogenates and malondialdehyde (MDA) were similar between Con and G4H$^{-/-}$ mice (Figure 1A, B). In isolated mitochondria, hydrogen peroxide concentrations and aconitase activity were unchanged as well (Figure 1C, D). Cytosolic aconitase activity was not altered in G4H$^{-/-}$ compared to Con mice (Figure 1E). While concentrations of GSH (reduced glutathione) in whole cardiac homogenates was unchanged in G4H$^{-/-}$ mice, the amount of

GSSG (oxidized glutathione) was significantly greater such that GSH:GSSG was lower in G4H$^{-/-}$ mice compared to Con (Figure 1F-H). In 5-week-old mice prior to the onset of cardiac hypertrophy, a similar pattern of increased GSSG leading to reduced GSH:GSSG ratios were observed (Table 1). Interestingly, at 8-weeks of age, the reduction in GSH:GSSG ratios were secondary to reduced GSH that was associated with a more modest increase in GSSG (Table 2). In isolated mitochondria an increase in GSSG and reduced GSH:GSSG ratios were observed in G4H$^{-/-}$ hearts (Table 3).

Tempol Treatment

Since increased cardiac oxidized glutathione concentrations preceded cardiac hypertrophy, we hypothesized that oxidative stress contributed to the hypertrophic phenotype in G4H$^{-/-}$ mice. To test this, the antioxidant tempol was administered as a 1 mM solution in drinking water. Overall water consumption was unchanged among treated vs. untreated mice (data not shown). Body weight and tibia length were also unchanged during the 4-week treatment period (Table 2). Tempol prevented cardiac hypertrophy in G4H$^{-/-}$ mice as evidenced by similar heart weight and heart:tibia length in tempol treated G4H$^{-/-}$ mice vs. control mice (Table 2). GSH:GSSG was lower in all G4H$^{-/-}$ compared to all Con, and tempol treatment did not alter this ratio (Table 2).

To assess if the changes in the GSH:GSSG ratio in G4H$^{-/-}$ hearts could be accounted for by upregulation of glutathione peroxidase (GPX) and/or downregulation of glutathione reductase (GR) we measured RNA and protein levels of these enzymes. There was no difference in either GR gene transcripts or protein in hearts of Con vs. G4H$^{-/-}$ mice (Figures 2a, 3a). There was also no change in gene expression of GPX4 and GPX1 in hearts of G4H$^{-/-}$ mice vs. Con (Figure 2a). Protein expression of GPX4 in untreated G4H$^{-/-}$ mice was inconsistent between cohorts used for the tempol and MnTBAP studies. For example, in the tempol study GPX4 was greater in G4H$^{-/-}$ mice vs. Con, but in the MnTBAP cohort, where both G4H$^{-/-}$ and Con received daily intraperitoneal injections of saline, GPX4 was similar (Figure 3). Although there were minor changes in GPX4 expression levels in tempol-treated G4H$^{-/-}$, protein levels were not modulated by tempol in control or G4H$^{-/-}$ mouse hearts, respectively. Similarly, small changes in glutathione reductase protein, in the MnTBAP cohort are unlikely to account for the differences observed in GSH:GSSG ratios.

NOX2 is a generator of cytosolic superoxide that is located on the cell membrane. Full activation of NOX2 requires assembly with p67^{phox} (also known as NOXA1) and p47^{phox} subunits before superoxide can be produced. Expression of the p67^{phox} gene was similar among all mice (Figure 2a). Expression of p47^{phox} remained similar among all groups (Figure 2a). The protein levels of p67^{phox} subunit of NOX2 in cardiac membrane fractions were markedly greater in G4H$^{-/-}$ vs. Con, suggesting an increase in NOX2 activation. Protein levels of p67^{phox} in the cytosol were unchanged (data not shown). The activation state of NOX2 remained unchanged in G4H$^{-/-}$+T mice as the membrane localization of p67^{phox} protein was similar to untreated G4H$^{-/-}$ (Figure 4). Thioredoxin (TXN2) expression, a redox-active molecule that is found in cardiac mitochondria, was increased in G4H$^{-/-}$ mice vs. Con, but, similar between Con+T and G4H$^{-/-}$+T (Figure 2a).

The reduction in cardiac hypertrophy resulting from tempol treatment in G4H$^{-/-}$ mice was accompanied by the reduction of some, but not all of the classic genetic markers of hypertrophy. In G4H$^{-/-}$ mice α-MHC, brain naturetic peptide (BNP), SERCA2a, and SERCA2b were unchanged compared to Con, and were not further affected by tempol treatment (Figure 2b). In contrast to the present study, our original study characterizing G4H$^{-/-}$ mice found increases in BNP[14]. We believe that the most likely basis for this difference is that our previous work used only male mice aged 13-14 weeks, and northern blots were employed to detect BNP expression, whereas the current study used a mixed

cohort of 8-week old males and females, and real time PCR was used to detect transcripts. Expression of atrial naturetic factor (ANF), on the other hand, was increased in G4H$^{-/-}$ mice vs. Con. While there was a trend present (p=0.06) toward increased β-myosin heavy chain (MHC) between Con and G4H$^{-/-}$ mice, this was not statistically significant (Figure 2b). β-MHC expression was similar in tempol treated Con and G4H$^{-/-}$ mice (Figure 2b). Consistent with previous reports [14], GLUT1 expression was higher in G4H$^{-/-}$ compared to Con (Figure 2b).

Glycogen synthase kinase (GSK) 3β inactivation has been reported to play a role in other diabetic models of cardiac hypertrophy [19]. To determine if tempol reduced cardiac hypertrophy in G4H$^{-/-}$ mice through an oxidative stress independent mechanism, we measured phosphorylated -(p) GSK3β and total GSK3β in hearts of Con and G4H$^{-/-}$ mice. Our data indicated that the ratio of p-GSK3β to total GSK3β was unchanged between Con and G4H$^{-/-}$ mice, and that tempol did not affect GSK3β activation in either group (Figure 5).

MnTBAP treatment

Isolated mitochondria of G4H$^{-/-}$ mice had greater GSSG and reduced GSH:GSSG compared to control mice (Table 3). To determine if a mitochondrial oxidative stress was a contributor to cardiac hypertrophy in G4H$^{-/-}$ mice, we used the mitochondrial targeted antioxidant MnTBAP. A 4-week treatment with MnTBAP increased mitochondrial GSH in G4H-/- mice compared to saline treated G4H$^{-/-}$. MnTBAP also lowered GSSG and increased GSH:GSSG in G4H$^{-/-}$ mice to levels similar to controls. However, this improvement in mitochondrial oxidative stress was not accompanied by a reduction in heart weight or cardiac hypertrophy (Table 3). These data indicate that while mitochondrial oxidative stress is present in G4H$^{-/-}$ mice, it is unlikely to be the basis for the development of cardiac hypertrophy. MntBAP treatment did not produce any change in mRNAs for GR, GPX1 or TXN2. There was also no effect of MnTBAP treatment on GPX4 protein and the small reduction in GR cannot account for changes in GSH:GSSG that was observed.

DISCUSSION

The major findings of this study are that G4H$^{-/-}$ mice have signs of low level oxidative stress in the heart since both whole cell homogenate and mitochondrial preparations had lower GSH:GSSG. The reduction in GSH:GSSG was seen in mice as young as 5 weeks old, prior to the presence of significant cardiac hypertrophy, suggesting a role for oxidative stress in the pathogenesis of hypertrophy. G4H$^{-/-}$ mice also had increased protein expression of p67^{phox}. Treatment with a general membrane permeable antioxidant, tempol, prevented cardiac hypertrophy, while the mitochondrial-targeted antioxidant, MnTBAP, did not affect development of hypertrophy. Tempol treatment did not reverse the membrane localization of p67^{phox} and did not normalize GSH:GSSG, so the possibility remains that the impact of tempol on cardiac hypertrophy could be an off target effect that is unrelated to oxidative stress. However, the MnTBAP experiments presented in this study strongly suggest that mitochondrial oxidative stress is unlikely to play a role in cardiac hypertrophy in G4H$^{-/-}$ hearts.

Our conclusion that G4H$^{-/-}$ mice have low-level oxidative stress is based on data indicating that there was no difference between many indicators of oxidative stress in Con vs. G4H$^{-/-}$ mice, whereas glutathione concentrations were consistently altered in G4H$^{-/-}$ mice. In agreement with our study, previous studies using rodents have also reported changes in glutathione ratio without alterations in other markers of oxidative stress, such as lipid peroxidation [20]. This suggests that the status of glutathione is a sensitive indicator of the redox state of the heart. Glutathione is a major antioxidant in the cell and it is well known that oxidative stress consumes reduced glutathione (GSH). Increases in oxidized cardiac

glutathione are widely reported in rodents with hypertrophy, heart failure, and/or diabetes [21-24]. Changes in the ratio of oxidized versus reduced glutathione, could be secondary to differences in the expression or activity of enzymes that mediate the interconversion of GSH and GSSH, or to differences in the concentration of proton donors or acceptors. GPX4 protein levels were actually lower and GR protein levels were unchanged between Con and $G4H^{-/-}$ mice. Thus enzyme levels cannot account for the differences observed, although changes in activity cannot be ruled out.

This current study follows a previous report by Ritchie et al. that used a different model wherein cardiac specific GLUT4 deletion was superimposed upon reduced GLUT4 expression in all tissues [15]. There were several points of similarity between our study and the aforementioned one. Ritchie et al. reported that tempol reduced both cardiac hypertrophy without any change in NOX2 mediated superoxide production [15]. Similarly, we also found that tempol attenuated hypertrophy and that translocation of $p67^{phox}$ in $G4H^{-/-}$ mice was not affected. However, there are also several important differences between the experimental design and methods of the current study and the one by Ritchie et al. For example, 1) the $G4H^{-/-}$ mouse used in our study is a specific model of cardiac specific GLUT4 deletion, whereas the model used by Ritchie et al. has very low, but not completely deleted levels cardiac GLUT4 protein that is superimposed on whole body GLUT4 knockdown, 2) our model does not have insulin resistance, whereas the model used by Ritchie et al. does, 3) we examined a number of markers of oxidative stress in both cytosol and mitochondria, 4) we conducted additional experiments with another antioxidant (MnTBAP) to evaluate the potential contribution of mitochondrial-specific oxidative stress to the development of cardiac hypertrophy. The effectiveness of MnTBAP to reduce mitochondrial superoxide has been previously demonstrated *in vitro* and *in vivo* using oxygen-glucose depleted cortical cell cultures [25] and SOD2 null mice [26]. Furthermore, since mitochondria have a negative resting potential, it is thought that they can accumulate positively charged antioxidant compounds such as MnTBAP (containing Mn^{3+}).

One of the aims of this study was to determine if the source of oxidative stress was mitochondrial or cytosolic in origin. General parameters of oxidative stress such as malondialdehyde levels, cytosolic aconitase, and whole cell superoxide production were unchanged. In isolated mitochondria, aconitase activity and hydrogen peroxide concentrations were also unchanged. In spite of this both whole cell and mitochondrial GSH:GSSG was lower. Taken together these data suggest that while overt oxidative stress does not exist, there is a degree of mild oxidative stress present in both mitochondria and whole homogenates. Use of two unique antioxidants (tempol and MnTBAP) allowed us to determine which ROS generating compartment was critical to the phenotype of cardiac hypertrophy. The fact that MnTBAP treatment lowered oxidized glutathione concentrations and improved GSH:GSSG in mitochondria without a concomitant reduction of cardiac hypertrophy, supports the conclusion that mitochondrial oxidative stress is not an important driver of the hypertrophy in $G4H^{-/-}$ mice. Tempol treatment, on the other hand, markedly attenuated hypertrophy. $G4H^{-/-}$ mice also displayed persistent translocation of $p67^{phox}$ (Figure 4) indicating activation of NOX2 and superoxide production. Given the importance of NOX2 as a major source of cytosolic oxidative stress and its role in cardiac hypertrophy [27-29], it is likely that NOX2 could be a cytosolic driver of cardiac hypertrophy in the $G4H^{-/-}$ mouse. However, tempol treatment did not alter the translocation of $p67^{phox}$, nor were any changes in NOX activity observed by Ritchie et al[15, 30]. Taken together, these studies suggest that cytosolic sources of oxidative stress, rather than mitochondrial, are contributing to the hypertrophic phenotype, although neither our study nor that of Ritchie et al. have specifically elucidated the precise source of cytosolic ROS that mediates this effect.

In spite of the reduction in hypertrophy after tempol treatment, whole cell GSH:GSSG was not improved in G4H$^{-/-}$+T mice. There are 2 possible explanations for the lack of change in glutathione concentrations. 1) Either tempol exerts it action due to an off target or nonspecific effect that is unrelated to oxidative stress, or 2) glutathione concentrations in whole homogenates may not specifically reflect cytosolic glutathione concentrations. With regard to the latter point, whole cardiac tissue was homogenized to obtain GSH and GSSG concentrations. Mitochondria extracts present in this whole homogenate contributed to the levels of GSSG measured. It is possible that if the dosage of tempol used in this study did not successfully reduce mitochondrial GSSG levels, then the whole cell assay may reflect a GSH:GSSG concentration that is unchanged due to the contribution from the mitochondria. In support of this, it has been reported that tempol does not have a strong affinity for accumulating in mitochondria, and is only weakly reduced by the mitochondrial CoQ pool [31]. In contrast, mitoTempol, a mitochondrial targeted version of tempol, accumulates 100 fold greater in mitochondria and is strongly reduced by the mitochondrial CoQ pool [31]. Interestingly, there are other reports of i.v. tempol infusion failing to alter GSH:GSSG in skeletal muscle homogenates [32], and that at least 4 weeks of tempol treatment (1mM in drinking water) were required prior to improvement of GSH:GSSG in the heart. Thus a limitation of this study is the fact that we did not measure cytosolic levels of GSH and GSSG.

Since it was possible that tempol attenuated hypertrophy in G4H$^{-/-}$ mice through off target mechanisms that may be unrelated to oxidative stress, we examined two alternative mechanisms; GLUT1 expression and GSK3β activation. GLUT1 is a related and constitutively active glucose transporter. A compensatory increase in GLUT1 expression could re-establish normal glucose supply and rescue the heart from GLUT4 deficiency. Though we confirmed our previous findings that G4H$^{-/-}$ mice have an increase in GLUT1 expression [14], we did not find that tempol treatment increased GLUT1 expression further, but instead decreased it. Alternatively, GSK activation has been reported to play a role in cardiac hypertrophy in other models of diabetes. For example, in diabetes produced by streptozotocin treatment, GSK3β is less phosphorylated (more active) in the hypertrophied heart of rodent models of diabetes [19]. Furthermore, when these rats are treated with tempol, the phosphorylation of GSK3β is increased, it becomes less active, and hypertrophy is subsequently reduced [19]. However, we did not observe any change in GSK3β activation in G4H$^{-/-}$ vs. Con mice, either before or after tempol treatment. In line with our data, Richie et al. found no change in GSK3β gene expression in hearts of mice with GLUT4 deletion superimposed on whole body GLUT4 suppression[15]. However, they also reported a significant increase in GSK3β expression following tempol treatment, whereas we saw no change. It is important to note that we assessed GSK3β status by measuring both total and phosphorylated GSK3β protein rather than changes in mRNA. While we can speculate that GSK3β activation may be altered earlier in the development phase of hypertrophy (i.e. between 4 and 7 weeks of age), our present data does not support a role for GSK3β in the G4H$^{-/-}$ mice. Another possible mechanism that could have mediated the observed reduction in cardiac hypertrophy in G4H$^{-/-}$ mice is reduction of blood pressure. A limitation of this study is that we did not measure blood pressure, however, it should be noted that there was no genetic manipulation of blood pressure regulating tissues such as arteries, kidneys, and brain, and that these mice had normal whole body metabolic profile, in spite of GLUT4 deletion in the heart. Therefore, we speculate that blood pressure was unlikely to be a factor.

The G4H$^{-/-}$ mouse was originally developed to study the importance of insulin mediated glucose uptake in cardiac function [14]. As such the development of cardiac hypertrophy due to GLUT4 deletion in this model was an unexpected finding. Data from our present study indicates that oxidative stress may contribute to the development of cardiac hypertrophy. While this notion has been confirmed by a previous study that used tempol to

reduce hypertrophy in a different model of cardiac specific GLUT4 deletion that was superimposed on whole body GLUT4 suppression, the cellular source of ROS that drove hypertrophy remained elusive. In the present study, both the increased membrane translocation of $p67^{phox}$ and the efficacy of tempol to prevent hypertrophy suggest that the source of ROS in $G4H^{-/-}$ mice appears to be cytosolic. Furthermore, we demonstrate that improving mitochondrial redox state through the use of MnTBAP does not reduce hypertrophy. Therefore, the contribution of mitochondrial ROS to cardiac hypertrophy in cardiac specific GLUT4 deletion may be ruled out.

MATERIALS AND METHODS

Animals and methods for initial evaluation of markers of oxidative stress

All protocols were approved by the University of Utah Institutional Animal Care and Use Committee. Mice with cardiac specific deletion of GLUT4 were generated as previously described ($G4H^{-/-}$) [14]. Littermates with normal GLUT4 expression were used as controls (Con). Mixed cohorts of male and female mice were used for all studies since both develop cardiac hypertrophy as previously described [14]. All animals were kept on a 12 h light : dark cycle, and all experiments were conducted on random fed mice. Adult Con and $G4H^{-/-}$ mice (25±3 weeks old) were first used to determine status of markers of oxidative stress prior to conducting antioxidant treatment experiments.

Aconitase activity

Cardiac aconitase activity was measured in cytosolic fractions and isolated mitochondria as previously described using succinate as a metabolic substrate [33, 34]. Aconitase activity has been previously shown to be sensitive to oxidative stress[34]. Assays were conducted in 50 mM Tris-HCl (pH 7.5) buffer containing 20 mM cis-aconitic acid. The rate of change of absorbance was followed for 10 min at 240 nm, with the activity expressed as mmol cis-aconitate used/min/mg of protein.

Hydrogen peroxide concentrations

Mitochondrial H_2O_2 generation was measured as previously described [35, 36]. This method uses a spectro-fluorophotometer (RF5301PC; Shimadzu, Columbia, MD) that monitors H_2O_2-induced fluorescence of homovanillic acid (excitation wavelength 312 nm, emission wavelength 420) in the prescence of horseradish peroxidase as a catalyst. Succinate (4 mmol/l) was used to stimulate ROS production after inhibition of the F_1F_0-ATP synthase with oligomycin (1 μmol). Rotenone (10 μmol /L) was then added to the mixture to stop complex I–mediated superoxide production. Data is expressed as μmol / L / min / mg mitochondrial protein.

Determination of reactive oxygen species

The presence of reactive oxygen species in cardiac homogenates were evaluated by using a method to measure the conversion of non-fluorescent dichlorodihydrofluorescein diacetate (DCFDA) to 2', 7' – dichlorofluorescein (DCF) upon oxidation with free radicals [37]. Briefly, cardiac homogenates were prepared by homogenizing 50 mg of tissue in buffer containing 50 mM phospohate buffer, 1mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin, 80 mg/L trypsin inhibitor at pH 7.4 and centrifuging as previously described [36]. Both DCFDA and H2DCFDA were added to cardiac homogenates to achieve a 25μM final concentration. Excitation at 485 nm and emission at 530 nm were then measured at 0 and 30 min at 37°C. Data is expressed as nmol DCF/mg protein/min.

Measurement of cardiac malondialdehyde (MDA) concentrations

MDA concentrations were measured using LPO-586™ assay (Oxis Research, Portland, OR). Heart tissue was weighed and homogenized in 6 volumes of ice-cold phosphate-buffered saline (PBS, 20 mM, pH 7.4) containing 10 ml 0.5 M BHT in acetonitrile (to prevent sample oxidation during homogenization). After brief sonication, the homogenate was centrifuged at 3000 g at 4°C for 10 min and supernatant used to determine the concentration of MDA by incubating with 650 μl of diluted chromogenic reagent, N-methyl-2-phenylindole, at 45°C for 60 min. After incubation with the chromogenic agent, samples were centrifuged at 15,000 g for 10 min to obtain a clear supernatant in which absorbance at 586 nm was measured. Results are reported as nmol / g heart tissue used.

Determination of glutathione concentrations in whole homogenates

Concentrations of oxidized (GSSG) and reduced (GSH) glutathione were determined using a kit from Oxis International (Foster City, CA), following the manufacturer's instructions. Both total GSSG and the ratio of GSH:GSSG was used as an index of oxidative stress. To determine whole heart GSH concentrations, approximately 25-35 mg of ventricular tissue was homogenized in 10 volumes of 5% ice cold metaphosphoric acid (MPA). For measurement of GSSG, a similar sized heart sample was homogenized with MPA and supplemented with 30μL M2VP to block the further oxidation of GSH to GSSG. All samples were done in duplicate and concentrations (nmol) of glutathione were normalized to ventricular tissue sample (g) used.

Antioxidant treatments

Four-week old mice were given free access to water (Con; n=6, $G4H^{-/-}$; n=7) or water with 1 mM tempol as previously described [15] (Con +T; n=7, $G4H^{-/-}$+T n=7) for 4 weeks. Previous studies have used a similar dosage of tempol in other models to successfully reduce oxidative stress [17, 38]. Water consumption was monitored daily during the treatment period to verify that the presence of tempol did not affect water intake. In a second experiment using an alternative antioxidant, 4-week old $G4H^{-/-}$ mice were treated every other day for 4 weeks with 10 mg/kg Mn(III) tetrakis (4-benzoic acid) porphyrin chloride (MnTBAP, i.p.) or saline-vehicle (Con + Veh; n=22, $G4H^{-/-}$+ Veh; n=19, Con + MnTBAP; n=26, $G4H^{-/-}$+ MnTBAP; n=23). At the end of both tempol and MnTBAP treatments, mice were anesthetized using chloral hydrate (90 mg/kg) and the heart was removed, rinsed and weighed. Hearts were then used to determine indices of oxidative stress from whole homogenates after tempol treatment or isolated mitochondria after MnTBAP treatments.

Determination of mitochondrial glutathione concentrations after MnTBAP treatment

Freshly prepared mitochondria from individual hearts were isolated using STE1 buffer (pH 7.4) [250mM sucrose, 5mM tris-HCl, and 2mM EGTA (Sigma-Aldrich, Inc., St. Louis, MO)] and STE2 buffer (pH 7.4) [STE1 plus, 0.5% bovine serum albumin, 5mM $MgCl_2$, 1mM ATP, and 2.5U/ml Sigma protease (Sigma-Aldrich, Inc., St. Louis, MO)] as previously described [39]. Ventricles were chopped in 4 ml ice-cold STE1 buffer 2 ~ 3 times to wash out blood. After a 4-min digestion period in ice cold STE2, the digested tissue was washed in 5ml STE1 buffer and then homogenized on ice. Homogenates were centrifuged at 8,000g (4°C) for 10 minutes, re-suspended in 4 ml ice-cold STE1 buffer, and centrifuged at 700g (4°C) for 10 minutes to separate mitochondria from other organelles. Finally, the supernatant (containing mitochondria) was centrifuged at 8,000g (4°C) for 10 minutes to wash out low-molecular weight impurities.

To determine glutathione concentrations, fresh mitochondria were suspended in 2.4 ml of ice-cold STE1 buffer, and aliquoted into 2 portions. Both aliquots were then centrifuged at

8000g (4°C), and the pellets recovered. For GSSG measurement, one aliquot was suspended in 0.45 volumes of ice-cold thiol- scavenger M2VP (Oxis International Inc., Portland, OR) plus 1.05 volumes of ice-cold 5% MPA (Sigma-Aldrich, Inc., St. Louis, MO). The other aliquot, used for determination of GSH, was suspended in 2 volumes of ice-cold 5% MPA. Pellets were then snap frozen in liquid nitrogen and then stored under -140°C for later analysis using a kit from Oxis International (Foster City, CA), following the manufacturer's instructions. Mitochondrial GSH and GSSG concentrations (nmol) were normalized to mitochondrial protein (mg) content.

Western blotting for $p67^{phox}$ protein expression after tempol treatment

Homogenization of hearts and western blotting was done as previously described [40, 41]. Antibodies directed against $p67^{phox}$, a subunit of NADPH oxidase, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Signals were visualized by enhanced chemiluminescence (Cell Signal Technology, Beverly, MA). After exposure, membranes were stripped and probed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control (Chemicon, Temecula, CA). Blots were prepared in triplicate and relative band densities were measured using a Kodak GL1500 gel imaging system.

Real time PCR (RT-PCR)

RNA extraction and quantitative RT-PCR was performed as previously described [42]. Total RNA was extracted from hearts with TRizol reagent (Invitrogen Corporation, Carlsbad, CA) and 3 μg of RNA were reverse transcribed using Superscript III Reverse Transcriptase (Invitrogen). Primers used for Brian Natiuretic Peptide (BNP), sarcoplasmic reticulum calcium ATPase (SERCA) 2a & 2b, α-myosin heavy chain (α-MHC), β-myosin heavy chain (β-MHC), atrial natriuretic factor (ANF), glucose transporter 1 (GLUT1), glutathione reductase (GR), glutathione peroxidase-1 (GPX1), glutathione peroxidase-4 (GPX4), thioredoxin-2 (TXN2), $p47^{phox}$, and $p67^{phox}$ are shown in Table 4. The resulting cDNA were subjected to quantitative real-time RT-PCR. All reactions were performed in triplicate. Relative quantification was performed by interpolating crossing point data on an independent standard curve. Product size was confirmed by melting curve and agarose gel electrophoresis with ethidium bromide staining. Data were corrected for loading relative to the levels of the invariant transcript cyclophilin (Table 4) and normalized to $GLUT4^{+/+}$ controls (a.u. = 1.0).

Statistics

A t-test was used to detect significant differences in between control and $G4H^{-/-}$mice during initial studies. A 2 way ANOVA (SPSS v.20) was used to determine significant differences among groups when treated with MnTBAP or Tempol. A Bonferroni post-hoc test was used when main effects were detected after ANOVA. Significance was accepted at $P<0.05$. All data is expressed as mean±SEM.

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Abbreviations

α-MHC	alpha myosin heavy chain
ANF	atrial natriuretic peptide

BNP	brain natriuretic peptide
DCFDA	dichlorodihydrofluorescein diacetate
FAO	fatty acid oxidation
GLUT4	glucose transporter 4 gene
G4H$^{-/-}$	cardiac specific GLUT4 deletion
GSH	reduced glutathione
GSSG	oxidized glutathione
GPX	glutathione peroxidase
GR	glutathione reductase
GSK	glycogen synthase kinase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
MnTBAP	Mn(III) tetrakis (4-benzoic acid) porphyrin chloride
MDA	malondialdehyde
MPA	metaphosphoric acid
NOX	NADP oxidase
ROS	reactive oxygen species
SOD	superoxide dismutase
TXN	thioredoxin

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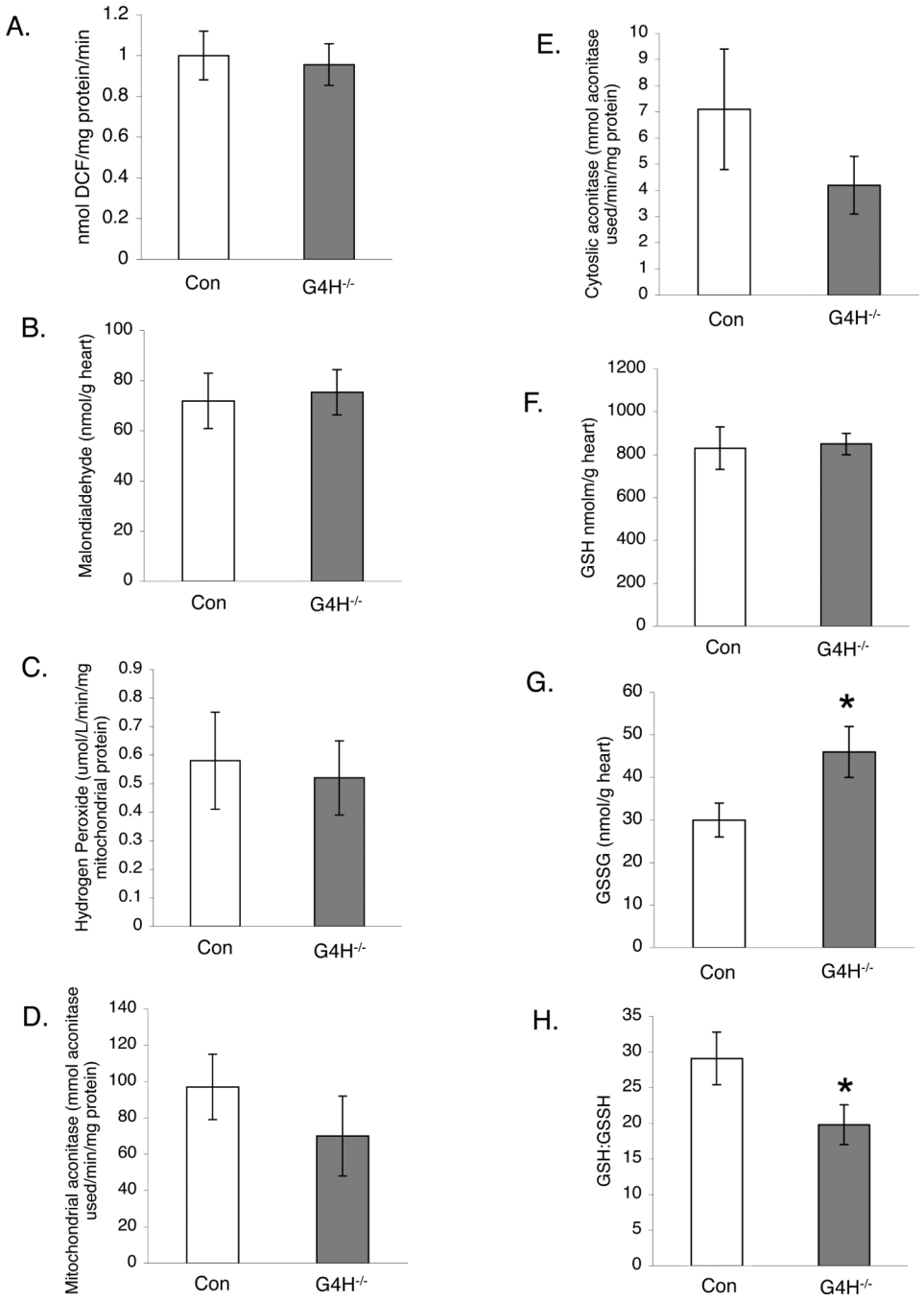

Figure 1.

Indices of oxidative stress in whole homogenates and isolated mitochondria from hearts of cardiac specific GLUT4 deleted ($G4H^{-/-}$) and control (Con) mice. All mice were 25±1 weeks old, except as indicated. **A)** Dichlorodihydrofluorescein diacetate (DCFDA) assay (nmol dichlorodihydrofluorescein (DCF) / mg protein/min), from mice 8.0±0.5 weeks old, $G4H^{-/-}$; n= 8, Con; n=8. **B)** Mitochondrial hydrogen peroxide production (μmol / L / min / mg mitochondrial protein), $G4H^{-/-}$; n= 6, Con; n=6. **C)** Malondialdehyde content (nmol / g heart), $G4H^{-/-}$; n= 9, Con; n=7. **D)** Mitochondrial aconitase activity (nmol aconitase used / min / mg mitochondrial protein), $G4H^{-/-}$; n= 6, Con; n=8. **E)** Cytosolic aconitase activity (nmol aconitase used / min / mg protein), $G4H^{-/-}$; n= 9, Con; n=7. **F)** Reduced (GSH)

glutathione (nmol / g heart), $G4H^{-/-}$; n= 11, Con; n=10. **G)** Oxidized (GSSG) glutathione (nmol / g heart), $G4H^{-/-}$; n= 11, Con; n=10. **H)** GSH : GSSG, $G4H^{-/-}$; n= 11, Con; n=10. Data are mean ± SEM. * Indicates significant differences at $p<0.05$.

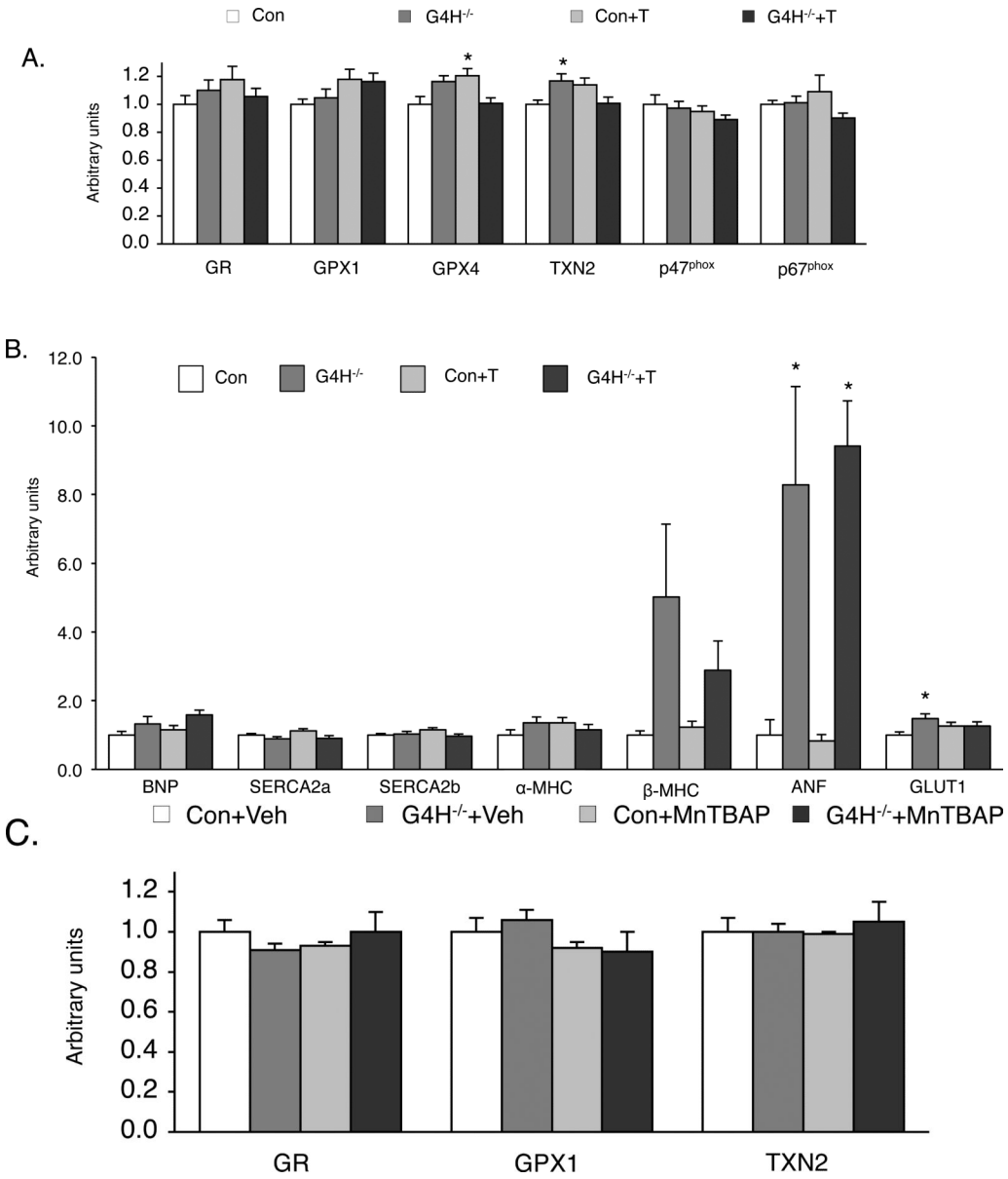

Figure 2a, b.

mRNA levels as determined by quantitative PCR in hearts of cardiac specific GLUT4 deleted ($G4H^{-/-}$, n=7), control (Con, n=8), tempol treated $G4H^{-/-}$ (+T, n=8, 1mM), and tempol treated Con mice (+T, n=9, 1mM). **2c,** mRNA levels as determined by quantitative PCR in hearts of $G4H^{-/-}$ and Con mice treated with (Mn(III)tetrakis(4-Benzoic acid) porphyrin Chloride (+MnTBAP, 10 mg/kg, ip) or Vehicle (+Veh, saline), n=4 for each group. All samples are normalized to cyclophilin expression and normalized to controls. Data are mean ± SEM. * Indicates significant differences at $p<0.05$. vs. Con.

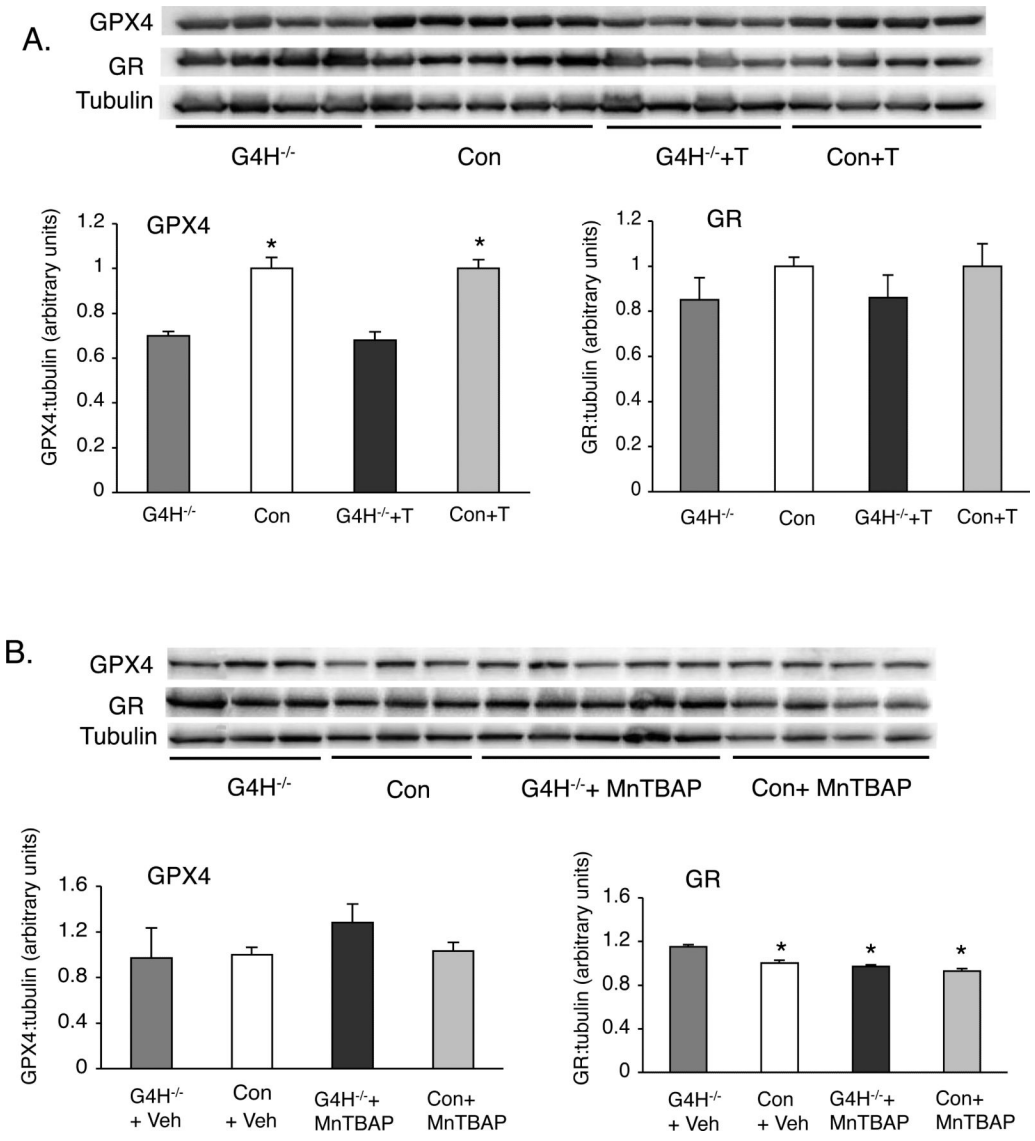

Figure 3a.

Glutathione peroxidase 4 (GPX4) and glutathione reductase (GR) protein levels in hearts of cardiac specific GLUT4 deleted ($G4H^{-/-}$, n=7), control (Con, n=8) mice, tempol treated $G4H^{-/-}$ (+T, n=8, 1mM), and tempol treated Con mice (+T, n=9, 1mM). **3b.** GPX4 and GR protein levels in hearts of $G4H^{-/-}$ (n=4) and Con (n=3) mice treated with (Mn(III)tetrakis(4-Benzoic acid) porphyrin Chloride (+MnTBAP, 10 mg/kg, ip, n=5) or Vehicle (+Veh, saline, n=4). Data are mean ± SEM. * Indicates significant differences at $p<0.05$. vs. Con.

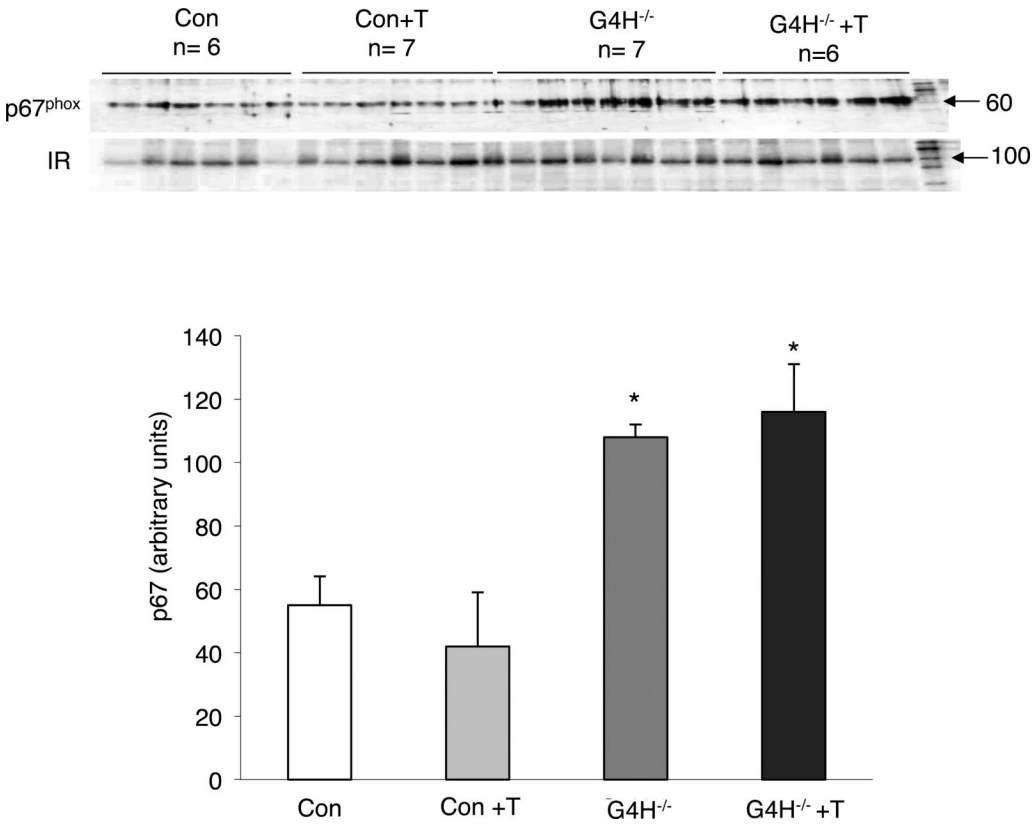

Figure 4.

p67^{phox} protein levels in membrane fractions of cardiac homogenates from cardiac specific GLUT4 deleted (G4H$^{-/-}$, n=7), control (Con, n=7) mice, tempol treated G4H$^{-/-}$ (+T, n=8, 1mM), and tempol treated Con mice (+T, n=7, 1mM). IR, insulin receptor, used as loading control for membrane bound proteins. Data are mean ± SEM. ***** Indicates significant differences at $p<0.05$ vs. Con.

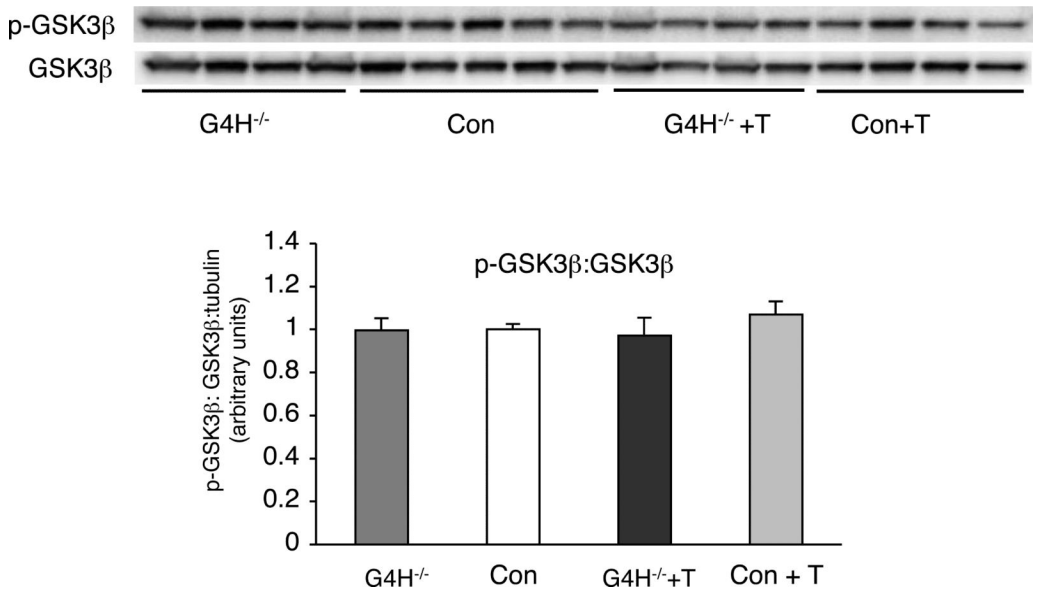

Figure 5.

Glycogen synthase kinase 3β (GSK) and phospho (p)-GSK3 β protein levels in hearts of cardiac specific GLUT4 deleted ($G4H^{-/-}$, n=7), control (Con, n=8) mice, tempol treated $G4H^{-/-}$ (+T, n=8, 1mM), and tempol treated Con mice (+T, n=9, 1mM). Data are mean ± SEM.

Table 1

Morphology of 5 week old and 25 week old mice

	Con	G4H$^{-/-}$
4 week old (n)	*7*	*7*
Body weight (g)	16.4±1.0	14.1±1.1
Heart weight (mg)	100±6	102±7
Tibia length (mm)	15.34±0.26	14.65±0.46
Heart : tibia (mg : mm)	6.51±0.31	6.94±0.36
GSSG	13.2±1.4	20.0±2.8*
GSH	798±38	887±44
GSH:GSSG	62.0±5.8	45.5± 3.9*
25 week old (n)	*11*	*11*
Body weight (g)	28.7±1.6	27.8±1.7
Heart weight (mg)	145±8	202±16*
Tibia length (mm)	18.1±0.1	18.2±0.2
Heart : tibia (mg : mm)	8.01±0.4	11.1±0.9*

Data are mean ± SEM. Adult mice aged 4.9±0.5 and 25±3 weeks old.

* Indicates significant differences at $p<0.05$. GSH and GSSG concentrations in 25-week-old cohort are shown in Figure 1.

Table 2

Tempol (1 mM) treated mice

	Con	G4H$^{-/-}$	Con + T	G4H$^{-/-}$ + T
n	*15*	*13*	*16*	*16*
Body weight (g)	20.5±0.8	22.3±0.8	20.9±0.7	19.4±0.7
Heart weight (mg)	110±4	146±5*	110±4	121±6
Tibia length (mm)	17.2±0.2	17.2±0.1	17.0±0.1	16.7±0.2
Heart : tibia (mg : mm)	6.40±0.23	8.43±0.40*	6.46±0.24	7.27±0.36
Whole homogenate glutathione concentrations (nmol / g tissue)				
n	*7*	*7*	*7*	*8*
GSH	799±34	634±36*	604±27*	598±31*
GSSG	11.1±1.6	13.5±1.0	9.9±2.6	12.7±2.0
GSH:GSSG^{a}	77±7	49±6	82±16	52±6

Data are mean ± SEM. GSH, reduced glutathione. GSSG, oxidized glutathione. Con (Control), G4H$^{-/-}$ (Cardiac specific GLUT4 deletion), T = tempol. Mice were treated for 4 weeks, starting at age 4-weeks and studied at the age of 8±0 weeks.

*Significant differences at $p<0.05$ vs. Con.

^{a}Main effects detected at $p<0.05$ for genotype (G4H$^{-/-}$ vs. Con), but interactions were not significant.

Table 3

MnTBAP (2 mg / kg) treated mice

	Con	G4H$^{-/-}$	Con+MnTBAP	G4H$^{-/-}$+MnTBAP
n	*26*	*23*	*30*	*28*
Body weight (g)	21.2±0.6	20.4±0.6	21.3±0.5	21.0±0.5
Heart weight (mg)	110±3	135±4*	111 ±3	133±3*
n	*17*	*17*	*19*	*22*
Tibia length (mm)	16.5±0.1	16.7±0.1	16.6±0.1	16.4±0.1
Heart : tibia (mg : mm)	6.74±0.23	8.22±0.23*	6.73±0.22	7.99±0.20*
Mitochondrial glutathione concentrations (nmol / mg mitochondrial protein)				
n	*10*	*12*	*14*	*11*
GSH	712±36	604±40	730±34	646±38
GSSG	19±3	34±3*	16±3	23±3
GSH:GSSG	40±3	22±4*	48±3	31 ±4

Data are mean ± SEM. GSH, reduced glutathione. GSSG, oxidized glutathione. Mice were treated for 4 weeks, starting at age 4-weeks and studied at the age of 8±0 weeks.

*Significant differences at $p<0.05$ vs. Con.

Table 4

Primer pairs used for RT-PCR of selected genes in heart.

Gene	Sequence (5’ - 3’)	Product size (bp)
BNP	GGATCTCCTGAAGGTGCTGT TTCTTTTGTGAGGCCTTGGT	205
SERCA2a	GAAACTACCTGGAACAACCCG CTTTTCCCCAACCTCAGTCA	138
SERCA2b	GAAACTACCTGGAACAACCCG CGACAGGGAGCAGGAAGAT	65
α-MHC	ACTGTGGTGCCTCGTTCC TTCCGTTTTCAGTTTCCGC	276
β-MHC	CATTCTCCTGCTGTTTCCTTAC CATGGCTGAGCCTTGGAT	140
ANF	ATGGGCTCCTTCTCCATCA CCTGCTTCCTCAGTCTGCTC	209
GLUT1	GCGGGAGACGCATAGTTACA CTCCCACAGCCAACATGAG	130
GPX1	GACTGGTGGTGCTCGGTT TCACCATTCACTTCGCACTT	151
GPX4	GCAGGAGCCAGGAAGTAATCAAGA GCATCGTCCCCATTTACACA	99
GR	GTGGCACTTGCGTGAATGTTG GCACTGCTGTGTTCCACATTACCTT	60
TXN2	GTGTGGGCTTCCCTCACCTCT GGCTGGGCTGGGCATTACTGTTAGA	81
p47^{phox}	CCTGATGACCTGAAACTGCCCA GGTTGCCACGGTCATCTCTGTT	174
p67^{phox}	GACTATCTGGGCAAGGCTACGGTT AGGGGCGAAGCCAGAAAAGTTG	69
Cyclophilin	AGCACTGGAGAGAAAGGATTTGG TCTTCTTGCTGGTCTTGCCATT	349