Effects of voluntary exercise duration on myocardial ischaemic tolerance, kinase signalling and gene expression

Boris P. Budiono[1], Louise E. See Hoe[2], Jason N. Peart[2], Jelena Vider[2], Kevin J. Ashton[3], Angela Jacques[4], Luke J. Haseler[4†] and John P. Headrick[2†]
1 Charles Sturt University, School of Community Health (Port Macquarie, NSW, Australia)
2 Griffith University, School of Medical Science (Gold Coast, QLD, Australia);
3 Bond University, Faculty of Health and Medicine (Robina, QLD,, Australia)
4 Curtin University, School of Physiotherapy and Exercise Science (Bentley, WA, Australia)
†, Co senior authors

Short title: Short to long-term exercise and cardioprotection

***Corresponding Author:**
Prof. John P. Headrick
School of Medical Science
Griffith University
Southport, QLD 4216
AUSTRALIA
Ph (Intl): +61 7 555 28292
Email: j.headrick@griffith.edu.au

ABSTRACT

Aim: Exercise is cardioprotective, though optimal interventions are unclear. We assessed duration dependent effects of exercise on myocardial ischemia-reperfusion (I-R) injury, kinase signalling and gene expression.

Methods: Responses to brief (2 day; 2EX), intermediate (7 and 14 day; 7EX and 14EX) and extended (28 day; 28EX) voluntary wheel running (VWR) were studied in male C57Bl/6 mice. Cardiac function, I-R tolerance and survival kinase signalling were assessed in perfused hearts.

Key findings: Mice progressively increased running distances and intensity, from 2.4±0.2 km/day (0.55±0.04 m/s) at 2-days to 10.6±0.4 km/day (0.72±0.06 m/s) after 28-days. Myocardial mass and contractility were modified at 14-28 days VWR. Cardioprotection was not 'dose-dependent', with I-R tolerance enhanced within 7 days and not further improved with greater VWR duration, volume or intensity. Protection was associated with AKT, ERK1/2 and GSK3□ phosphorylation, with phospho-AMPK selectively enhanced with brief VWR. Gene expression was duration-dependent: 7 day VWR up-regulated glycolytic (*Pfkm*) and down-regulated maladaptive remodeling (*Mmp2*) genes; 28 day VWR up-regulated caveolar (*Cav3*), mitochondrial biogenesis (*Ppargc1a*, *Sirt3*) and titin (*Ttn*) genes.

Interestingly, I-R tolerance in 2EX/2SED groups improved *vs.* groups subjected to longer sedentariness, suggesting transient protection on transition to housing with running wheels.

Significance: Cardioprotection is induced with as little as 7 days VWR, yet not enhanced with further or faster running. This protection is linked to survival kinase phospho-regulation (particularly AKT and ERK1/2), with glycolytic, mitochondrial, caveolar and myofibrillar gene changes potentially contributing. Intriguingly, environmental enrichment may also protect via similar kinase regulation.

Keywords: Cardioprotection, exercise, myocardial ischemia, survival kinase, voluntary activity, wheel-running

1. INTRODUCTION

Regular physical activity is a simple, economical and highly effective preventative measure against cardiovascular disease (CVD), and may also confer direct 'cardioprotection' against the effects of acute I-R or infarction. Unfortunately, despite clear evidence of benefit (and conversely, of detriment with sedentariness), a majority of adults and increasing numbers of children in developed countries fail to meet recommended minimum daily activity levels.[1,2] This may be exacerbated by confusion engendered by debates regarding optimal exercise interventions and dietary recommendations.[3] Characterizing activity-dependent cardioprotection and the mechanisms involved can not only facilitate consensus regarding exercise prescription, but may lead to more effective strategies for reducing the risk and subsequent impacts of ischemic heart disease.[4,5]

Animal studies reveal powerful exercise dependent protection that may prime the heart against injurious stressors such as hypoxia or ischaemia.[6] Importantly, physical activity together with calorie restriction appear to be the only consistently protective interventions in aged hearts/subjects, and in the presence of co-morbidities including diabetes, hypertension and dyslipidemia: exercise restores protective signaling and responses in older and diseased hearts.[7-10] Exercise is a physiological stressor activating multiple mechanisms across organ systems. Multiple endogenous protective systems appear sensitive to exercise stimuli, with investigations of cardioprotection focused on roles of heat shock proteins, anti-oxidants, NO

and cyclooxygenase-2.[4,11-14] Nonetheless, as Powers and colleagues have highlighted,[15] despite consistent evidence for anti-oxidant involvement, important questions remain regarding roles (and the necessity) of heat shock proteins, NO and cyclooxygenase-2 pathways in exercise-dependent protection. The basis of this powerful effect still awaits delineation. Importantly, relatively few studies have assessed signaling network changes with exercise, from agonism of membrane G protein-coupled receptors (GPCRs) or growth factor receptors through to survival and injury kinase modulation (including the 'reperfusion injury salvage kinase' or RISK path).[16] These signaling mechanisms may contribute to multiple adaptations, including stress-resistance, physiological hypertrophy (also thought to involve the 'RISK' proteins PI3K/AKT) and shifts in substrate and energy metabolism (involving AKT and AMPK).[17,18]

These signaling paths and protective outcomes may in turn be influenced by factors including the duration, intensity and mode of physical activity, together with associated stress or emotional responses.[19] Understanding these influences is important in optimizing the benefits of exercise, and its utility in CVD. Our recent data highlight acute sensitivity of myocardial I-R tolerance to physical activity level, with stress-resistance changing within 3-7 days of low-intensity VWR (or inactivity), independently of cardiac function or mass, and paralleling shifts in kinase phosphorylation and protective EGFR protein expression.[20,21] Other studies support even more rapid protection with high intensity forced forms of exercise.[22,23] Whether the duration or volume of low-intensity VWR governs the degree of cardiac protection, and whether such changes are paralleled by survival kinase signaling, is presently unclear. Thus, the goals of the current study were to test the dependence of cardioprotection on duration of low-stress activity (VWR), and whether shifts in cardiac stress-resistance are consistently linked with putative determinants of ischemic tolerance, including phosphorylation of AKT, AMPK, ERK1/2 and GSK3□. We hypothesized that: i) a cardioprotective phenotype would be observed within 7 days of commencement of VWR, and progressively improve with exercise duration; and ii) this cardioprotection would be underpinned by parallel shifts in survival kinase signaling and cardiac gene expression as exercise duration increased.

2. Materials and methods

2.1 - Ethical approval and animals

All studies were approved by and performed in accordance with the guidelines of the Animal Ethics Committee of Griffith University, which is accredited by the Queensland Government, Department of Primary Industries and Fisheries under the guidelines of *"The Animal Care and Protection Act 2001, Section 757"*. Male C57Bl/6 mice were sourced from the Central Animal House, University of Queensland, and housed in the Griffith University Animal Facility for the duration of the study. Animals were acclimated to 12:12h light/dark cycles and housed at 23±2°C (40% humidity) in communal cages (4/cage) fitted with corn-cob bedding and nesting materials (two Kleenex tissues). Mice were provided standard rodent chow (Specialty Feeds, Glen Forest, Australia) and water *ad libitum*.

2.2 - Experimental design

Male mice 8-12 weeks of age were introduced into large individual cages (40 x 37.5 x 17.5 cm) containing locked rodent running-wheels (20 cm dia/6.5 cm wide Wodent Wheels; Transoniq, Flagstaff AZ, USA) for a period of 3 days before random allocation to 8 experimental groups: 2EX, 7EX, 14EX, 28EX *vs.* time-matched sedentary groups 2SED, 7SED, 14SED and 28SED (n=20-21 per group). Wheels were unlocked for the EX groups and locked for respective sedentary (SED) groups. Bidirectional wheel running was recorded daily via calibrated bicycle computers activated by a reed-switch mechanism (model BC-560, Sigma Sport, Olney, IL), permitting analysis of daily running distances and time spent running. Free-roaming activity outside of wheels and in control cages was not measured.

At the end of each experimental period mice were anaesthetized with 60 mg/kg sodium pentobarbital administered intraperitoneally, hearts rapidly removed, washed briefly in ice-cold isotonic Krebs solution and either cryogenically frozen for proteomic (n=6 per group) and transcriptional analyses (n=6 per group), or perfused for assessment of cardiac function and I-R tolerance *ex vivo* (n=8-9 per group). Running wheels were locked 24 hrs prior to cardiac sampling, which was undertaken over a 4 hr period from 10 am - 2 pm.

2.3 - Ischaemia-reperfusion in Langendorff perfused hearts

Hearts were isolated and perfused via the aorta in a Langendorff mode, as detailed previously.[21,24,25] Contractile function was monitored via a water-filled balloon in the left ventricle, inflated to an initial end-diastolic pressure of ~5 mmHg and connected to a pressure transducer. Coronary flow was monitored with an ultrasonic flow-probe proximal to the aortic cannula and connected to a T206 flowmeter (Transonic Systems Inc, Ithaca, NY, USA). All functional data were recorded at 1 KHz on a Powerlab 4/30 system (ADInstruments, Castle Hill, Australia) connected to an Apple iMac computer. The left ventricular pressure signal was digitally processed to yield peak systolic and end-diastolic pressures, +dP/dt, -dP/dt and heart rate.

Following 20 min stabilization hearts were switched to ventricular pacing at 420 bpm. Baseline measurements were made after a further 10 min before initiating a 25 min period of global normothermic ischaemia followed by 45 min aerobic reperfusion. Ventricular pacing was terminated on initiation of ischaemia and resumed after 1.5 min of reperfusion. Hearts were excluded from study after pre-ischemic stabilization if they met one of the following criteria: i) coronary flow >5 ml/min, ii) unstable contractile function, iii) left ventricular systolic pressure <100 mmHg, or iv) significant arrhythmias.

2.4 - Analysis of myocardial kinase expression

Since there were no changes in myocardial I-R tolerance between 7 and 28 days VWR, subsequent molecular analyses (protein, gene) were limited to tissues from 2-, 7- and 28-day groups. Ventricular lysates for 2-, 7- and 28-day EX and SED mice were prepared from frozen hearts, and samples containing 30 μg of total protein loaded onto 10% acrylamide gels and separated at 150 V for ~1.5 hrs. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes and blocked in 5% skim milk powder in TBST for 60 min. Membranes were incubated with primary antibody (total and phosphorylated AKT (Ser^{473}), ERK1/2 (Thr^{202}/Tyr^{204}), GSK3□ (Ser^{9}) and AMPK (Thr^{172}), and □-actin; 1:1000, Cell Signaling Technology Inc., Danvers, MA, USA) overnight at 4°C. Following 3 washes in TBST, membranes were incubated with HRP-congjugated secondary antibody and visualized on a

ChemiDoc XRS system (Bio-Rad, Hercules, CA, USA).

2.5 - RNA isolation and quantitative reverse transcription PCR (RT-qPCR) analysis

To further characterize molecular adaptations to VWR we assessed gene expression responses to 2-, 7- and 28-days of VWR. Ventricular myocardium of EX and SED groups was homogenized in TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) and total RNA isolated according to manufacturer's guidelines. Total RNA was further purified using RNeasy spin columns (Qiagen, Maryland, USA). Total RNA yield and purity via 260/280 nm and 260/230 nm ratios (≥ 1.8) were determined using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA).

Two-step RT-qPCR, utilizing SYBR Green I, was employed to assess differential gene expression for 6 transcripts (see **Supplementary Table 1** for primer details). Briefly, 500 ng total RNA was used to synthesize cDNA using the Superscript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) using manufacturer's protocols. Each 10 µL RT-qPCR reaction contained 1× SYBR Green Supermix (Bio-Rad, Hercules, CA, USA), 50 nM of each primer and 5 µL of a 1:20 dilution of cDNA, and was assayed on a CFX96 RT-qPCR system (Bio-Rad, Hercules, CA, USA). Optimal RT-qPCR cycling conditions consisted of an initial denaturation at 95°C for 3 min followed by 40 cycles of 95°C for 15 sec / 62°C for 60 sec. After the final cycle, reactions underwent melt curve analysis to detect non-specific amplicons. Reactions were performed in triplicate and expression levels normalized to phosphoglycerate kinase 1 (*Pgk1*) as a reference gene. Changes in gene expression relative to control sedentary hearts were calculated using the $2^{-\Delta\Delta Ct}$ method**.** [26]

2.6 - Statistical analysis

Unless stated otherwise, physiological, proteomic and transcriptional data are expressed as means ± *S.E.M*. The design consisted of 3 control and intervention groups (Group A: 2SED *vs.* 2EX, Group B: 7SED *vs.* 7EX, and Group C: 28SED *vs.* 28EX). For each group, independent t-tests were used to compare outcomes between control (SED) and intervention (EX). Outcomes included cardiac functional data, myocardial kinase expression and

transcriptional responses. Linear regression analysis was performed to determine associations between mean functional parameters of ischemic tolerance (LVDP and EDP) and mean myocardial kinase phosphorylation. Coefficient of determination (R^2) was used to measure the proportion of variance explained by predictor variables in the linear models. Data analysis was performed using GraphPad Prism 6 and significance was considered at a P-value <0.05

3. Results

3.1 - Animal and VWR characteristics

Baseline body weights were comparable on commencement of studies, with no discernible pattern of change arising between sedentary and exercised groups. Heart weights were significantly increased by VWR in the 14EX (131 *vs*. 117 mg; P<0.05) and 28EX (140 *vs.* 119 mg; P<0.05) groups, with heart:body ratios also significantly increased (**Table 1)**. In terms of running behavior, mice exhibited a training response to VWR, progressively increasing daily running distances and speeds (**Figure 1, Table 2**).

Figure 1 and **Table 2** present running data for all exercise groups, including cumulative and daily distances and velocities of running. Each group exhibited overlapping patterns of daily and cumulative running, confirming uniformity of VWR across the different running-time cohorts. Data reveal a progressive increase in running distances and velocities over the course of the study: the 2EX group ran on average 2.4 km/day (mean speed of 0.55 m/s), increasing to 10.5 km/day in the 28EX group (mean speed of 0.72 m/s) (**Figure 1, Table 2**). Thus, running intensity (speed and daily distance) also rose modestly as VWR duration or volume increased.

3.2 - Effects of VWR on cardiac function and I-R tolerance

Exercise did not induce major shifts in *ex vivo* cardiovascular function, though there was evidence of modest improvements in contractile function with 7 and 28 days of VWR (in terms of dP/dt), and moderately improved coronary flow in these same groups (**Table 3**). Following ischemic insult sedentary hearts exhibited elevations in end-diastolic pressure

(EDP) of ~25 mmHg with 60-70% reductions in left ventricular pressure development (LVDP) and +dP/dt, compared with pre-ischaemia (**Figure 2, Table 3**). The exercise groups (7-28 day VWR) exhibited lower diastolic dysfunction (~18 mmHg) and significantly improved recoveries of LVDP and dP/dt. Recoveries for the 2SED and 2EX groups did not differ, and curiously were similar to those for the longer duration VWR groups.

3.3 - Effects of VWR on stress and survival kinase phospho-regulation

Both cytosolic and membrane levels of phospho-AKT were increased with 7-28 days of running **(Figure 3)**. A trend to increased cytosolic and membrane GSK3□ phosphorylation was also evident, though only achieved statistical significance after 28 days for cytosolic levels and at 7 and 28 days for membrane levels. Similarly, cytosolic phospho-ERK1/2 was increased in 7 and 28 day running groups whereas phospho-AMPK was elevated in the 7-day running group. Overall a pattern of increased kinase phosphorylation is evident in run *vs.* sedentary hearts, however there was little clear relationship with exercise duration or volume. To explore potential contributions of kinase phospho-regulation to cardioprotection, mean expression values were plotted against I-R outcomes for SED and EX groups **(Figure 4)**. These data suggest cytosolic phospho-AKT and phospho-ERK1/2 most consistently correlate with I-R tolerance, whereas changes in AMPK appear poorly related.

3.4 - Effects of VWR on cardiac gene expression

We assessed a suite of genes spanning protective caveolar elements (*Cav3*), myofibrils (*Ttn*), substrate metabolism (*Pfkm*), mitochondrial biogenesis (*Ppargc1a, Sirt3*) and remodeling (*Mmp2*) **(Figure 5)**. Changes in *Pfk1* and *Mmp2* were consistent with early VWR-dependent cardioprotection: *Pfk1* was up-regulated and *Mmp2* repressed with 7 and 28 days VWR. In contrast, *Cav3, PPargc1a, Sirt3* and *Ttn* were only significantly induced after more prolonged 28 days of VWR.

4. Discussion

Whether cardiac benefits of exercise are augmented with more prolonged or intense forms of physical activity is unclear, and may well differ for specific cardiac end-points. For example,

there is evidence for both comparable[27] and distinct[28,29] myocardial outcomes with high *vs.* low intensity exercise. Whether myocardial ischemic or hypoxia tolerance is strongly dependent upon the duration or volume and intensity of exercise is unclear. In the current study mice exhibited a training response to running, with distance run and velocity increased over a 20-day period before plateauing **(Figure 1)**. However, contrary to our hypothesis, moderate levels of VWR induce a cardioprotected phenotype that is not dose-dependent in terms of duration or volume of activity - maximal protection appears to arise within 7 days with no further improvements over greater running distances. There is indication, however, of increased cardiac growth in the 14EX and 28EX groups (**Table 1**). Early induction of protection confirms our observations that the heart is acutely sensitive to variations in activity/inactivity, and as little as 3-7 days of VWR induces a cardioprotected state.[20, 21]

VWR activity was highly consistent and reproducible (**Figure 1**), an important consideration when studying the effect of exercise duration, and counter to the perception of VWR as highly variable.[30] Changes in cardiac I-R tolerance (and growth and functional state) were associated with modulation of survival kinase signaling, together with early and late transcriptional responses benefiting mitochondrial, caveolar and contractile phenotypes. Intriguingly, evidence also suggests that a brief period of environmental enrichment may induce similar (albeit transient) cardioprotection and kinase signaling changes. Further investigation is required, as a necessary control group to confirm this 2 day protective stimulus is lacking (ie. cages without enrichment of a locked running wheel). Nonetheless, it is clear I-R tolerance in 7-28 day sedentary controls is invariant, lower than in 2EX and 2SED groups, and consistent with outcomes from the same I-R insult in hearts of control (non-running) mice across our prior studies.[21,24,25,31]

4.1 - Myocardial molecular response to running

Cardiac molecular changes are consistent with emergence of stress-resistance with VWR:

phospho-activation of AKT, ERK1/2 and AMPK, and phospho-inhibition of GSK3β are evident with 7-28 days of running, and each is predicted to enhance stress-resistance and cell survival **(Figure 3)**. These changes are consistent with the notion that protective 'conditioning' stimuli act via modulation of survival kinase signalling.[16,24] Data indicate that cytosolic phospho-AKT and -ERK1/2 levels most consistently correlate with ischemic tolerance, whereas there appears to be little correlation between cardioprotection and the phosphorylation of AMPK and GSK3b (**Figure 4**). Our prior work also suggests activity-dependent protection may be dissociated from AMPK phosphorylation, though this study also revealed a dissociation from AKT phosphorylation.[21] Thus, although patterns of AKT and ERK1/2 phosphorylation (and less consistent shifts in GSK3□ and AMPK phosphorylation) are broadly congruent with a protected state, specific mechanistic contributions warrant more detailed interrogation. While post-translation modification via phosphorylation is important in acute or short-term regulation of protein functionality, longer lived shifts in cardiac kinase or associated receptor phosphoylation may modify intrinsic activities, and are evident for example during cardiac develpment[32] and aging[24,33,34], and with caloric restriction[34] and exercise[20,21] (including augmenting baseline AKT phosphorylation in aged hearts[35]).

Cardiac transcriptional changes were also consistent with a protected phenotype **(Figure 5)**, and with adaptations reported in other exercise models. For example, reduced *Mmp2* expression agrees with an exercise-dependent reduction in extracellular MMP2 associated with cardiac protection.[15] The enzyme phosphofructokinase (encoded by up-regulated *Pfkm*) is a key rate-limiting enzyme in glycolysis[36] and this is one the most highly induced genes following 7 days of running.[20] Prior studies also indicate skeletal muscle expression is reliant on an intrinsic molecular clock to regulate metabolism,[37] and VWR is known to reinforce circadian rhythm.[38,39] Transcription of the titin gene was significantly and progressively increased with 7-28 days of VWR, supporting exercise-dependent sarcomeric adaptation with even relatively brief activity. Titin is a primary determinant of myocardial elasticity, Frank-Starling responses and potentially exercise tolerance.[40] In a recent study aerobic exercise was found to up-regulate myocardial titin and improve diastolic function in the hearts of diabetic

rats,[41] while earlier work supports shifts in titin phosphorylation with acute exercise, consistent with initially increased myofilament stiffness.[42] Induction of titin in a duration-dependent manner may reflect adaptive expression of myofibrillar proteins with physiological hypertrophy, together with modulation of myocardial elasticity (influenced by isoform ratios and site-specific phosphorylation). Somewhat surprisingly, *Cav3*, *PGC1a* and *Sirt3* were only up-regulated after 28 days of VWR, supporting distinct molecular changes with prolonged *vs.* brief (or high *vs*. low volume) exercise. Products of these genes are implicated in a variety of protective mechanisms, including mitochondrial biogenesis, membrane microdomain and cardioprotective signalling.[43-47] Transcriptional data thus suggest early induction of carbohydrate metabolism and repression of detrimental remodeling genes, followed by later induction of caveolar (*Cav3*) and myofibrillar elements (*Ttn*), and promoters of mitochondrial biogenesis and function (*Ppargc1a*, *Sirt3*).

Collectively, data indicate the extent of cardiac protection and Akt phosphorylation with VWR appears independent of exercise duration (7 to 28 days running) and volume (33 to 278 km run), and are not modified with a 2-fold increase in intensity (~5 km/day at 0.55 m/s after 7 days *vs.* ~11 km/day at 0.72 m/s after 28 days). On the other hand, induction of *Cav3*, *Ppargc1a*, *Sirt3* and *Ttn* transcripts appears duration (or intensity) dependent. These latter changes are consistent with evidence of exercise intensity-dependent modulation of cardiac mitochondrial function, substrate metabolism, energetics and autophagy.[28,29] These transcriptional responses, and the post-translational effects of even brief exercise (AKT and ERK1/2 phospho-regulation), are all predicted to improve myocardial stress-resistance, although specific roles require further investigation.

4.2 - Evidence of protection in 2 day experimental groups

The 2-day EX and SED groups are of interest since both exhibited molecular changes akin to those with 7-28 days of VWR, and both exhibited increased I-R tolerances (equivalent to 7-28 days VWR, and surpassing that with 7-28 days of sedentary behavior and reported for non-run mice in our prior work[24,31]). This effect thus appears largely independent of running.

Perhaps the simplest interpretation is that an acute change in housing and environment (2 day individual housing with fixed or free running wheels) induces transient cardiac stress-resistance, though confirmation of this effect is necessary. Ischemic tolerance was consistent with common elevations in phospho-AKT and phospho-ERK1/2 in the 2EX and 2SED groups (equivalent to levels observed in 28EX hearts). Prior studies report a variety of beneficial molecular and phenotypic responses to environmental enrichment in mice, particularly at the level of the nervous system and neurohumoral control,[48,49] and also in the context of experimental traumatic brain injury.[50] However, an early cardioprotective effect has not been previously reported. These intriguing observations warrant investigation, potentially highlighting the importance of psychological state or mood in dictating myocardial phenotype. For example, chronic stress and depression impairs infarct tolerance in rodents and humans.[51] This observation may also have relevance to animal welfare, raising questions regarding the behavioral status in 'control' caged rodents often deprived of environmental enrichment.

4.3 - Limitations and future studies

The current study assesses exercise cardioprotection in healthy young adult mice, whereas ischemic heart disease/AMI is age-dependent, prominent in patients >55 yrs old in association with multiple co-morbidities (including diabetes and hypertension). We and others have shown that survival kinase signalling responses may be disrupted with aging,[24,34] and such effects coupled with the negative influences of disease may limit cardioprotection via widely studied pre- and post-conditioning interventions.[52] Whether exercise mediated protection is similarly impaired or better preserved with age and disease is unclear and worthy of further study. Of note, exercise does beneficially influence cardiac protein expression[9,10] and appears to be cardioprotective in aged animals.[35] Sex is an additional factor warranting study: we have assessed responses in males, while it is known that females

enjoy relative protection against ischemia-reperfusion injury. Whether this involves sex-dependent differences in the kinase pathways addressed here, and whether exercise induces common or distinct response in females *vs.* males awaits further study.

Finally, we have assessed a select sub-set of proteins involved in the protective RISK path,[16] encomapssing key upstream mediators (AKT and ERK1/2) and a downstream determinant of mitochondrial permeabilisation (GSK3b), together with AMPK (protective sensor of adipokines, metabolic stress and exercise, linking protective pathways). Additional elements implicated in these paths, including PKC, PKG and nitric oxide synthase, could be assessed to better localise exercise-sensitive sites within these signal cascades. Candidate targets identified in PCR analysis should also be further tested for expression, and where relevant, phosphorylation changes. Despite widespread focus on gene expression in the literature, based on a general link between tranacription and protein expression and the ease, low cost and automation of gene analysis, it is nonetheless important to confirm the impact of these transcriptional responses on protein levels and pathway function.

5. Conclusions

The current data indicate that VWR produces significant cardiac protection that is not dose-dependent in terms of exercise duration or volume. Rather, benefit arises with as little as 7 days running, with no improvements to 28 days of running. This may have implications for exercise prescription and notions of benefit in myocardial infarction, with our recent analysis of activity and inactivity also suggesting that recent physical activity may be more relevant to cardioprotection than a history of exercise.[21] The cardioprotected phenotype is associated with beneficial changes in kinase signaling, including phospho-activation of survival kinases (AKT, ERK1/2) and phospho-inhibition of injurious GSK3b, with AKT and ERK1/2 phosphorylation the most consistently correlated with stress-resistance). Transcriptional changes support distinct adaptations with brief *vs*. more prolonged running, including early induction of metabolic and repression of remodeling genes *vs*. later induction of determinants

of caveolar, myofibrillar and mitochondrial structure and function.

Availability of data and material

All data is available from the corresponding author upon reasonable request.

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Declaration of competing interest

The authors declare there are no conflicts of interest.

Author contributions

BPB, LJH and JPH – conception, design, acquisition and interpretation of data, drafting and editing of the manuscript; LJH and JPH are the guarantors of this work.

KJA, JV, LES - acquisition and analysis of data, and reviewed and edited the manuscript

AJ and JNP - analysis and interpretation of data, review and editing of the manuscript

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Table 1. Body and Heart Masses for 2-28 Day Run and Sedentary Mice.

Group	**Body Mass** (g) **Pre-EX**	**Post-EX**	**Δ Mass** (g)	**Heart Mass** (mg)	**Heart:Body Wt** (mg/g)
2SED *n=14*	23.3±0.5	24.5±0.1	+1.2	109+4	4.4±0.3
2EX *n=14*	25.9±0.4	24.2±0.5	-1.7	113±3	4.7±0.1
7SED *n=15*	24.6±0.7	22.0±0.1	-2.6	112±4	4.6±0.2
7EX *n=15*	23.9±0.3	23.0±0.2	-0.9	114±2	4.8±0.2
14SED *n=15*	23.8±0.7	23.9±0.8	+0.1	117±4	4.9±0.3
14EX *n=15*	23.9±0.4	24.3±0.3	+0.4	131±4 *	5.4±0.4 *
28SED *n=14*	24.0±0.5	24.4±0.7	+0.4	119±3	4.9±0.2
28EX *n=14*	24.1±0.3	23.9±0.2	-0.2	140±6 *	5.8±0.3 *

Data are expressed as means ± S.E.M. *, denotes P<0.05 for EX *vs*. respective SED group.

Table 2. Running Properties for Mice Undertaking 2-28 Days of Wheel-Running.

Group	**VWR Rate** (km/day)	**VWR Velocity** (m/s)	**Cumulative Distance** (km)

2EX *(n=20)*	2.4±0.2	0.55±0.04	5±7
7EX *(n=21)*	5.3±0.3	0.83±0.07	33±7
14EX *(n=21)*	6.9±0.9	0.67±0.04	96±7
28EX *(n=20)*	10.6±0.4	0.72±0.06	278±7

VWR properties for 2-, 7-, 14-, and 28-day groups, including running rate (km/day), velocity (m/s) and cumulative distance (km). Data are expressed as means ± S.E.M.

Table 3. Baseline (normoxic) Functional Parameters for Hearts From Sedentary and VWR Mice.

Group	**EDP** (mmHg)	**Systolic Pressure** (mmHg)	**Heart Rate** (bpm)	**+dP/dt** (mmHg/s)	**-dP/dt** (mmHg/s)	**Flow** (ml/min)
2SED *(n=8)*	3±1	142±6	427±3	4389±217	-2337±247	3.7±0.7
2EX *(n=8)*	5±1	143±6	418±3	4354±244	-3210±529	3.5±0.3
7SED *(n=9)*	5±1	128±10	405±10	5397±637	-3682±409	3.5±0.4
7EX *(n=9)*	3±1	165±6*	413±6	7595±321*	-4710±523*	4.5±0.4*
14SED *(n=9)*	4±1	142±5	423±2	5171±266	-3099±162	3.6±0.3
14EX *(n=9)*	2±1	142±4	410±9	5303±270	-3792±205*	3.4±0.3
28SED *(n=8)*	6±2	146±9	419±6	5398±311	-3430±408	3.2±0.3
28EX *(n=8)*	4±1	164±7*	417±3	6099±287*	-2995±180	3.8±0.2*

Data were acquired after 25 min aerobic perfusion (at a fixed pacing rate of 420 bpm). Data are means ± S.E.M. EDP, end-diastolic pressure; +dP/dt, rate of increase in LV systolic pressure (rate of LV contraction); -dP/dt, rate of decline in LV systolic pressure (rate of LV relaxation). Data are expressed as means ± S.E.M. *, $P<0.05$ *vs*. respective SED group.

Fig. 1. Running characteristics of 8-wk male C57BL/6 mice. Voluntary wheel running distances, daily (*top panel*) and cumulative (*bottom panel*), for male C57Bl/6 mice over 2-days (*n*=20), 7-days (*n*=21), 14-days (*n*=21) and 28-days (*n*=20). Means±S.E.M.

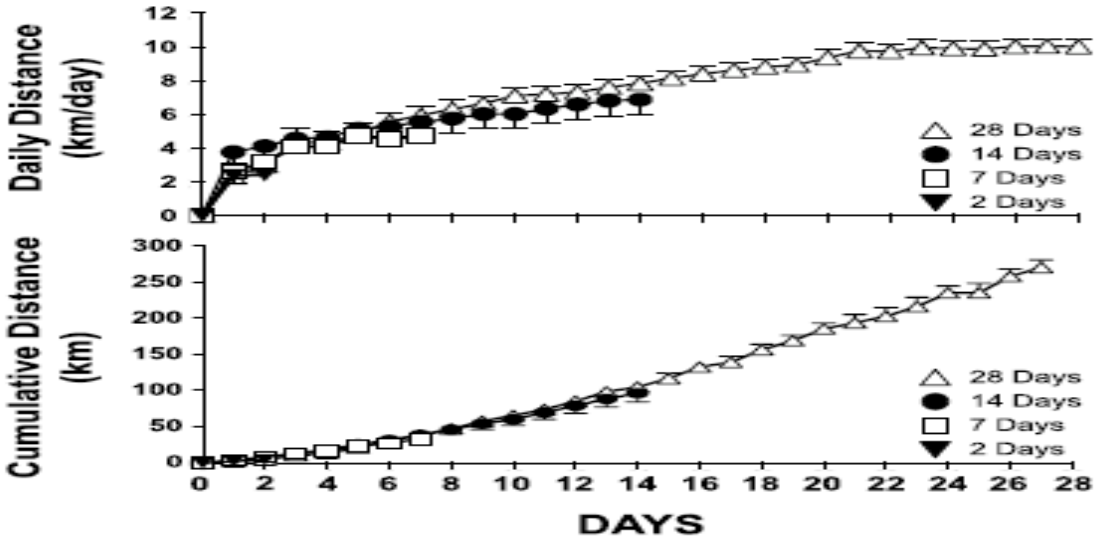

Fig. 2. Myocardial functional tolerance to ischaemia-reperfusion is improved with VWR. Data are shown for final recoveries following 25 min of ischaemia and 45 min of reperfusion. **A)** left ventricular developed pressure (% pre-ischaemia); **B)** left ventricular end-diastolic pressure (mmHg); and **C)** coronary flow rate (% pre-ischaemia). Means±S.E.M. (*n*=8-9 per group). *, P<0.05; **, P<0.01; and ***, P<0.001 *vs*. respective SED group.

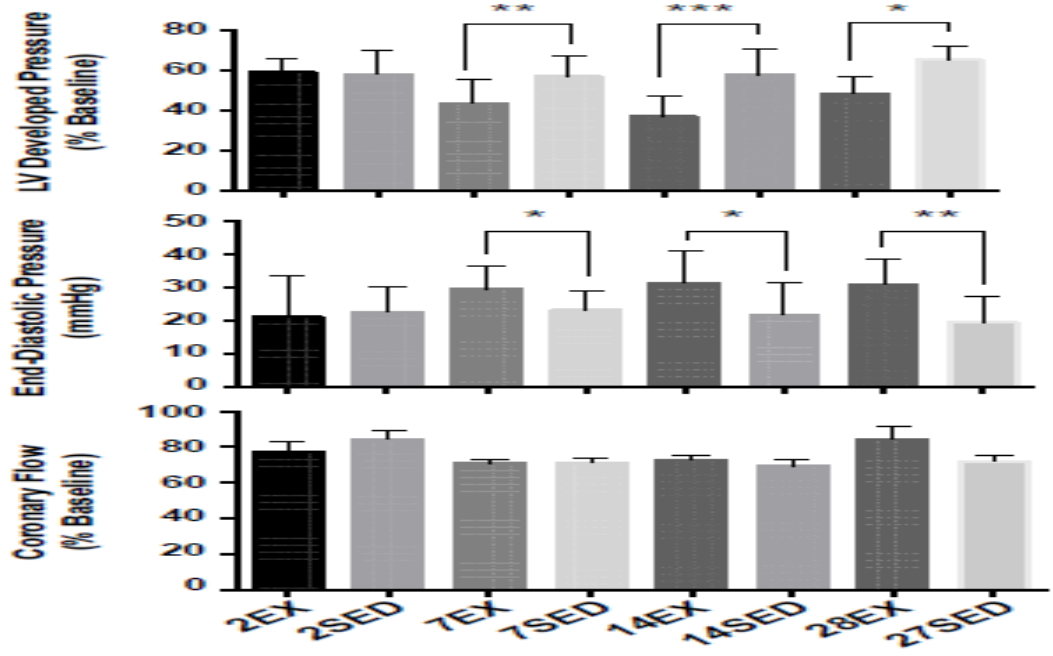

Fig. 3. Myocardial protein kinase phosphorylation after 2-28 days of VWR. Phosphorylation of AKT, GSK3β, ERK1/2 and AMPK was assessed in cytosolic or membrane fractions from

hearts of exercised and sedentary mice. All expression data were initially normalized to β-actin levels. Means±S.E.M. (*n*=6 per group). *, P<0.05 *vs*. respective SED group.

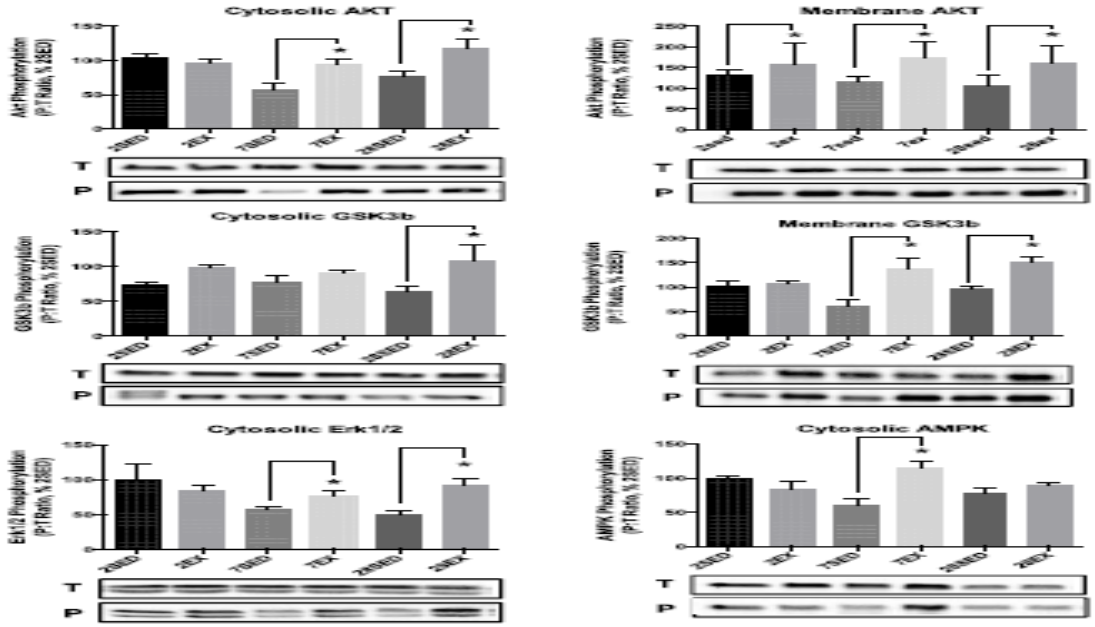

Fig. 4. Relationships between kinase phosphorylation and ischemic tolerance in hearts from VWR and sedentary mice. Data for ischemic tolerance (mean recoveries of LVDP and EDP) are plotted against mean phospho-kinase levels determined in normoxic myocardium. Regression analyses for mean data support correlations between I-R tolerance and both phospho-AKT and phospho-ERK1/2 levels, an intermediate correlation with phospho-GSK3□, and poor correlation with phospho-AMPK. Means±S.E.M.

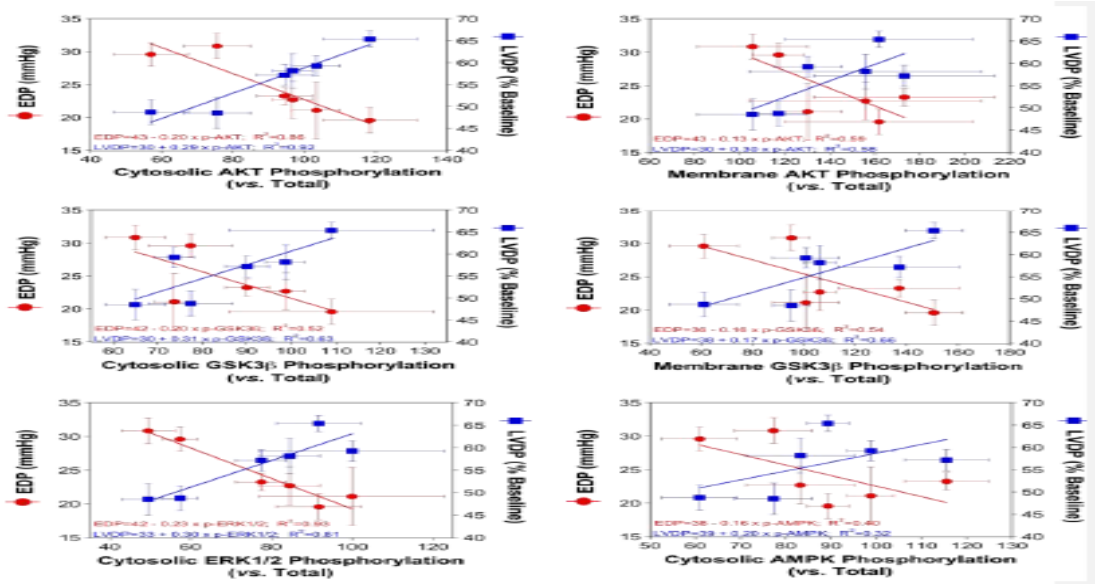

Fig. 5. Cardiac transcriptional changes with 2-28 days of VWR. Tissues was analyzed for expression of transcripts for *Pfkm* (phosphofructokinase, muscle), *Cav3* (caveolin-3), *Mmp2* (matrix metalloprotease 2), *PGC1α* (peroxisome proliferator-activated receptor gamma,

coactivator 1 alpha), *Sirt3* (sirtuin 3) and *Ttn* (titin). Means±S.E.M. (*n*=6 per group). *, P<0.05; **, P<0.01; and ***, P<0.001 *vs*. respective SED group.