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Coupling of myocardial stress-resistance and -signalling to voluntary activity and inactivity

Boris P. Budiono, Louise E. See Hoe, Athena R. Brunt, Jason N. Peart, John P. Headrick†, Luke J. Haseler†*

Menzies Health Institute Queensland, Griffith University, Gold Coast, QLD 4222, Australia

Short Title: Cardiac Stress-Resistance With Activity And Inactivity

†, Co-senior authors

***Corresponding Author:**
John P. Headrick
Menzies Health Institute Queensland
Griffith University Gold Coast
Southport, Q 4222
AUSTRALIA

Ph (Intl): +61 7 5552 8802
FAX (Intl): +61 7 5552 8292
E-mail: j.headrick@griffith.edu.au

ABSTRACT

Aims: We examined coupling of myocardial ischaemic-tolerance to physical activity and inactivity, and whether this involves modulation of survival (AKT, AMPK, ERK1/2, HSP27, EGFR) and injury (GSK3β) proteins implicated in ischaemic preconditioning and calorie restriction.

Methods: Proteomic modifications were assessed in ventricular myocardium, and tolerance to 25 min ischaemia in *ex vivo* perfused hearts from C57Bl/6 mice subjected to: 14 days voluntary activity in running-naïve animals (*Active*); 7 days of subsequent inactivity (*Inactive*); brief (3 day) restoration of running (*Re-Active*); or time-matched inactivity.

Results: *Active* mice increased running speed and distance by 75-150% over 14 days (to ~40 m/min and 10 km/day), with *Active* hearts resistant to post-ischaemic dysfunction (40-50% improvements in ventricular pressure development, diastolic pressure and dP/dt). Cardioprotection was accompanied by ~2-fold elevations in AKT, AMPK, HSP27 and

GSK3β phosphorylation and EGFR expression. Ischaemic tolerance was reversed in *Inactive* hearts, paralleling reduced EGFR expression and GSK3β and ERK1/2 phosphorylation (AKT, AMPK, HSP27 phosphorylation unaltered). Running characteristics, ischaemic tolerance, EGFR expression and GSK3β phosphorylation returned to *Active* levels within 1-3 days of restored activity (without changes in AKT, AMPK or HSP27 phosphorylation). Transcriptional responses included activity-dependent *Anp* induction *vs. Hmox1* and *Sirt3* suppression, and inactivity-dependent *Adora2b* induction.

Conclusions: Data confirm sensitive coupling of ischaemic tolerance to activity: voluntary running induces cardioprotection that dissipates within 1 wk of inactivity yet recovers rapidly upon subsequent activity. While exercise in naïve animals induces a molecular profile characteristic of preconditioning/calorie restriction, only GSK3β and EGFR modulation consistently parallel activity- and inactivity-dependent ischaemic tolerance.

Key Words: cardioprotection; exercise; inactivity; ischaemia-reperfusion; survival kinase

Introduction

Physical activity is one of the strongest determinants of both risk of ischaemic heart disease, and resultant morbidity/mortality (Kohl 2001; Erbs *et al.* 2006; Booth *et al.* 2012; Pierre-Louis *et al.* 2014). Animal studies also reveal powerful exercise cardioprotection - activity-dependent myocardial tolerance to ischaemia-reperfusion (I-R) and other insults (Starnes & Taylor, 2007). Low physical activity levels in post-industrial societies thus promote heart disease prevalence and impacts (Booth *et al.* 2012), while activity-related interventions have significant preventative and therapeutic potentials. However, unknowns remain regarding the broad phenomenon of *exercise cardioprotection*, including the optimal stimulus for inducing cardiac protection, and the mechanistic basis of activity (and inactivity) dependent changes in myocardial stress phenotype.

In terms of interventions, relatively brief and low-intensity activities appear very effective protective stimuli, and particularly suited to clinical exploitation. Reductions in ischaemic disease risk may be optimal with only 1-3 bouts of moderate activity per week (Wisloff *et al.* 2006), and reductions in mortality near optimal with a single weekly bout of moderate exercise (Moholdt *et al.* 2008). Protection against myocardial I-R injury itself is inducible with single or brief (1-7 day) periods of low- to moderate activity (Taylor *et al.* 1999; Hamilton *et al.* 2001; Lennon *et al.* 2004b; Budiono *et al.* 2012; McGinnis *et al.* 2015; Miller *et al.* 2015), although threshold levels may be necessary for cardiac benefit (Starnes *et al.* 2005). This mode of cardioprotection is rapid, powerful, and free of potential untoward

effects; and the acute mechanisms involved (Starnes & Taylor, 2007; Calvert *et al.* 2011; Powers *et al.* 2014; McGinnis *et al.* 2015) may be more amenable to therapeutic manipulation than transcriptional, translational and post-translational elements triggered with longer or intense exercise (Powers *et al.* 2014). Nonetheless, the acute protective mechanisms engaged with short-term/low-intensity activities remain ill defined (Powers *et al.* 2014). One intriguing possibility is that activity bouts are a mild stressor inducing a molecular phenotype common to ischaemic preconditioning (Marongiu & Crisafulli, 2014).

Transient ischaemia and exercise clearly differ in fundamental ways, including the severity of metabolic perturbations with global (albeit brief) ischaemia *vs*. exercise, and their hemodynamic, neurohumoral and immunomodulatory impacts. Moreover, while exercise induces early and delayed windows of protection (Domenech *et al.* 2002) characteristic of ischaemic preconditioning (Heusch *et al.* 2015), mechanisms of late protection differ, with iNOS and cyclo-oxygenase only induced with ischaemia (Powers *et al.* 2014; Heusch, 2015). On the other hand, the acute signalling and molecular impacts of exercise and ischaemia do suggest a common 'hormesis' response (Marongiu & Crisafulli, 2014). Both acutely activate protective GPCRs and sarcolemmal and mitochondrial K_{ATP} channels (Murphy & Steenbergen, 2008; Cai *et al.* 2012; Heusch *et al.* 2015; McGinnis *et al.* 2015; Miller *et al.* 2015), and specific analyses of brief or voluntary activity implicate opioid, adrenergic and cytokine receptors, kinase pathways and effector molecules (Calvert *et al.* 2011; Budiono *et al.* 2012; Ji *et al.* 2013; McGinnis *et al.* 2015; Miller *et al.* 2015) that appear common to ischaemic preconditioning (Murphy & Steenbergen, 2008; Heusch, 2015). Engagement of similar effectors with short-term caloric restriction (Noyan *et al.* 2015), further suggests induction of a common molecular phenotype by diverse conditioning stimuli. Nonetheless, whether exercise is a physiologic corollary of these preconditioning responses, and whether detrimental *inactivity* modulates the same or distinct molecular paths, remain to be established.

We here test the hypothesis that myocardial stress-resistance is coupled to physical activity via molecular effectors common to ischaemic and calorie restriction stimuli. Specifically, we test whether: *i)* myocardial stress-resistance is consistently coupled to short-term (3-14 day) elevations and reductions in low-intensity activity; and *ii)* this cardiac coupling can be explained by reversible control of kinase (AKT, ERK1/2, AMPK, GSK3ß), heat shock (HSP27) and membrane receptor (EGFR) proteins common to preconditioning via ischaemia/calorie restriction and associated opioid and cytokine stimuli (Murphy & Steenbergen 2008; Lorita *et al.* 2010; Williams-Pritchard *et al.* 2011; Cai *et al.* 2012; Ji *et al.* 2013; Heusch *et al.* 2015; McGinnis *et al.* 2015; Noyan *et al.* 2015).

Methods

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.

Animals and experimental design

Male C57Bl/6 mice (8-10 wks) were individually housed at 23±2°C (12:12 hr light-dark cycle; lighting on at 0600h, off at 1800h) in cages equipped with plastic composite solid-surface running wheels (20 cm dia., 6.5 cm width; Silent Spinner; Pets International Ltd, Elk Grove Village, USA), as outlined previously (Budiono *et al.* 2012). The experimental protocol involved allowing running-naïve mice 14 days of free wheel running (*Active*; *n*=20), 14 days of running followed by 7 days of locked wheels (*Inactive*; *n*=14), or 14 days of running, 7 days of locked wheels and 3 days of restored wheel running (*Re-Active*; *n*=14) (**Fig. 1**). Time-matched sedentary controls were maintained under identical conditions with running wheels locked for 14 (*Control 1*; *n*=20), 21 (*Control 2*; *n*=14) or 24 days (*Control 3*; *n*=14). A similar wheel-lock approach has been applied previously to assess effects of inactivity (Kump *et al.* 2005). Running activity (distance and duration of bidirectional running) was tracked via reed-switch activated bicycle computers (model BC-509; Sigma Sport, Olney, IL). Incidental activity outside of wheels (and in sedentary controls) was not measured. At the end of each experimental period mice were terminally anaesthetised with 60 mg/kg sodium pentobarbital (*i.p*) and hearts either excised into ice-cold Krebs solution for Langendorff perfusion (*n*=7-13 per group) or frozen in liquid N_2 and stored at -80°C for molecular analyses (*n*=6-7 per group).

Ischaemia-reperfusion in Langendorff-perfused hearts

Hearts were perfused in a Langendorff mode with modified Krebs-Henseleit solution (at 80 mmHg), and contractile and coronary function monitored as described in detail previously (Headrick *et al.* 2001). Functional data were recorded at 1 kHz via a PowerLab 4/30 system (ADInstruments Pty Ltd., Bella Vista, Australia). Ventricular pressure signals were digitally processed to yield heart rate, peak systolic and end-diastolic pressures, +dP/dt and -dP/dt. After a 20 min period of stabilisation at intrinsic heart rates all hearts were

switched to 10 min of ventricular pacing at 420 bpm (Grass SD9 stimulator; Natus Neurology Inc., Warwick, RI, USA). Baseline measures were then made before subjecting hearts to 25 min global normothermic ischaemia and 45 min aerobic reperfusion. Pacing was stopped in ischaemia and resumed at 1.5 min reperfusion. No hearts warranted exclusion based upon previously detailed functional criteria (Headrick *et al.* 2001).

Immunoblot analysis of protein expression

Ventricular myocardial lysates were prepared as described previously (Williams-Pritchard *et al.* 2011; Budiono *et al.* 2012), and samples containing 30 μg of protein loaded onto 10% acrylamide gels and separated at 150 V for 60 min. Proteins were transferred to LF-PVDF membranes and blocked in Li-COR blocking buffer for 90 min before incubation overnight (at 4°C) with 1:1,000 dilutions of primary antibodies (Cell Signaling Technology, Danvers, MA) for total and phosphorylated AKT (Ser^{473}), ERK1/2 (Thr^{202}/Tyr^{204}), AMPK (Thr^{172}) and GSK3β (Ser^{9}), total EGFR and phosphorylated HSP27 (Ser^{82}), with GAPDH assayed for purposes of normalisation. Following 3 washes in TBST buffer, membranes were incubated with fluorescent secondary antibody and scanned on a Li-COR Odyssey system (Li-COR Biosciences, Lincoln, NE, USA). Protein expression was normalised to GAPDH (assayed on each membrane), and phosphorylation of AKT, ERK1/2, AMPK and GSK3β determined from the ratio of phosphorylated:total protein. For purposes of comparison, protein data (**Fig. 3**) were expressed relative to the *Active* experimental group.

PCR analysis of gene expression

Ventricular RNA was isolated and expression of select genes assessed via RT-quantitative PCR (RT-qPCR), as detailed previously (Headrick *et al.* 2001; Budiono *et al.* 2012). Reactions were performed in triplicate and expression normalised to phosphoglycerate kinase 1 (*Pgk1*) as a reference. Changes in expression were calculated relative to time-matched control (inactive) hearts using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). Primer sequences for *Anp* (atrial natriuretic peptide; marker of hypertrophic remodelling), *Adora2b* (A_{2B} adenosine receptor; mediating adaptive cardiac stress-resistance), *Hmox1* and *Sirt3* (heme oxygenase 1 and the deacetylase sirtuin 3; both implicated in adaptive stress-resistance and metabolic remodelling), and *Calm2* (calmodulin 2 phosphorylase kinase; major cardiac form of this key orchestrator of Ca^{2+} signalling) are provided in **Table 1**.

Statistical analyses

Unless stated otherwise all physiological and proteomic data are expressed as means ± SEM. To compare outcomes across groups an ANOVA was performed, with post-hoc analysis via a Tukey's test. Where appropriate, specific comparisons between data for intervention (*Active*, *Inactive* or *Re-Active*) *vs.* time-matched control groups were made via two-tailed Student *t-test*. In all tests significance was accepted for $P<0.05$.

Results

Animal characteristics

Initial body weights did not differ across groups at baseline, whereas differences were evident at the end of the experimental protocols: weight gain in time-matched sedentary controls significantly exceeded that for *Active* mice (**Table 2**). Transition to 7 days of inactivity and a subsequent 3-day period of activity did not modify body weight beyond the effects of the initial running period. Heart mass and heart:body mass ratios were not modified after transitions to either activity or inactivity (**Table 2**).

Running characteristics for all groups are depicted in **Fig. 1**. Mice increased running speeds by ~1 m/min per day, and daily distances by 0.7 km/day, over the initial 14 day *Active* period. Recovery of running following the 7 day *Inactive* period was extremely rapid: running distance and speed recovered within the 1st day to levels attained after 7-14 days of initial activity (**Fig. 1**). Mice ran 226 km (range: 159-305 km) over the total experimental period, averaging 13.3 km/day (9.4-17.9 km) at a mean speed of 32.7 m/min (26.4-37.2 m/min).

Cardiac function and I-R tolerance

Baseline functional parameters for perfused hearts did not differ across groups (**Table 3**), though there was a trend to slightly higher +dP/dt (5-10%) in *Active* and *Re-Active vs. Control* hearts (albeit insignificant). Following I-R insult all groups of hearts failed to recover contractile function and coronary flow to normoxic (baseline) levels (**Fig. 2**). However, 14 days of activity markedly enhanced functional outcomes from I-R, with up to 50% improvements in left ventricular end-diastolic pressure, pressure development and ±dP/dt *vs.* time-matched controls (**Fig. 2**). Protection was not associated with shifts in systolic function (or coronary reflow), evidencing a primary impact of activity on determinants of diastolic function and lusitropic and inotropic states. A 7-day *Inactive* period negated this cardiac protective effect, while a 3-day return to activity restored I-R tolerance to the level of the

Active group (**Fig. 2**).

Myocardial proteomic and transcriptional responses

Myocardial expression/phosphorylation of kinases implicated in promoting (AKT, ERK1/2, AMPK) or reducing (GSK3β) myocardial stress-resistance was assessed in each group, together with expression of stress-responsive p-HSP27 and protective EGFR (**Fig. 3**). Consistent with induction of a broadly beneficial stress phenotype, 14 days of voluntary running significantly enhanced the phospho-activation of protective AKT and AMPK, phospho-inhibition of GSK3β, and expression of p-HSP27 and EGFR (**Fig. 3**). In contrast, ERK1/2 phosphorylation was unaltered during initial activity. Neither 7 days of physical inactivity nor a 3-day return to activity further influenced AKT or AMPK phosphorylation (both remaining consistently above sedentary control levels). Expression of p-HSP27 also remained elevated during the *Inactive* period, though declined modestly with restoration of activity. Interestingly, ERK1/2 phosphorylation declined significantly during the *Inactive* period, rising again with subsequent running (coupled with insensitivity to initial running, this suggests specific sensitivity of ERK1/2 signalling to inactivity). In contrast to these variable responses, phospho-inhibition of GSK3β and expression of EGFR increased with initial activity, declined during inactivity and recovered again with subsequent restoration of activity (**Fig. 3**). Total cardiac expression levels for AKT, ERK1/2, AMPK and GSK3ß were not significantly altered by transitions in activity/inactivity (data not shown).

Analysis of cardiac gene expression revealed a mix of changes (**Fig. 4**), including: induction of *Anp* with activity that was reversed with inactivity (yet was unresponsive to a subsequent return to activity); reversible induction of *Adora2b* with inactivity, relative insensitivity of *Calm2*, and apparent repression of *Hmox1* and *Sirt3* with activity/inactivity.

Discussion

The current study demonstrates sensitive coupling of myocardial stress-resistance to brief and moderate shifts in activity, with distinct molecular responses in mice naïve *vs*. previously subjected to wheel-running. Cardiac molecular effects of activity in running-naïve mice correspond with mechanisms implicated in ischaemic preconditioning and calorie restriction (phospho-regulation of AKT, AMPK, GSK3β), and support a significant stress-response (up-regulated *Anp*, p-HSP27). However, many of these mediators are insensitive to

subsequent shifts in activity, with only GSK3ß phosphorylation and EGFR expression consistently paralleling cardiac stress-resistance (**Fig. 5**). Involvement of GSK3β is congruent with prior analysis (Budiono *et al.* 2012), whereas activity-dependent EGFR expression is a novel and intriguing observation. The latter may contribute to dynamic control of stress-resistance given the receptors protective functions (Pareja *et al.* 2003; Lorita *et al.* 2010; Williams-Pritchard *et al.* 2011).

Mouse running behaviour. Wheel-running presents a useful model for studying physiological impacts of activity without complications of stressful exercise-inducement. Prior studies show animals willingly run high distances, even exceeding those in the wild (Sherwin 1998). Here mice initially naïve to wheel-running substantially increase speeds and distances over the 14 day *Active* period (**Fig. 1**). Consistent with a low intensity, myocardial mass, heart:body weight and cardiac function were unaltered during 3-14 day transitions in activity (**Table 2**). Interestingly, while naïve mice progressively increased running over 14 days, those subjected to a subsequent cycle of inactivity/activity immediately return to these levels in the 1st day of running (**Fig. 1C)**. These observations suggest a conditioning or memory effect, with running behaviour rapidly restored in animals exposed to prior exercise. Thought to involve learning effects within the CNS, this may also involve local mechanisms including acquisition of inactivity-resistant myonuclei (Bruusgaard *et al.* 2010). This immediate return of physical activity may contribute to prompt restoration of cardioprotection: cardiac stress-resistance (together with EGFR expression and GSK3ß phosphorylation) rapidly recovered within 1-3 days of restored activity.

Distinct molecular responses to initial vs. subsequent activity. While myocardial ischaemic tolerance improves with running in both naïve (*Active*) and previously active (*Re-Active*) mice, the responses are associated with unique molecular profiles, with only the former group displaying changes akin to ischaemic preconditioning or calorie restriction (**Fig. 5**). This initial response agrees with reports of exercise-dependent activation of AKT or AMPK (Coven *et al.* 2003; Kemi *et al.* 2008; Calvert *et al.* 2011; Ma *et al.* 2013), inhibition of GSK3β (Budiono *et al.* 2012) and induction of HSPs (Esposito *et al.* 2011). Nonetheless, although each change might be predicted to enhance ischaemic tolerance, most are not apparent with subsequent activity transitions (**Fig. 5**) and are thus unlikely to participate in active coupling of stress-resistance to activity.

Transcriptional changes further differentiate myocardial responses to initial and subsequent running. We previously reported a mixed transcriptomic response to running,

involving shifts in inflammatory/immune and sarcomeric elements without changes in canonical protective paths (Budiono *et al.* 2012). Here, *Anp* is induced with initial activity, consistent with changes with more intense exercise (Pan 2008). However, expression dissipates with inactivity and is unaltered on subsequent running. Activity-dependent stress-resistance in non-cardiac tissues has also been linked to heme oxygenase-1 and sirtuin 3, yet neither *Hmox1* nor *Sirt3* was induced in *Active* hearts, and indeed appear to be suppressed. Interestingly, Niess *et al.* (1999) found exercise training lowered heme oxygenase-1 expression in other cell types, a potentially adaptive response to regular activity.

Taken together, differential induction of *Anp* and stress-sensitive p-HSP27 with initial (not subsequent) running, and sustained phospho-activation of AKT and AMPK, support a more profound stress-response to exercise in naïve mice. Though resembling the molecular profile for ischaemic preconditioning and caloric restriction (Marongiu & Crisafulli, 2014; Noyan *et al.* 2015), subsequent activity transitions more selectively influence GSK3β phosphorylation and EGFR expression.

Roles for EGFR and GSK3β in dynamic stress-resistance. This is the first report of rapid, reversible control of cardiac EGFR levels via a physiological stimulus such as running - a 'sensor' more responsive to activity/inactivity than AKT, AMPK or HSP27 phosphorylation. Protective functions of this receptor (Pareja *et al.* 2003; Lorita *et al.* 2010), and evidence of involvement in ischaemic preconditioning and adenosinergic protection (Williams-Pritchard *et al.* 2011), suggest this regulation will impact stress-resistance. Similarly, GSK3β phospho-inhibition was sensitive to activity and inactivity and paralleled ischaemic tolerance, consistent with prior observations (Budiono *et al.* 2012) and the putative role of the kinase in stress-resistance (Murphy & Steenbergen, 2008; Lal *et al.* 2015).

Relative insensitivities of AKT, AMPK and HSP27 signalling to inactivity and subsequent activity are inconsistent with roles in the reversible control of cardiac stress-resistance, however they may contribute to effects of more stressful exercise (*eg*. in naïve animals). Prior studies also question the role of heat shock proteins in activity-dependent protection (Powers *et al.* 2014), and whereas AKT is implicated in diverse protective responses, acute activation may fail to protect hearts (Moreira *et al.* 2015) and expression/activation can inhibit I-R tolerance (Kohl 2001) and protective conditioning responses (Fullmer *et al.* 2013). While current and previous data (Coven *et al.* 2003; Kristiansen *et al.* 2009) confirm AMPK is activated with exercise, and the kinase may mediate anti-fibrotic effects of intense activity (Ma *et al.* 2015), studies have yet to confirm

causal involvement of AMPK in activity-dependent ischaemic tolerance.

Persistence of protection, and molecular effects of inactivity. The persistence of exercise protection is unclear. Effects of up to 3 mo of exercise on myocyte and myocardial structure, function and autonomic control are lost within 2-4 wks of inactivity (Kemi *et al.* 2004; Mostarda *et al.* 2009; Bocalini *et al.* 2010; Waring *et al.* 2015). The current and prior studies collectively suggest a dose-response relation between duration/intensity of activity and persistence of protection. We show effects of 14 day low-intensity running are lost within 7 days of inactivity (**Fig. 2**), while prior work shows protection with 8 days of exercise (final 3 at 70% VO_{2max}) is lost after 18 days (Lennon *et al.* 2004a); that with 4 wks voluntary running is lost after 4 wks (Calvert *et al.* 2011); while that with 10 wks of high-intensity exercise (80% VO_{2max}) is partly retained after 4 wks of inactivity (Esposito *et al.* 2011).

Whether inactivity effects on the heart reflect simple reversal of activity-dependent mechanisms, or select modulation of additional processes (as evidenced in other tissues; Booth *et al.* 2012), is also unclear. Previous studies suggest at least partial reversal of activity-dependent changes: loss of protection after 8 days of running is associated with partial reversal of HSP72 and catalase induction (Lennon *et al.* 2004b); loss of protection with 4 wks of voluntary running is paralleled by reversal of CuZnSOD, AMPK and β_3-adrenergic receptor induction (and resulting NO bioavailability) (Calvert *et al.* 2011); and loss of protection after 10 wks of high-intensity exercise parallels reversal of HSP70 and MnSOD induction (without altering oxidative stress) (Esposito *et al.* 2011). The only activity responses consistently reversed with inactivity here are GSK3β phosphorylation and EGFR expression, whereas distinct effects of inactivity *vs.* activity were apparent (**Fig. 5**): inactivity did not influence activity-dependent shifts in AKT, AMPK or HSP27; ERK1/2 phosphorylation was unaltered with initial activity yet repressed with inactivity; and A_{2B} adenosine receptor transcript (*Adora2b*) was also unaltered with initial activity, whilst induced with inactivity. The importance of ERK1/2 in exercise protection is uncertain, with divergent responses reported, including evidence the stress-kinase is insensitive to (Konhilas *et al.* 2004) or in fact down-regulated with exercise (Gosselin *et al.* 2006). The A_{2B} receptor, transcriptionally sensitive to inactivity here, is implicated in adaptive cardioprotection and influences myocardial repair, remodelling and inflammation (Eckle *et al.* 2012; Eltzschig *et al.* 2013). Translation at the protein level, and the physiologic relevance of this inactivity response, deserve further analysis.

Study limitations. Three limitations are worth noting. Absent direct measures of cell

death we cannot ascertain contributions of improved cellular function *vs*. survival to activity-dependent protection. This is also relevant to prior studies reporting exercise protection against reversible 'stunning' (Hamilton *et al.* 2001; Lennon *et al.* 2004a) despite use of models involving combined contractile dysfunction and cell death. We show≥20 min ischaemia does induce cell death in the current model (Headrick *et al.* 2001). Though we document a strong correlation between oncotic death and diastolic dysfunction in this model (Peart & Headrick, 2003), and the latter is modified by activity/inactivity (**Fig. 2A**), specific impacts on stunning *vs.* death await delineation. A second limitation is that activity outside running wheels was not recorded - whether incidental activity of experimental *vs*. *Control* mice varied during wheel-lock periods is not known. Finally, mice were not placed on a reverse light cycle with experimentation undertaken in daylight hours, potentially influencing outcomes. Myocardial ischaemic tolerance follows a circadian pattern, minimal at sunrise and maximal around midnight (a response sensitive to A_{2B} receptors, shown here to be transcriptionally sensitive to inactivity) (Eckle *et al.* 2012). While our findings reveal significant activity-dependent protection when hearts are at their most susceptible to injury, the degree of benefit during night-time hours is unknown and deserving of study.

Perspective

The present observations have several implications. First, the high plasticity of myocardial stress-resistance, paralleling brief and low-intensity shifts in activity, indicates recent rather than historic patterns of physical activity may be particularly relevant to cardiac protection. Second, data suggest physical activity in naïve (untrained) animals may be sufficiently stressful to invoke a molecular response consistent with transient ischaemia or calorie restriction. However, subsequent transitions in activity induce more select changes, implicating EGFR expression and GSK3β phosphorylation in dynamic control of stress-resistance. This raises a key question: how do these translational and post-translational responses arise? Dissociation from AKT, ERK1/2 or AMPK phospho-activation implicates other pathways. Other kinases phosphorylating GSK3β(Ser9) include PKA, p70/p85 and p90-ribosomal S6 kinases, mitogen- and stress-activated protein kinases, and serum/glucocorticoid-regulated kinases (Sugden *et al.* 2008). Expression of EGFR (ErbB-1, HER1) may be regulated by HIF-2α, with hypoxic induction involving a recently identified internal ribosome entry site within its 5'-untranslated region (Webb *et al.* 2015). These mechanisms deserve further attention, as do precise roles of GSK3β and EGFR in coupling

cardiac phenotype to physical activity. From a therapeutic perspective, GSK3β is already highlighted as a potentially valuable target (Lal *et al.* 2015), whereas little attention has been paid to EGFR. Unravelling the mechanisms controlling expression of this protective receptor tyrosine kinase (Pareja *et al.* 2003; Lorita *et al.* 2010) may reveal novel approaches to manipulating myocardial stress-resistance.

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Conflict of Interest

No conflicts of interest, financial or otherwise, are declared by the authors.

Author Contributions

BPB, JPH, LJH: conception and design of research; BPB, LES, JPH, ARB: performed experiments; BPB, JPH, ARB: analysed data; BPB, JPH, LJH, JNP: interpreted experimental data; BJP, JPH: prepared figures; BPB, JPH: drafted manuscript; BPB, LJH, JPH, JNP edited and revised manuscript; BPH, LES, ARB, JNP, JPH, LJH: approved final version of manuscript.

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Tables

Table 1 Primer sequences for RT-qPCR analyses

Symbol	Gene Name	Forward Sequence	Reverse Sequence
Anp	Atrial natriuretic peptide	ATTTCAAGAACCTGCTAGACC	TCAGTCTGCTCACTCAGG
Adora2b	A_{2B} adenosine receptor	TCCCGCTCAGGTATAAAGGT	GGCACTGTCTTTACTGTTCCA
Calm2	Calmodulin 2	CCTGAATTTCTGACAATGATGG	CATTGCCATCCTTATCAAACAC
Hmox1	Heme oxygenase 1	ACCAAGGAGGTACACATCCA	CCATCACCAGCTTAAAGCCT
Sirt3	Sirtuin 3	AGGACTAGTGTTACAGGTGG	AAACTTCTTCTCACTGCTTCC

Shown are forward and reverse primer sequences for RT-qPCR analysis of indicated cardiac transcripts.

Table 2 Body and heart weights in *Active*, *Inactive* and *Re-Active* mice (*vs*. time-matched controls).

GROUP	**Body Weight** (gm)		**Heart Weight** (mg)	**Heart:Body Weight Ratio** (mg/gm)
	Pre-Experiment	**Post-Experiment**		
Active (*n*=20)	22.9±0.3	24.0±0.5*	119±8	5.0±0.3
Control 1 (*n*=20)	23.2±0.3	25.0±0.3	118±5	4.7±0.2
Inactive (*n*=14)	23.0±0.8	23.7±0.9*	113±7	4.8±0.2
Control 2 (*n*=14)	21.9±1.0	26.9±0.7	115±8	4.7±0.5
Re-Active (*n*=14)	23.2±0.1	24.3±0.2*	112±2	4.6±0.1
Control 3 (*n*=14)	23.0±1.1	26.8±0.9	110±3	4.6±0.1

Values are shown as means ± SEM (*n* values shown). *, P<0.05 *vs*. respective sedentary *Control* groups.

Table 3 Baseline functional properties of hearts from *Active*, *Inactive* and *Re-Active* mice (*vs*. time-matched controls).

GROUP	**LV EDP**	**LV Systolic Pressure**	**+dP/dt** (mmHg/s)	**-dP/dt** (mmHg/s)	**Coronary Flow**

	(mmHg)	(mmHg)			(ml/min)
Active (*n*=13)	2±1	143±7	5883±464	-3890±508	3.7±0.5
Control 1 (*n*=13)	4±1	142±7	5261±525	-2296±120	3.7±0.4
Inactive (*n*=7)	5±2	141±7	3874±504	-2621±250	3.6±0.5
Control 2 (*n*=7)	8±2	137±8	4702±410	-3484±242	3.7±0.3
Re-Active (*n*=7)	2±1	134±8	6184±549	-3194±237	3.8±0.4
Control 3 (*n*=7)	5±1	128±5	5869±414	-3218±181	3.7±0.4

Data were acquired after 30 min aerobic perfusion (hearts paced at 420 bpm). LV, left-ventricular; EDP, end-diastolic pressure; +dP/dt, rate of increase in left ventricular systolic pressure; -dP/dt, rate of decline in LV systolic pressure. No significant differences were detected between groups ($P > 0.05$). Values are shown as means ± SEM (*n* values shown).

Figure Captions

Figure 1 Outline of the study design, and running characteristics of C57BL/6 mice. **A)** Mice were given free access to running wheels for 14 days (*Active*), with a subsequent 7 day period of locked running wheels (*Inactive*), and a 3 day period of wheel running (*Re-Active*). Time-matched sedentary controls (wheels locked throughout) were also assessed (*Control 1*, *Control 2*, *Control 3*). Cardiac tissue was sampled at time points indicated by arrows. **B)** Mice progressively increased running from ~4 km/day on day 2 to ~10 km/day between days 10-14, in association with increased running speeds. **C)** Initial activity characteristics for *Active* and *Re-Active* groups on transition to running (demonstrating rapid recovery of running in previously active mice). Data are means ± SEM (*n*=14-20 per group).

Figure 2 Effects of variations in voluntary activity on myocardial recovery from 25 min global normothermic ischaemia and 45 min reperfusion. Data are shown for recoveries of left ventricular: **A)** end-diastolic pressure; **B)** systolic pressure; **C)** developed pressure; **D)** +dP/dt; **E)** -dP/dt; and **F**) coronary flow rate. Data are means ± SEM (*n*=7-13). *, P<0.05 *vs*. time-matched sedentary *Control*; †, P<0.05 *Inactive vs. Active*; ‡, P<0.05 *Re-Active vs. Inactive*.

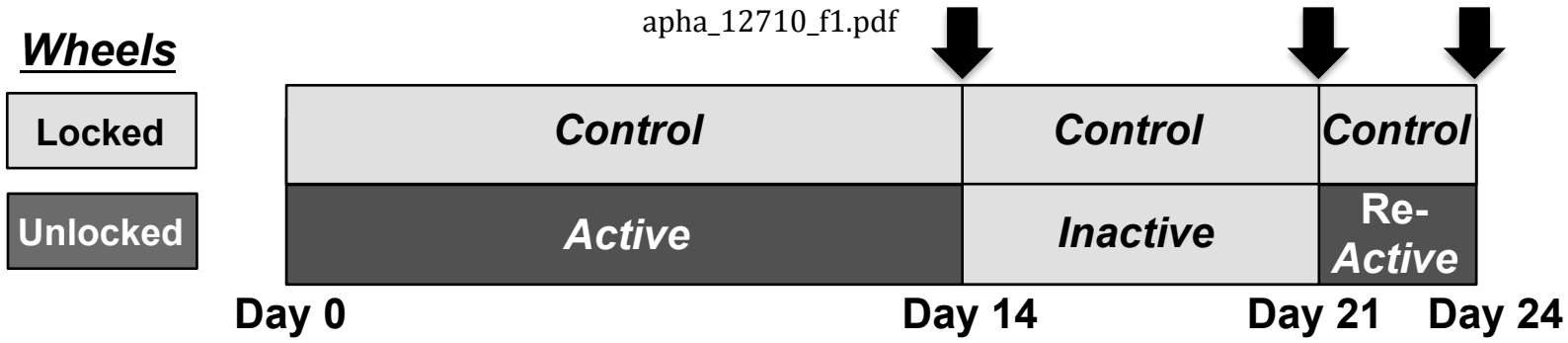
Figure 3 Effects of variations in voluntary activity on myocardial expression/ phosphorylation of proteins influencing stress-resistance. Data are shown for phosphorylation states of: **A)** AKT; **B)** GSK3β; **C)** ERK1/2; and **D)** AMPK; and expression of **E)** EGFR; and **F)** p-HSP27. Data are means ± SEM (*n*=6 per group). *, P<0.05 *vs*. time-matched sedentary *Control*; †, P<0.05 *Inactive vs. Active*; ‡, P<0.05 *Re-Active vs. Inactive*.

Figure 4 Cardiac transcriptional effects of variations in voluntary activity. Data are shown for expression of transcripts for atrial natriuretic peptide (*Anp*), A2B adenosine receptor (*Adora2b*), calmodulin-2 (*Calm2*), heme-oxygenase 1 (*Hmox1*) and sirtuin 3 (*Sirt3*). Data are means ± SEM (*n*=7 per group). *, P<0.05 *vs*. time-matched sedentary

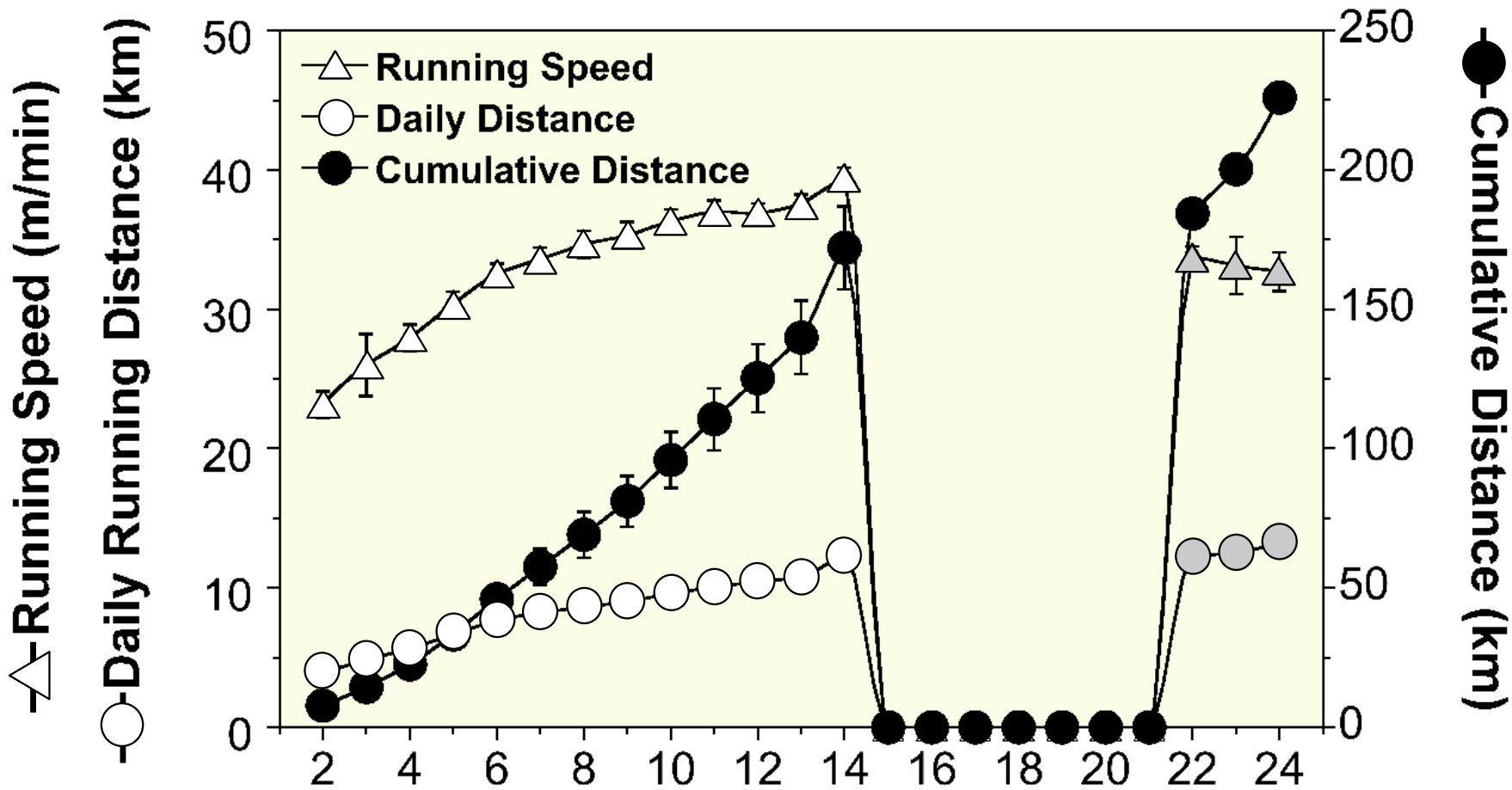
Control; †, P<0.05 *Inactive vs. Active*; ‡, P<0.05 *Re-Active vs. Inactive*.

Figure 5 Summary of distinct molecular responses to initial activity, inactivity and subsequent restoration of activity. Initial activity in running-naïve mice triggers a broad protective profile similar to that in ischaemic preconditioning (encompassing up to 2-fold elevations in AKT, AMPK, GSK3β and HSP27 phosphorylation, and EGFR expression, while ERK1/2 is unaltered). Subsequent inactivity selectively reduces EGFR expression and GSK3β (and ERK1/2) phosphorylation, with AKT, AMPK and HSP27 phosphorylation insensitive to inactivity. Restored running selectively elevates EGFR expression and GSK3β (and ERK1/2) phosphorylation, while p-HSP27 declines modestly (AKT and AMPK phosphorylation again insensitive).

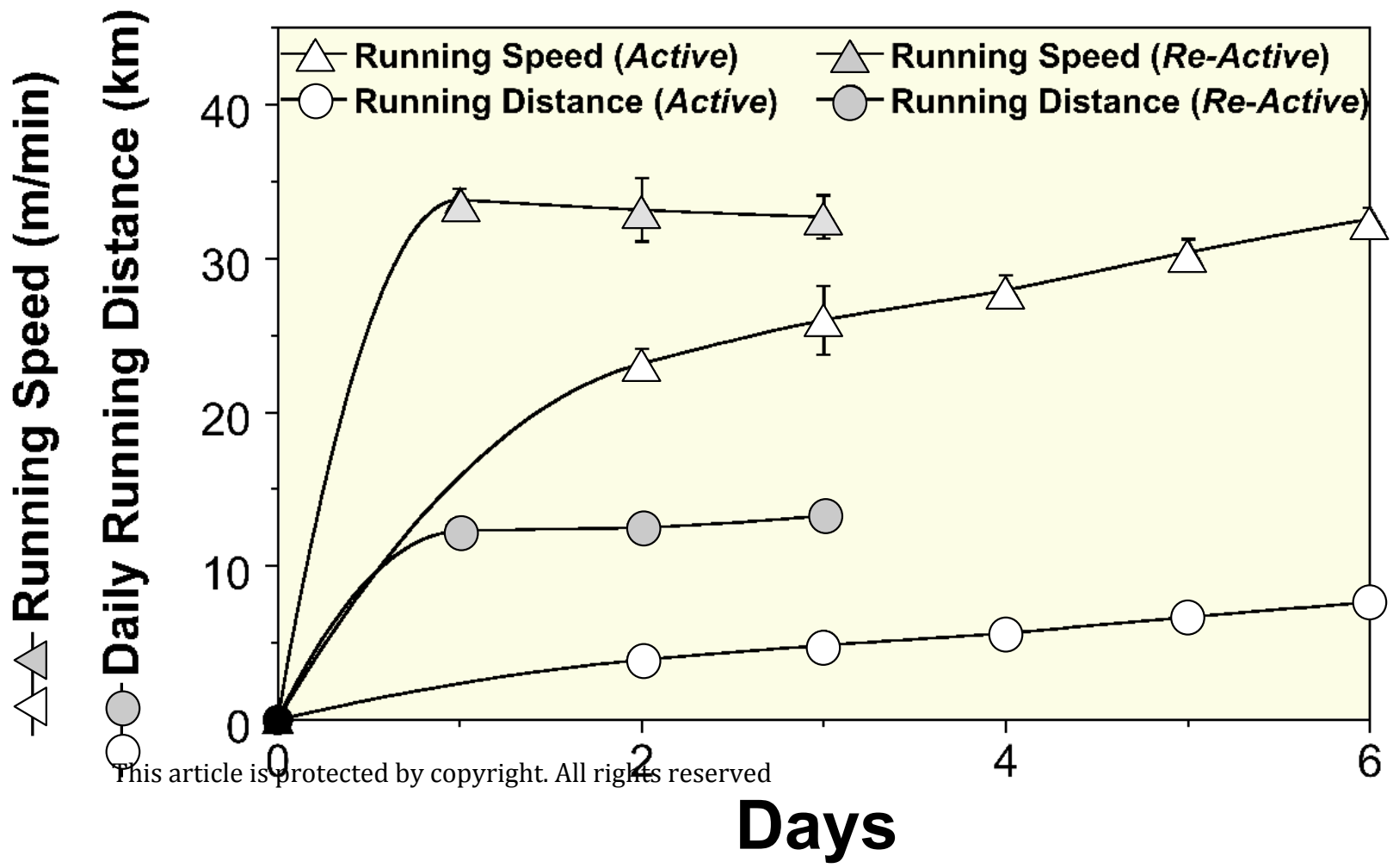
A)

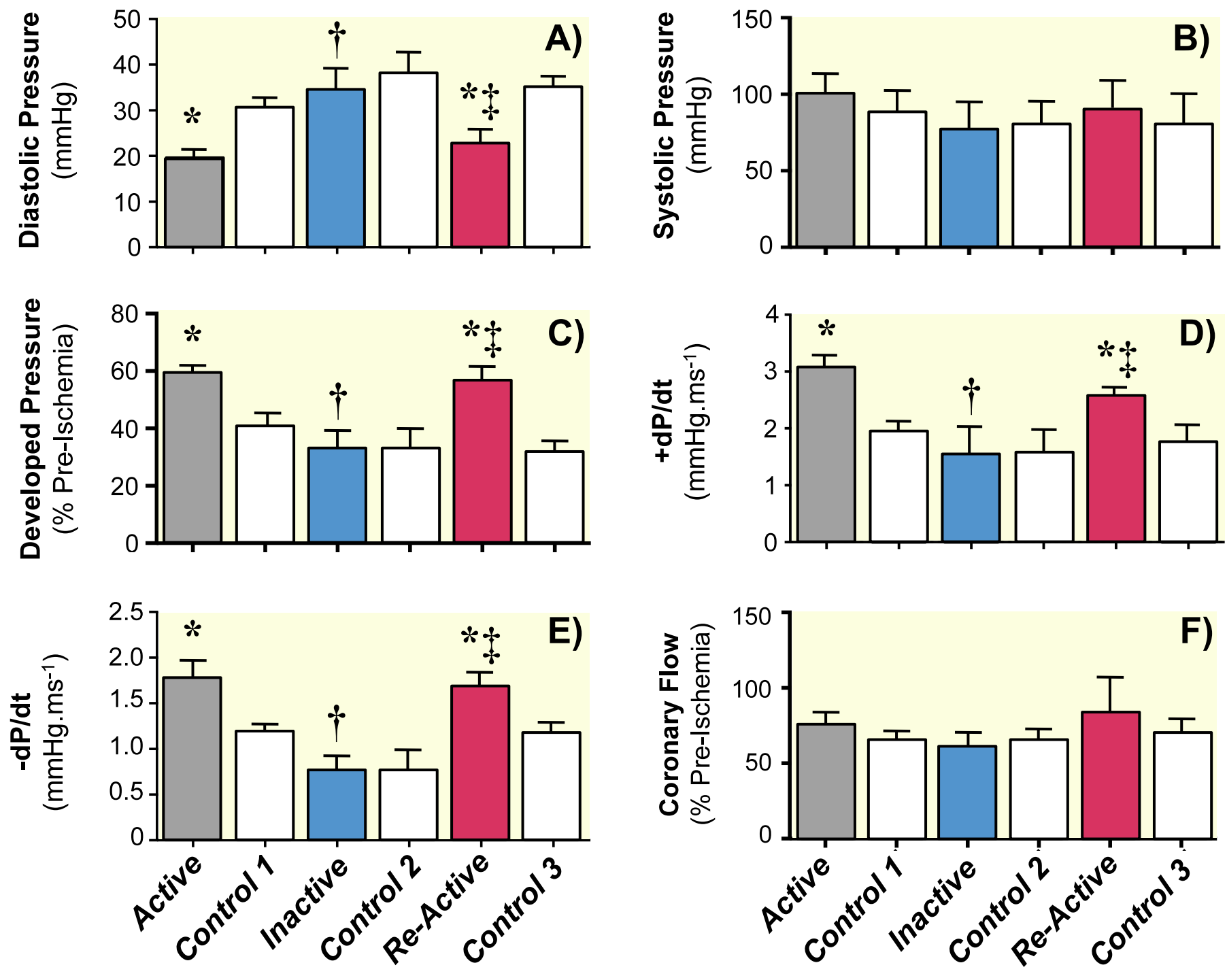


B)



C)




A)
Diastolic Pressure
(mmHg)
50
40
30
20
10
0
B)
Systolic Pressure
(mmHg)
150
100
50
0
C)
Developed Pressure
(% Pre-Ischemia)
80
60
40
20
0
D)
+dP/dt
(mmHg.ms-1)
4
3
2
1
0
E)
-dP/dt
(mmHg.ms-1)
2.5
2.0
1.5
1.0
0.5
0
F)
Coronary Flow
(% Pre-Ischemia)
150
100
50
0
Active
Control 1
Inactive
Control 2
Re-Active
Control 3