Figures and figure supplements

Myocardial NADPH oxidase-4 regulates the physiological response to acute exercise

Matthew Hancock et al
Figure 1. Nox4 mediates myocardial Nrf2 activation and is essential in the acute response to exercise. (A) Changes in myocardial Nox4 mRNA and protein levels after acute moderate exercise (Ex) compared to sedentary controls (Sed). *p<0.05, **p<0.01, 2-tailed t-test (n = 4–6/group). (B) Exercise capacity of Nox4-null mice (Nox4KO) and littermate wild-types (WT) measured by maximal running distance and running time. **p<0.01, 2-tailed t-test (n = 5–8/group). (C) Ratio of glutathione/glutathione disulfide (GSH/GSSG) in Nox4KO and WT mouse hearts before (Sed) and after exercise (Ex). n = 6–9/group. (D) Protein levels of 4-hydroxynonenal (4-HNE) adducts in the heart. n = 3–4/group. (E and F) Protein and mRNA levels of major Nrf2 targets. n = 3–6/group *p<0.05, **p<0.01 vs respective sedentary controls (Sed); #p<0.05, ##p<0.01 vs WT/Ex, 1-way ANOVA followed by Tukey post hoc analysis. CAT: catalase, GSTA2: glutathione S-transferase A2, GCLC: glutamate cysteine ligase catalytic subunit, TXNRD1: thioredoxin reductase 1.

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**Figure 1—figure supplement 1.** Full images of Western blots in Figure 1.

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(Nox4Tg: Heart tissue lysate from cardiomyocyte-specific overexpression of Nox4 mouse as positive control)
Figure 1—figure supplement 2. Myocardial Nox2 is unchanged during acute exercise. Protein levels of total Nox2 (A) and p47\textsuperscript{phox} in membrane fraction (B) in normal mouse hearts after acute exercise (Ex). GAPDH and cadherin were used as cytosolic and membrane protein marker respectively. 
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Figure 1—figure supplement 3. Quantification of Western blots in Figure 1E. Protein levels of Nrf2 and its main targets in the hearts of Nox4KO and WT mice before (Sed) and after exercise (Ex). *p < 0.05, **p < 0.01 vs respective sedentary control. #p < 0.05, ##p < 0.01 vs WT/Ex, 1-way ANOVA followed by Tukey post hoc analysis (n = 4–6/group). GSTA2: glutathione S-transferase A2, GCLC: glutamate cysteine ligase catalytic subunit.

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Figure 2. Cardiac Nox4 deficiency impairs heart function and exercise capacity due to lack of Nrf2 activation. (A) Expression of Nrf2-related genes in hearts of cardiomyocyte-specific Nox4KO (csNox4KO) and littermate controls after exercise (Ex). n = 3–4/group. **p<0.01 vs respective sedentary controls (Sed); #p<0.05, ##p<0.01 vs Control/Ex. (B) Maximal exercise distance and time. n = 3–5/group. *p<0.05 vs Control/Ex; #p<0.05, ##p<0.01 vs csNox4KO/Ex, 1-way ANOVA followed by Tukey post hoc analysis. (C) Fractional shortening (FS) evaluated by conscious echocardiography in control and csNox4KO mice immediately after the exercise capacity test. Some animals were treated with sulforaphane (SFN) prior to exercise. n = 4–12/group. **p<0.01 vs Sed; #p<0.01 vs Control/Ex, 2-way ANOVA followed by Tukey post hoc analysis. Cat: catalase, Gsta2: glutathione S-transferase A2, Gclc: glutamate cysteine ligase catalytic subunit, Txnrd1: thioredoxin reductase 1.

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Figure 2—figure supplement 1. csNox4KO mice exhibit reduced cardiac function at peak exercise. Cardiac function was evaluated by awake echocardiography prior to (sedentary, Sed) and after the exercise capacity test (Ex). Some animals were treated with sulforafane (SFN). Mean data of heart rate (HR), left ventricular end-systolic dimension (LVESD) and left ventricular end-diastolic dimension (LVEDD) (A to C). *p<0.05, **p<0.01 vs respective sedentary controls, ##p<0.01 vs Control/Ex, 2-way ANOVA followed by Tukey post hoc analysis (n = 4–12/group).
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Figure 3. Cardiomyocyte-targeted Nrf2 knockout mice have impaired mitochondrial function and reduced heart performance upon exercise. (A) Targeting strategy for generation of Nrf2(lox/lox) mice (Flox). The endogenous Nrf2 locus is shown at the top and the targeting vector at the bottom. Exons 2–5 were flanked by LoxP sites which are represented by blue triangles; FRT (Neo) and F3 (Puro) sites are shown by double red triangles. Cre-mediated recombination deletes a 5.5 kb fragment including most of the open-reading frame and the 3' UTR. (B) Southern blot analysis of genomic DNA from selected ES cell clones, performed after excision at HincII sites, showing correct 3' homologous recombination in all clones. WT indicates wild-type. (C) Nrf2 mRNA levels (n = 6/group) and (D) protein levels (n = 3/group) in the hearts of csNrf2KO and Flox control mice. **p<0.01, 2-tailed t-test. (E) Increase in Nox4 mRNA levels in csNrf2KO and control mouse hearts after acute moderate exercise. **p<0.01 vs respective sedentary controls, 2-tailed t-test (n = 6/group). (F) Fractional shortening (FS) by echocardiography immediately after the exercise capacity test. *p<0.05 vs sedentary (Sed) Flox control mice, 1-way ANOVA followed by Tukey post hoc analysis (n = 6–7/group). (G) Respiration rates in isolated cardiac mitochondria of csNrf2KO and control mice after exercise. Both ADP-limited oxidative phosphorylation (OXPHOS_{Lim}) and maximal oxidative capacity (OXPHOS_{Max}) were measured (see Materials and methods for details). *p<0.05 vs csNrf2KO/Sed, # p<0.05 Flox/Ex, 1-way ANOVA followed by Tukey post hoc analysis (n = 4–6/group). (H) Citrate synthase (CS) activity in the heart. n = 4–6/group. DOI: https://doi.org/10.7554/eLife.41044.008
Figure A: Western blot analysis showing expression levels of Nrf2 and GAPDH in Flox and csNrf2KO cells. The bands are labeled with molecular weights in kDa.

Figure B: Quantitative analysis of Nrf2 mRNA levels in different tissues (Lung, Liver, Skel Mus) for Flox and csNrf2KO cells. The results show no significant difference (n.s.) in mRNA levels between the two groups.
Figure 3—figure supplement 1. Identification of csNrf2KO mouse. (A) Full images of Western blot of Nrf2 using whole heart lysates from csNrf2KO mice and their littermate Flox controls. (B) Nrf2 mRNA levels by real-time PCR in different tissues including lung, liver and skeletal muscle (Skel Mus). n.s: no significance. n = 5/group.

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Figure 3—figure supplement 2. csNrf2KO mice exhibit normal cardiac morphology and function at baseline. (A) Heart weight/body weight ratio (HW/BW), and (B) Ejection fraction (EF). n.s: no significance. n = 5-6/group.

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Figure 3—figure supplement 3. Respiratory rates in isolated cardiac mitochondria of csNrf2KO mice after exercise. (A) The relationship between molecules of ADP phosphorylated and oxygen consumed (P/O ratio). (B) Respiratory coupling ratio (RCR). (C) Cytochrome C was added after oxidative capacity measurement to investigate mitochondrial membrane integrity (OXPHOS_{Cyt}). (D) Cardiac mitochondrial function evaluated by a substrate-uncoupler-inhibitor-titration (SUIT) protocol. Mitochondrial proton leak using malate and palmityl carnitine as substrates (Leak_{FA}), maximal oxidative phosphorylation with a saturated amount of ADP (OXPHOX_{FA}), following the addition of complex I substrates, pyruvate and glutamate (OXPHOX_{FA+CI}), and following the addition of complex II substrate, succinate (OXPHOX_{FA+CI+CII}). Maximal electron transport chain (ETC) capacity was measured following the addition of carbonyl cyanide m-chlorophenyl hydrazone (ETC_{FA+CI+CII}), and rotenone was added to measure ETC capacity using complex II substrates (ETC_{CII}). *p<0.05 vs respective sedentary controls, # p<0.05 vs Flox/Ex, 1-way ANOVA followed by Tukey post hoc analysis (n = 4–6/group). DOI: https://doi.org/10.7554/eLife.41044.011
Figure 4. Cardiomyocyte Nrf2 is required for increase in mitochondrial antioxidant capacity with exercise. (A) Western blots for peroxiredoxin III (PRXIII), thioredoxin reductase-2 (TRXR2) and superoxide dismutase-2 (SOD2) in heart of csNrf2KO and Flox controls after acute exercise (Ex). Mean data from n = 4–6/group are shown on the right. *p<0.05 vs respective sedentary controls (Sed); #p<0.05, ##p<0.01 vs Flox/Ex, 1-way ANOVA followed by Tukey post hoc analysis. (B) In vivo mitochondrial H₂⁻O₂ levels assessed by the MitoP/MitoB ratio in hearts of csNrf2KO mice and controls after acute exercise. **p<0.01 vs Flox/Sed, 1-way ANOVA followed by Tukey post hoc analysis (n = 5–6/group). (C) MitoQ treatment improved cardiac contractile performance at peak exercise in csNrf2KO mice. n = 6–7/group. (D) and (E) The effect of MitoQ treatment on maximal running distance and time. n = 7–11/group. *p<0.05, **p<0.01 vs Flox/Ex, #p<0.05, ##p<0.01 vs csNrf2KO/Ex, 2-way ANOVA followed by Tukey post hoc analysis.

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Figure 4—figure supplement 1. Full images of Western blots in Figure 4.
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Figure 4—figure supplement 2. Nox4 is required for increase in mitochondrial antioxidant capacity with exercise. Peroxiredoxin III (PRXIII), thioredoxin reductase-2 (TRXR2) and superoxide dismutase-2 (SOD2) in the hearts of Nox4-null and littermate wild-type control mice (WT) after acute exercise (Ex). Mean data from n = 4–6/group are shown on the right. *p<0.05, **p<0.01 vs respective sedentary controls (Sed); #p<0.05, ##p<0.01 vs WT/Ex, 1-way ANOVA followed by Tukey post hoc analysis.

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Figure 4—figure supplement 3. Mitochondrial-targeted antioxidant MitoQ improves cardiac response to exercise in csNrf2KO. Cardiac function was evaluated by conscious echocardiography prior to (sedentary, Sed) and after exercise (Ex) with or without MitoQ treatment. Mean data for heart rate (HR), left ventricular end-systolic dimension (LVESD) and left ventricular end-diastolic dimension (LVEDD) (A to C). *p<0.05, **p<0.01 vs respective sedentary controls, ##p<0.01 vs Flox/Ex, 2-way ANOVA followed by Tukey post hoc analysis (N = 6–7/group).
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Figure 5. Schematic illustrating the role of the cardiomyocyte Nox4-Nrf2 pathway in enhancing mitochondrial and cardiac contractile function during acute physiological exercise. Cardiac Nox4 levels increase in response to acute exercise and induce an activation of Nrf2. The Nrf2-dependent gene program includes endogenous antioxidants that are critical in maintaining mitochondrial redox balance during peak exercise. The maintenance of mitochondrial redox balance is essential to achieve maximal mitochondrial respiration and cardiac contractile function at peak exercise. The sites of action of the Nrf2 activator sulforaphane and the mitochondria-targeted antioxidant MitoQ are shown.

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