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Acute exercise activates p38 MAPK and increases the expression of telomere protective genes in cardiac muscle

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Abstract

Age is the greatest risk factor for cardiovascular disease. Telomere length is shorter in the hearts of aged mice compared to young mice, and short telomere length has been associated with an increased risk of cardiovascular disease. One year of voluntary wheel running exercise attenuates the age-associated loss of telomere length and results in altered gene expression of telomere length maintaining and genome stabilizing proteins in heart tissue of mice. Understanding the early adaptive response of the heart to an endurance exercise bout is paramount to understanding the impact of endurance exercise on heart tissue and cells. To this end we studied mice before (BL), immediately post (TP1) and one-hour following (TP2) a treadmill running bout. We measured the changes in expression of telomere related genes (shelterin components), DNA damage sensing (*p53, Chk2*) and DNA repair genes (*Ku70, Ku80*), and MAPK signaling. TP1 animals had increased TRF1 and TRF2 protein and mRNA levels, greater expression of DNA repair and response genes (*Chk2 and Ku80*), and greater protein content of phosphorylated p38 MAPK compared to both BL and TP2 animals. These data provide insights into how physiological stressors remodel the heart tissue and how an early adaptive response mediated by exercise may be

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None.

maintaining telomere length/stabilizing the heart genome through the up-regulation of telomere protective genes.

Introduction

Age is the number one risk factor for both males and females in the development of cardiovascular diseases (Dominguez *et al.*, 2006). Prevention of cardiovascular diseases (CVD) is associated with lifestyle choices such as cessation of smoking, reduction in body mass, and maintaining physical activity (Dominguez *et al.*, 2006). The long-recognized positive benefits or 'cardioprotective' effects of exercise on reduction of CVD risk include reduced resting blood pressure, lowered basal heart rate, reduced cholesterol levels and improved vascular health (e.g., reduced atherosclerosis) (Neufer *et al.*, 2015). However, these improvements following regular endurance exercise training do not fully explain the cardioprotective effects of exercise (Neufer *et al.*, 2015) and questions about the biological underpinnings of the effects of exercise remain unanswered.

Certain cell types in the adult heart can regenerate and are proliferative (van Berlo & Molkentin, 2014; Bergmann *et al.*, 2015). Neonatal mouse hearts can fully regenerate after partial surgical resection or induced myocardial infarction (Porrello *et al.*, 2011). However, the ability of the heart to regenerate in this fashion is rapidly lost in the first week of post-natal life and is concurrent with massive changes in gene expression of the heart tissue (Porrello *et al.*, 2011). Further, the regenerative capacity of the heart is reduced with age (Bergmann *et al.*, 2015) and the mutational load of the heart cells increases with age as well, leading to genome instability and tissue dysfunction (Dolle *et al.*, 2000). Thus, understanding how age and aging per say influence the heart and finding means to slow, prevent, or reverse age-related changes in the heart tissue is paramount.

A hallmark of molecular aging is the progressive shortening of telomeres with advancing age (Lopez-Otin *et al.*, 2013; Bar *et al.*, 2014). Telomeres are repetitive DNA elements (5'-$TTAGGG_n$) at the ends of linear chromosomes that when sufficiently long prevent DNA ends from being recognized as DNA double strand breaks (Shay & Wright, 2010). Telomeres shorten with each cell division until the protective effect of telomere DNA is diminished, thus telomere length limits the regenerative capacity of a cell/tissue (Bodnar *et al.*, 1998). Short telomere length is associated with many age-related diseases and is an independent risk factor for mortality (Ludlow & Roth, 2011; Blackburn *et al.*, 2015). Short telomeres in human and mouse tissues and cells are also associated with increased genome instability and tissue dysfunction (O'Sullivan & Karlseder, 2010). Importantly, several lines of evidence indicate that human telomeres shorten at nearly equal rates across tissues with aging, even in low turnover tissues such as the heart (Daniali *et al.*, 2013). A recently identified additional function of telomere length in cells is that of telomere length dependent chromosome looping that regulates gene expression or telomere position effect over long distances (Robin *et al.*, 2014). Telomeres can also shorten due to unrepaired DNA damage at the telomeres caused by oxidative stress or other genotoxic insults (Ludlow *et al.*, 2014). Thus, understanding how exercise can maintain telomere length in cardiac tissue is

important from several vantage points including genome stability and gene expression regulation.

Telomere length is maintained in some cells by the reverse transcriptase enzyme telomerase that consists of two main components, a protein component, telomerase reverse transcriptase (TERT), and an RNA template, telomerase RNA component (TERC; (Shay & Wright, 2007)). Telomere length and genome stability are also regulated by a DNA binding complex found at the ends of chromosomes called shelterin (de Lange, 2010), which consists of six proteins that bind to telomere DNA and regulate telomerase at the telomeres (de Lange, 2010). Telomere repeat binding factors 1 and 2 (TRF1 and TRF2) bind to double stranded telomere DNA and regulate telomere length, while protection of telomeres 1 (POT1) regulates telomerase action at the telomere. Additionally, DNA damage response proteins that transiently associate with telomeres (KU70/80, CHK2 and p53) can aid in genome stability and modify the telomere end structure.

Previously, we showed that long-term (44 weeks) voluntary wheel running in rodents maintained telomeres at a similar length compared to young animals and were significantly longer compared to age-match sedentary animals in heart tissue (Ludlow *et al.*, 2012b). In a follow up study we showed that treadmill running induces an early adaptive response of the telomere length maintenance system in the highly activated skeletal muscles (plantaris) but not the lesser activated muscles (tibialis anterior) driven in part by activation of p38 MAPK (Ludlow *et al.*, 2012a). Whether a similar phenomenon of upregulation of genes related to maintenance of telomeres occurs in the cardiac tissue following an acute bout of endurance type exercise is currently unknown.

To begin to understand the early adaptive response of endurance type exercise on the telomere maintenance system in rodent cardiac tissue we determined the effects of an acute bout of treadmill running on gene expression and protein content of telomere-related proteins in mouse cardiac muscle. Further, we investigated MAPK (p38, ERK, and JNK) activation as potential pathways through which exercise may result in telomere length maintenance in cardiac tissue. We hypothesized that acute exercise would increase the expression of telomere length-maintaining proteins in parallel with increased activation of MAPKs in mouse myocardium.

Methods

Ethical Approval

The procedures used in the present study were approved by the University of Maryland Institutional Animal Care and Use Committee and conformed to the National Institutes of Health's Guide for the Use and Care of Laboratory Animals (NIH Pub. No. 85-23, revised, 1996). The authors of this study understand the ethical principles of the journal and we made every attempt to ensure that this work complies with the animal ethics checklist. C57Bl/6J mice were assigned to a baseline (BL) group that was not exposed to exercise or to one of two groups exposed to an acute treadmill running bout and sacrificed immediately (TP1) or one hour (TP2) following the exercise. Animals were kept on an *ad libitum* diet and on a standard 12 hours light : 12 hours dark schedule. Animals were first anesthetized by

isoflurane and euthanized by cardiac excision. Cardiac gene expression of telomere-related proteins (*Trf1, Trf2, Pot1a, Pot1b, Tert, Ku70, Ku80, p53 and Chk2*) was analyzed via RT-PCR and TRF1, TRF2 and p38 MAPK protein content was analyzed via immunoblotting.

Animals

Twenty-two female C57Bl/6J mice were purchased from Jackson Laboratory (Bar Harbor, Maine) and housed at 25°C on a 12 hour light-dark cycle with access to standard laboratory mouse chow (Prolab RMH 3000, 5P00, LabDiet by Purina, Nestlé S.A., Vevey, Switzerland) and water *ad libitum*. We choose to use only female C57Bl/6J mice because they have a greater propensity to run compared to male mice (Lightfoot *et al.*, 2004; Turner *et al.*, 2005). Mice from the two exercise groups were acclimated to the treadmill for two weeks before the tests were performed, beginning at 6 weeks of age. The acclimation period was comprised of 7 progressive exercise sessions, the first involving a 5 min running bout at 2 m/min, no shock and the last one involving a 10 min running bout at 15–20 m/min, moderate shock (details shown in Table 2). All mice were sacrificed at 8 weeks of age to limit the amount of influence age could have on telomere-related variables. Data on the skeletal muscles from these animals has been published (Ludlow *et al.*, 2012a).

Incremental treadmill exercise test

Forty-eight hours after the last acclimation session animals from the exercise groups were subjected to an incremental exercise test for assessment of their peak treadmill running speed. First, the mouse was placed for 2 min on the stationary treadmill belt. Thereafter, the treadmill speed was set at 6m/min and increased 3m/min every 2 minutes until refusal to run (animal seated on the shock pad for more than 30 seconds). The speed of the last stage completed was recorded as the peak treadmill running speed.

Acute treadmill exercise bout

Forty-eight hours after the incremental exercise test, the mice were exposed to 30 minutes of treadmill running at approximately 70% of their peak speed and were sacrificed immediately (TP1; n = 8) or 1h following (TP2; n = 8) the running bout. The duration and intensity of the treadmill exercise for the mice were chosen to mimic the current exercise recommendations of the American College of Sports medicine for maintenance of human health (Balady GJ, 2000). The treadmill was kept at 7° incline throughout the experiment. To encourage the animals to continue to run a shock grid at the back of the treadmill was used (detail shown in Table 1). All animals completed the 30-minute treadmill bout without stopping.

Cardiac muscle collection and preparation

At the specified time points, the animals were anesthetized (3% isoflurane) and euthanized by cardiac excision. The hearts were flash frozen and powdered with a mortar and pestle in liquid nitrogen prior to separation into four vials for RNA, DNA, protein, and telomerase enzyme activity. The samples were stored at −80°C until further analysis.

RNA extraction and reverse transcription–polymerase chain reaction

Total RNA was extracted from the frozen samples using TRIzol reagent (Invitrogen Technologies, San Diego, CA) on the basis of previously described techniques (Chomczynski & Sacchi, 1987). One μg of total RNA was reverse transcribed using the High Capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA). Gene expression analyses were performed for all target genes using 1μL of each reverse transcription reaction product and normalized to *18S* and Glycerol-aldehyde 3-dehydrogenase (*Gapdh*); however, expression of the target genes did not differ based on normalization to *Gapdh* or to *18S* (data not shown). Specific primer sequences are shown in Table 2. The PCR products were separated on a 2.0% agarose gel and visualized using ethidium bromide. Band intensities were analyzed by densitometry using Image J software (Rasband, 1997–2011).

Protein Expression

Protein was extracted using a lysis buffer (50 mM Hepes (pH 7.4), 0.1% Triton X-100, 4 mM EGTA, 10 mM EDTA, 15 mM $Na_4P_2O_7 \bullet H_2O$, 100 mM – glycerophosphate, and a protease inhibitor cocktail - complete mini EDTA-free tablet (Roche, Indianapolis, IN)) and protein content was determined using bicinchoninic acid protein assay (Pierce, Rockford, IL). Fifty micrograms of total protein was prepared and resolved on 10% (TRF1 and 2) or 7.5% (MAPKs) SDS-PAGE gels, transferred to PVDF membranes, and blocked in 5% non-fat dry milk for 30 min. Immunoblotting was performed for TRF1 (1:200, C-19, SC 1977, Santa Cruz Biotechnologies, Santa Cruz, CA), TRF2 (1:200, H-300, SC 9143, Santa Cruz Biotechnologies, Santa Cruz, CA), phosphorylated p38 MAPK (1:500, Cell Signaling 9211, Thr180/Tyr182, Danvers, MA), total p38 MAPK (1:500, Cell Signaling 9212, Danvers, MA), phosphorylated ERK1/2 (P44 ERK1/ p42 ERK2, Thr202/Tyr204, Cell Signaling 9101, 1:1000), total ERK1/2 (Cell Signaling 9102, 1:1000), phosphorylated SAPK/JNK1/2 (p46 JNK1, p54 JNK2, Thr183/Tyr185, Cell Signaling 9251, 1:1000), total SAPK/JNK1/2 (Cell Signaling 9251, 1:1000) and GAPDH (1:1000, Cell signaling, 14C10 Rabbit mAb # 2118, Danvers, MA). Products were visualized using species appropriate horseradish-peroxidase linked secondary antibodies and visualized on an enhanced chemiluminescence (ECL) imager (Syngene Bio Imaging, Fredrick, MD). Band intensities were analyzed by densitometry using Image J software (Rasband, 1997–2011). For protein content, GAPDH was used as a loading reference.

Telomerase

Telomerase enzyme activity was measured using a commercially available kit utilizing the telomere repeat amplification protocol (TRAP; Quantitative Telomerase Detection Kit; US Biomax, Rockville, MD). Protein concentration was determined (as above) and 1 μg of protein was added to the reaction according to the recommendations of the manufacturer and as previously performed in our lab (Ludlow *et al.*, 2008). In addition to the standards provided in the kit we assayed heat-treated samples as a negative control. Heat-treated samples were concluded to be telomerase negative if the mean of the critical threshold (Ct) for the heat-treated sample duplicates was three standard deviations above that of the telomerase positive sample (this criteria was also used to determine a telomerase positive

sample). We also assayed a human cancer cell line known to be telomerase positive (HeLa, ATCC, CCL-2, Manassas, VA) to ensure sensitivity of the assay. Due to sample processing and limitations in the assay (heat treated negative control was not three standard deviations different from lysate), we had to exclude two animals from the post exercise groups. This exclusion reduced the power of our analysis and may have hindered the detection of biologically relevant and statistically significant differences.

Statistical analysis

All values are presented as means ± standard deviation of the mean. One-way analysis of variance (ANOVA) with Tukey's honest significant difference was performed for all analyses. Statistical significance was accepted at $p \leq 0.05$.

Results

Body mass, peak and submaximal treadmill running speeds attained for each group are presented in Table 3.

Cardiac shelterin gene expression and protein content increase in response to acute exercise

Acute treadmill running resulted in a significant up-regulation of cardiac gene expression of shelterin components (Figure 1). TP1 animals showed increases in *Trf1* (+61%, p = 0.01) and *Trf2* (+47%, p = 0.04) mRNA while TP2 animals showed increases in *Pot1a* mRNA expression (+35%, p = 0.04) when compared to BL. There were no differences at any time point for *Pot1b* gene expression (Figure 1).

Acute treadmill running induced an increase in protein content of shelterin components at TP1. TRF1 protein content was greater in TP1 animals compared to TP2 animals (+58%, p = 0.002). TRF2 protein content was greater in TP1 animals compared to both BL (+97%, p = 0.05) and TP2 animals (+95%, p = 0.03; Figure 2a and b).

Telomerase enzyme activity and *mTert* gene expression following acute exercise

m*Tert* gene expression regulates telomerase activity (Armstrong *et al.*, 2000) and we hypothesized that an increase in *mTert* gene expression would correlate with an increase in telomerase enzyme activity. ANOVA analysis of m*Tert* mRNA expression did not detect an effect (p = 0.1, Figure 3b.). Telomerase enzyme activity was not different in the ANOVA analysis (p = 0.2, Figure 3a.).

DNA damage repair and response gene expression in cardiac muscle increases following acute exercise

Previous literature has implicated the accumulation of DNA damage and p53 signaling in the aging heart, therefore we tested the influence of acute exercise on DNA damage and repair and DNA damage signaling genes. The expression of DNA damage repair protein *Ku70* was not different (p = 0.08, Figure 4a), while *Ku80* gene expression was significantly increased in TP1 and TP2 animals compared to BL (p = 0.01; and p = 0.02, Figure 4b, respectively). Expression of the mRNA of DNA damage response protein *p53* was not different between

groups (p = 0.17, Figure 4c), but *Chk2* mRNA expression was significantly increased at TP1 compared to BL animals (p = 0.03, Figure 4d).

p38 MAPK phosphorylation is altered in parallel with changes in gene expression

To test if mitogen-activated protein kinases were activated during acute exercise in parallel with alterations in shelterin components we measured the phosphorylation status of three MAPKs: p38 MAPK, ERK1/2 and JNK1/2. ERK1 (p44) phosphorylation levels were significantly reduced immediately following exercise (p = 0.02, Figure 5) and were similar to BL at TP2, while ERK2 (p42) phosphorylation levels were not significantly different at any time point (p = 0.16). p38 MAPK phosphorylation levels were greater in TP1 animals compared to BL animals (p = 0.04), and tended to be greater in TP2 compared to BL animals (p = 0.10), but were not different between TP1 and TP2 (p = 0.63, Figure 5). Phosphorylated levels of JNK1 (p46) were not different at any time point (p = 0.59), but levels of phosphorylated JNK2 (p54) were significantly reduced at TP2 following exercise compared to both BL and TP1 (p = 0.001).

Discussion

Our laboratory has previously determined that chronic voluntary wheel running exercise in mice resulted in telomere length maintenance and increased expression of shelterin and DNA damage response and repair genes in cardiac tissue (Ludlow *et al.*, 2012a; Ludlow *et al.*, 2012b). The purpose of the current study was to begin to elucidate the acute effects of exercise on cardiac tissue gene expression of shelterin proteins and DNA damage response and repair genes. We describe for the first time in C57Bl/6J mice that an acute bout of treadmill exercise results in upregulation of cardiac telomere length maintaining proteins that occurs in parallel with activation of the p38 MAPK signaling pathway. These data provide important insights into a potential pathway mediating telomere length maintenance and genome stability in response to chronic exercise in cardiac tissue. We also show that key DNA damage response and repair proteins are increased following acute exercise. Together, these results support a ‘telomere length-protective’ effect of exercise in cardiac tissue and indicate that the initial adaptation may be associated with altered p38 MAPK signaling (Spallarossa *et al.*, 2009). Maintenance of longer telomeres in cardiac tissues/cell types may result in a ‘youthful’ gene expression program and longer healthy heart function with chronological aging (Robin *et al.*, 2014; Robin *et al.*, 2015). We hypothesize that the chronic effects of exercise on telomere length may be the cumulative effect of each bout and that understanding the pathways associated with the adaptive response to exercise may lead to improved therapeutics/exercise prescription for the prevention of cardiovascular disease.

The regenerative capacity of the heart cell types, particularly the cardiomyocyte, is limited (van Berlo & Molkentin, 2014); however, recent reports indicate that rare neonatal-like adult cardiomyocytes can divide (Porrello *et al.*, 2011; Canseco *et al.*, 2015; Kimura *et al.*, 2015). DNA damage signaling (p53, p21 and p16) and mitochondrial dysfunction occurs with aging in the heart and leads to pathological conditions such as heart failure and myocardial infarcts, indicating that the turnover of heart cells is not sufficient to replace cells under physiological wear and tear (Keller & Howlett, 2016; Narasimhan & Rajasekaran, 2016).

Further, it has been shown that telomere length in the adult human heart and in mouse heart tissue does shorten with aging, suggesting that some level of cellular turnover and/or telomeric DNA damage is occurring with age (Werner *et al.*, 2008; Werner *et al.*, 2009; Wong *et al.*, 2010; Ludlow *et al.*, 2012b). Exercise training is associated with substantial cardiovascular health benefits and we recently showed that long-term wheel running in mice maintained telomere length and increased expression of telomere length maintaining proteins in heart tissue of active animals compared to controls (Ludlow *et al.*, 2012b).

Previous research has indicated that exercise training is associated with increased gene expression of shelterin in cardiac tissue (Werner *et al.*, 2008; Werner *et al.*, 2009; Ludlow *et al.*, 2012b). In our previous chronic exercise study, we observed statistically significant decreases in *Trf1, Trf2, Pot1a* and *Pot1b* mRNA levels in hearts of aged mice that were attenuated by exercise training (Ludlow *et al.*, 2012b). TRF1 and TRF2 are double-stranded telomere binding proteins that regulate telomere length (de Lange, 2010). POT1a and POT1b interact with the single-stranded G-rich overhang of telomeres and interact with another shelterin protein TPP1, that together recruit telomerase to telomeres and control telomere repeat processivity (the ability of telomerase to successively add telomere repeats to chromosome ends) (Nandakumar *et al.*, 2012; Nandakumar & Cech, 2013; Schmidt & Cech, 2015). Maintenance of shelterin component expression levels is critical to maintaining telomere length homeostasis and to prevent DNA damage signaling at the telomere. Here, consistent with our previous data that shelterin adapts to exercise training, we show that a single bout of exercise is able to increase the expression of TRF1, TRF2 (both protein and mRNA), and *Pot1a* (mRNA), but not *Pot1b*. These data indicate that exercise stress related signaling results in an up regulation of shelterin components in cardiac muscle and that with training (cumulative effect of each individual exercise bout) mRNA levels may be maintained over time. The immediate response of TRF1 and TRF2 protein level increase is likely due to an increase in protein stability or a reduction in protein degradation, while the increase in mRNA levels are an adaptive response caused by early adaptive signaling events. Briefly, studies of master's athletes have shown increased TRF2 levels (protein and mRNA) compared to age-matched healthy, sedentary individuals (Werner *et al.*, 2009). A study of ultra-endurance athletes' white blood cells (WBCs) 24 hours following seven marathons in seven days showed an increase in *TRF1* and *TRF2* mRNA levels compared to baseline (Laye *et al.*, 2011). To the contrary, an acute endurance exercise bout (30 min cycling, 80% max HR) was associated with reduced *TRF2* mRNA levels in human WBCs 60 min after exercise (Chilton *et al.*, 2014). The authors also measured miRNAs and found several upregulated miRNAs that could target *TRF2*, indicating the exercise induced regulation of *TRF2* could be very tightly controlled and depend on the timing of measurement and intensity and duration of the exercise stimulus. *Pot1a* mRNA levels increased one-hour following exercise. POT1a in rodents suppresses the ATR-dependent DNA damage response at telomeres (Palm *et al.*, 2009). These findings also extend our previous results that chronic voluntary wheel running resulted in increased *Pot1a* gene expression in hearts of 1-year old animals compared to sedentary age-matched animals (Ludlow *et al.*, 2012b). Laye et al. (Laye *et al.*, 2011) also showed an increase in *POT1* levels compared to baseline levels in WBCs following the ultra-marathon event described above. Thus, the upregulation of shelterin with acute exercise may accumulate over time (i.e., a training effect or repeated bout effect)

resulting in increased shelterin and aiding in the telomere length maintenance phenotype observed in chronically trained animals.

The major telomere length maintenance enzyme complex, telomerase, has also been shown to be upregulated following short term (three weeks) and long-term (six months) exercise training in rodent heart tissue (Werner *et al.*, 2008; Werner *et al.*, 2009). We observed a slight but not statistically significant increase in telomerase activity and in m*Tert* (mouse telomerase reverse transcriptase) gene expression following exercise. Our data indicate that a single acute bout of exercise does not significantly increase telomerase activity levels and that the effect of exercise on telomerase may be a repeat bout effect, with telomerase adapting after several bouts of exercise. Several human studies to date have observed mixed results concerning the acute effects of exercise on *hTERT* gene expression. In a study of human WBCs, *hTERT* mRNA levels increased 60 minutes following a bout of cycling compared to resting levels (Chilton *et al.*, 2014). While in the ultra-marathon study mentioned above, no change in *hTERT* mRNA levels was observed in WBCs (Laye *et al.*, 2011). Most recently, a study of WBCs in young healthy individuals following a 30 min treadmill run showed an increase in telomerase activity compared to the rested state (Zietzer *et al.*, 2016). These mixed results point to the need for more research on exercise and telomerase. Several considerations about how exercise could be influencing telomerase and *hTERT* should be made as follows. In order for telomerase to maintain telomeres two different steps must occur, first there must be active enzyme (i.e., *TERT* must be expressed) and second the active enzyme must be recruited to the telomeres. Thus, an increase in either process could initiate telomere length maintenance. These data could suggest that the initial adaptation to exercise may be to increase telomerase recruitment to telomeres and the training adaptation is to increase telomerase enzyme activity as observed in previous studies (Werner *et al.*, 2008; Werner *et al.*, 2009). However, further research into the activation, assembly, and recruitment of telomerase (i.e., TPP1 levels) and m*Tert/hTERT* gene expression regulation as well as the cell cycle/division kinetics and types of cells expressing telomerase following exercise on telomere length maintenance in cardiac tissue of rodents and humans is warranted.

Since longer telomeres are associated with increased genome stability and previously we have shown an upregulation of DNA damage response and repair genes in cardiac tissue of trained animals, we investigated the effects of acute exercise on DNA damage response and repair genes. We observed that *Ku80* and *Chk2* gene expression increased immediately following exercise and returned to baseline levels by one-hour post exercise. Ku80 is a heterodimer protein that has been shown to transiently associate with telomeres and is involved in the DNA damage response (Lopez *et al.*, 2011; Pfingsten *et al.*, 2012). Further, (Laye *et al.*, 2011) observed increased gene expression of *Ku70/Ku80* in both immune cells and skeletal muscle in humans after running 7 marathons in 7 days. Moreover, we have previously observed greater *Ku70/Ku80* gene expression in cardiac tissue of one-year old mice that voluntarily ran for 44 weeks compared to sedentary aged-matched animals (Ludlow *et al.*, 2012b). Combined, these data indicate that exercise training and acute exercise in both trained and untrained individuals increases gene expression related to enhanced DNA damage response and repair. We hypothesize that improved DNA damage response and repair, in concert with increased shelterin expression and telomerase activity,

would result in telomere length maintenance and an anti-apoptotic and anti-senescent cellular environment in exercise-trained compared to sedentary individuals.

To address possible signaling mechanisms, we measured a common stress response pathway in relation to the observed changes in gene expression. We measured activation of p38MAPK, ERK1/2 and JNK1/2, which are known to be a part of cardiac growth pathways (Rose *et al.*, 2010). We observed a significant upregulation in p38MAPK phosphorylation following exercise in conjunction with gene expression changes, while we observed decreased activation (phosphorylation) of ERK1/2 and JNK1/2. We recently reported that activation of p38 MAPK in skeletal muscle was directly related to altered levels of shelterin components (Ludlow *et al.*, 2012a). Similar to our skeletal muscle data and other reports in cardiac muscle we observed significant p38 MAPK phosphorylation following an acute bout of exercise. Activation of these three MAPKs has been associated with cardiac disease and pathophysiological hypertrophy (Asrih *et al.*, 2013). Further, MAPKs have been associated with exercise and aging responses, as well as expression of telomere binding proteins in cardiac tissue (Iwasa *et al.*, 2003; Iemitsu *et al.*, 2006; Collado *et al.*, 2007; Spallarossa *et al.*, 2009). In a model of artificially selected rats bred for low or high ability to run, an acute treadmill running bout activated all three MAPKs (Hunter *et al.*, 2008). These discrepant results may be due to the exercise stress or the rodent model used; however, both interventions resulted in significant activation of p38MAPK. Activation of p38 MAPK results in changes in activation and localization (i.e., cytoplasmic versus nuclear) of a variety transcription factors (Rose *et al.*, 2010) that are likely related to the altered gene expression of shelterin components. While only descriptive in nature in the current experiment, we and others have previously shown links between p38MAPK and shelterin gene expression (Spallarossa *et al.*, 2009; Ludlow *et al.*, 2012a). The response of p38 MAPK is likely due to transient increases in calcium levels, oxidative stress and local concentration of trophic factors (growth hormone and IGF-1) in the cardiac tissue and cells (Rose *et al.*, 2010). While activation of the MAPKs has been implicated as being related to pathophysiological cardiac remodeling of the heart, we propose that activation of p38 MAPK alone may trigger an early adaptive response to increase genome stabilizing gene expression. Future experiments investigating the exercise associated activation of p38 MAPK, which isoform is specifically activated, and the regulatory roles that the p38MAPK stress response pathway plays in cardiac telomere length maintenance related gene expression are necessary.

This study is not without limitations that must be considered for appropriate interpretation of the presented data. We did not separate out the different p38 MAPK isoforms to determine which specific isoform is responsible to the observed changes in shelterin expression following acute exercise. To help prevent acute bout effects from carrying over from the acclimation or testing period, animals were placed in their normal cages for 48hrs prior to the acute exercise bout and sacrifice, which should minimize the effects of the acclimation and testing, but these effects cannot be ruled out. Additionally, we were limited by sample availability and assay sensitivity (qPCR based TRAP assay), thus while we were close to significance in several instances, we failed to detect differences due to these limitations. Future studies with greater sample sizes and more robust measures of telomerase activity may detect significant differences. Further, translation of these data from the rodent model to humans should be done with caution, as laboratory mice have significantly longer telomeres

compared to human telomeres. That being said the regulation of shelterin *in vivo* in humans and mice appears to be similar, thus providing evidence for the translational value of shelterin and telomere data obtained in mice (Werner *et al.*, 2008; Werner *et al.*, 2009; Laye *et al.*, 2011). Again, we emphasize that this is the first report describing a novel gene expression phenotype and potential association between shelterin and p38 MAPK in cardiac tissue. We did not measure telomere length, as we would not anticipate such a short-term exercise stimulus to alter telomere length in a meaningful or detectable fashion.

Understanding how telomere length is regulated in cardiac tissue with exercise training and how each bout of exercise results in a cardioprotective phenotype is important research for improving the healthspan of humans. Telomere length is one aspect of aging tissues that regulates genomic stability and, as elucidated recently, chromatin structure and gene expression. How exercise results in longer telomeres and perhaps influences gene expression with aging in cardiac tissue and how the altered gene expression effects specific cell types and the increase in fibrotic cells in the heart with aging makes these findings and future research particularly important to human cardiovascular health. Ultimately, understanding the positive benefits of exercise training on these phenotypes may lead to novel therapies in preventative and personalized medicine.

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New Findings

A positive association between telomere length and exercise training has been shown in cardiac tissue of mice. It is currently unknown how each bout of exercise influences telomere length regulating proteins. We sought to determine how a bout of exercise altered the expression of telomere length regulating genes and a related signaling pathway in cardiac tissue of mice.

Acute exercise altered the expression of telomere length regulating genes in cardiac tissue and may be related to altered MAPK signaling. These findings are important in understanding how exercise provides a cardio-protective phenotype with aging.

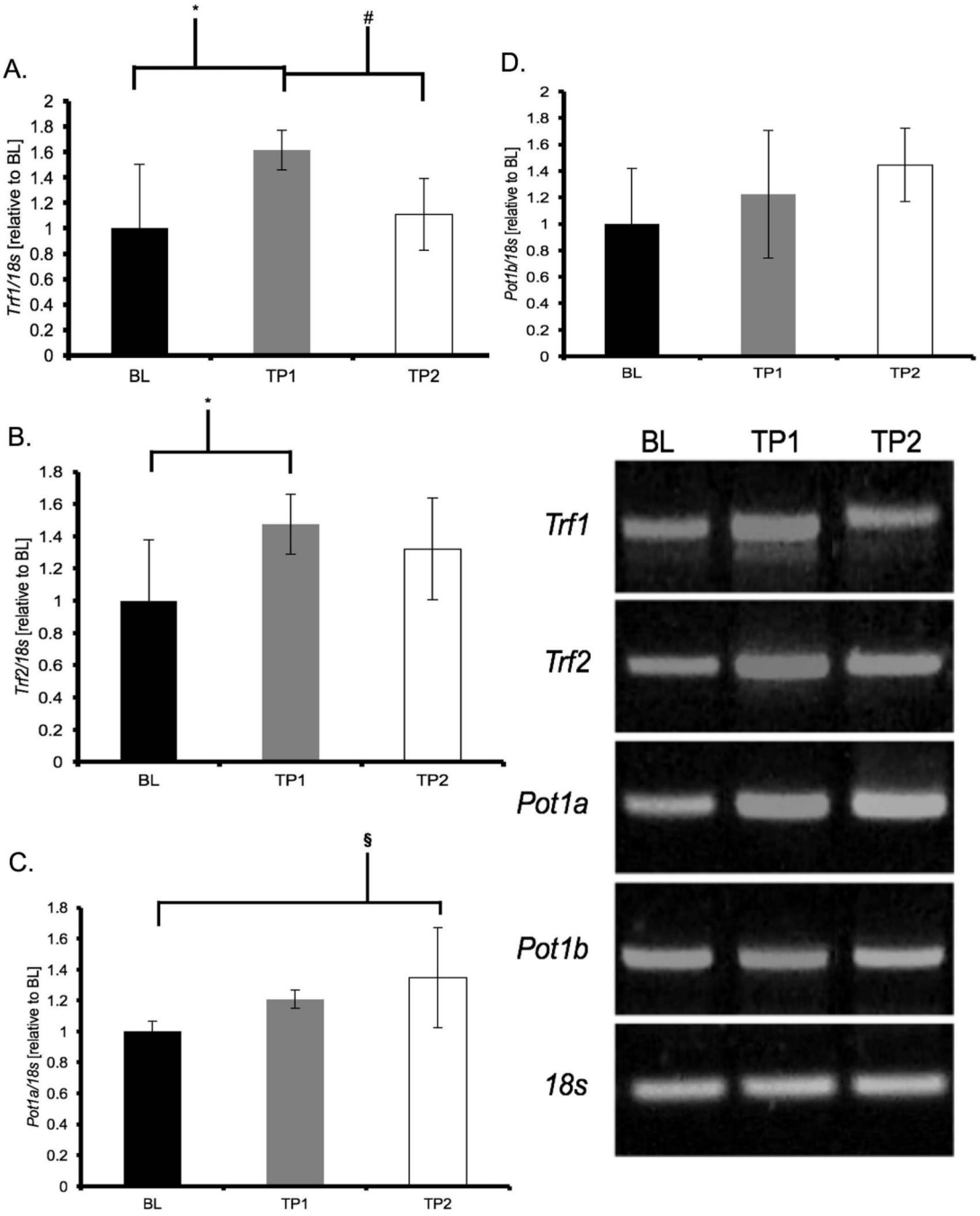

Figure 1. Acute treadmill running increased shelterin gene expression in cardiac muscle

mRNA abundance was assessed by RT-PCR and target genes were normalized to *18S* and expressed relative to BL. Results of densitometric analysis are shown. Data are presented as means ± S.D. BL: baseline (n = 6); TP1: immediately after the exercise bout (n = 8); TP2: one-hour after the exercise bout (n = 8). * TP1 significantly different than BL ($p < 0.05$). # TP1 significantly different than TP2 ($p < 0.05$). § TP2 significantly different than BL ($p < 0.05$).

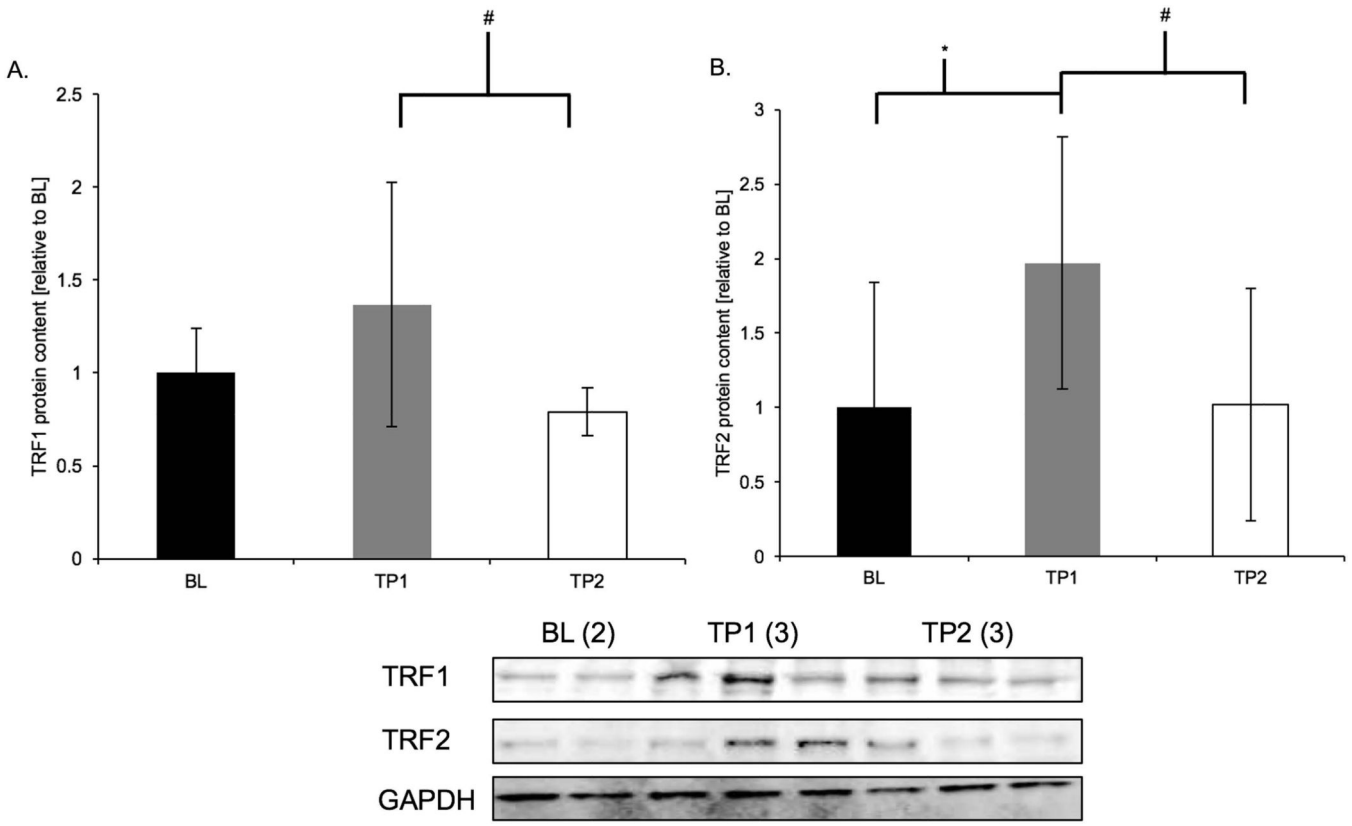

Figure 2. TRF1 and TRF2 protein content were increased following acute treadmill running

Results of densitometric analysis and representative immunoblots of TRF1, TRF2, and GAPDH are shown; GADPH was a loading reference. Data are presented as means ± S.D. BL: baseline (n = 6); TP1: immediately after the exercise bout (n = 8); TP2: one-hour after the exercise bout (n = 8). GAPDH: glyceraldehyde 3-phosphate dehydrogenase. * TP1 significantly different than BL ($p < 0.05$). # TP1 significantly different than TP2.

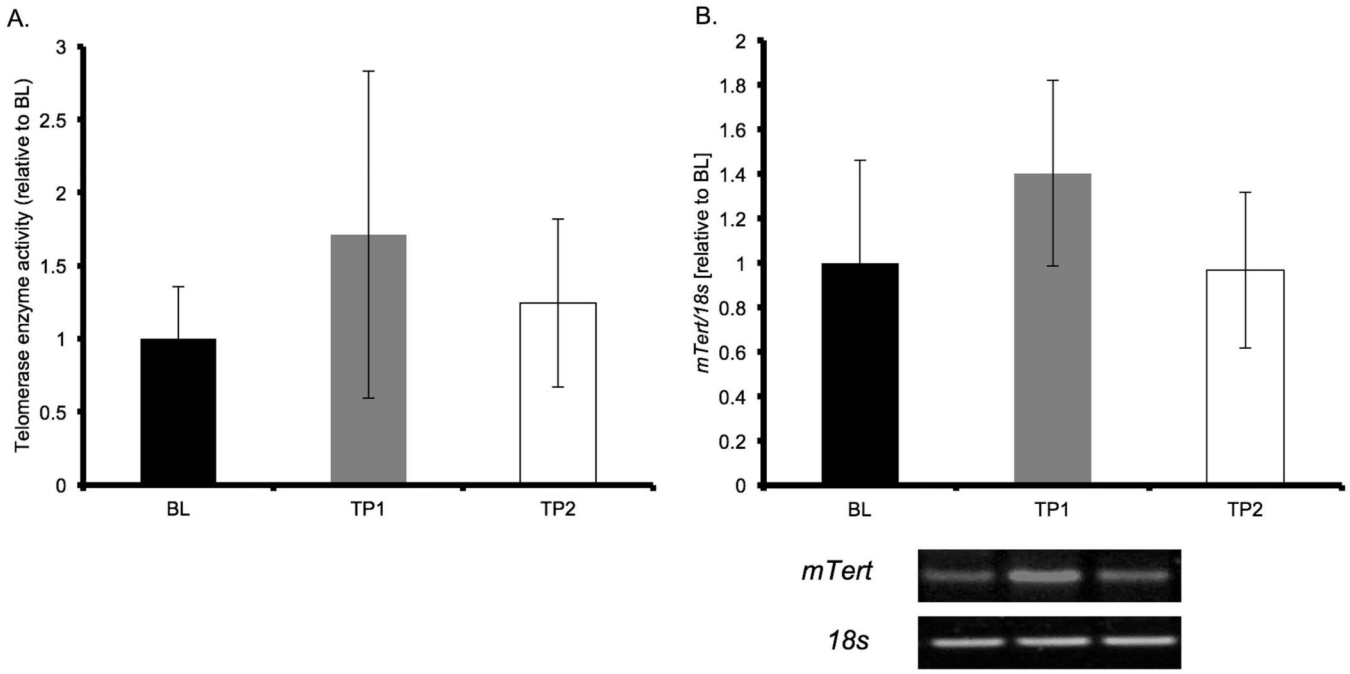

Figure 3. Activity and expression of telomerase increased after acute treadmill running

Telomerase enzyme activity (left) and *mTert* gene expression (right) are shown. mRNA abundance for *mTert* was assessed with RT-PCR, normalized to *18S* and expressed relative to BL. Results of densitometric analysis are shown. Data are presented as means ± S.D. BL: baseline (n = 4); TP1: immediately after the exercise bout (n = 6); TP2: one-hour after the exercise bout (n = 6). *mTert* = mouse telomerase reverse transcriptase.

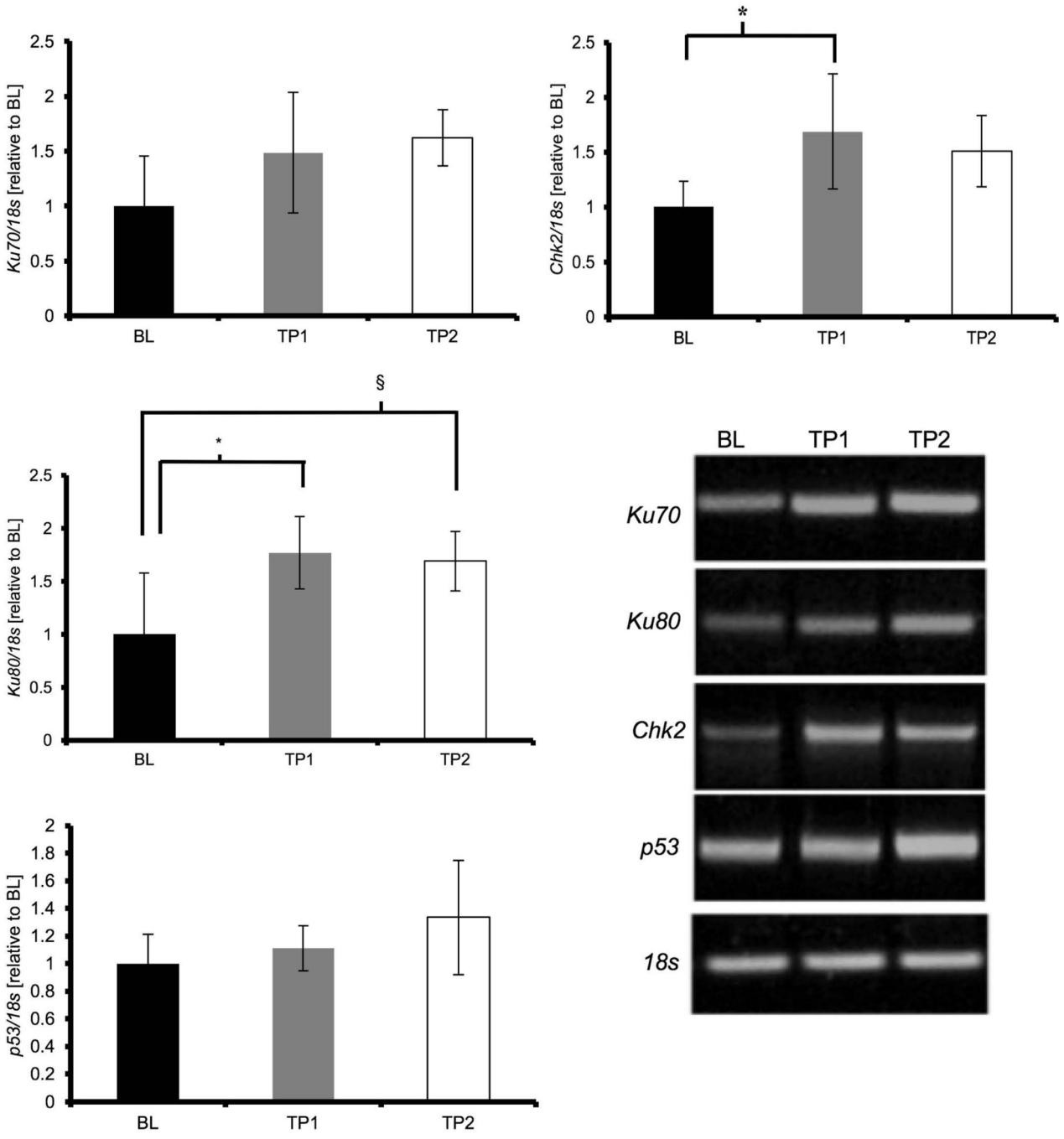

Figure 4. Acute exercise increases gene expression of DNA damage repair and response factors

mRNA abundance was assessed by RT-PCR and target genes were normalized to *18S* and expressed relative to BL. Results of densitometric analysis are shown. Data are presented as means ± S.D. BL: baseline (n = 6); TP1: immediately after the exercise bout (n = 8); TP2: one-hour after the exercise bout (n = 8). * TP1 significantly different than BL ($p < 0.05$). § TP2 significantly different than BL ($p < 0.05$).

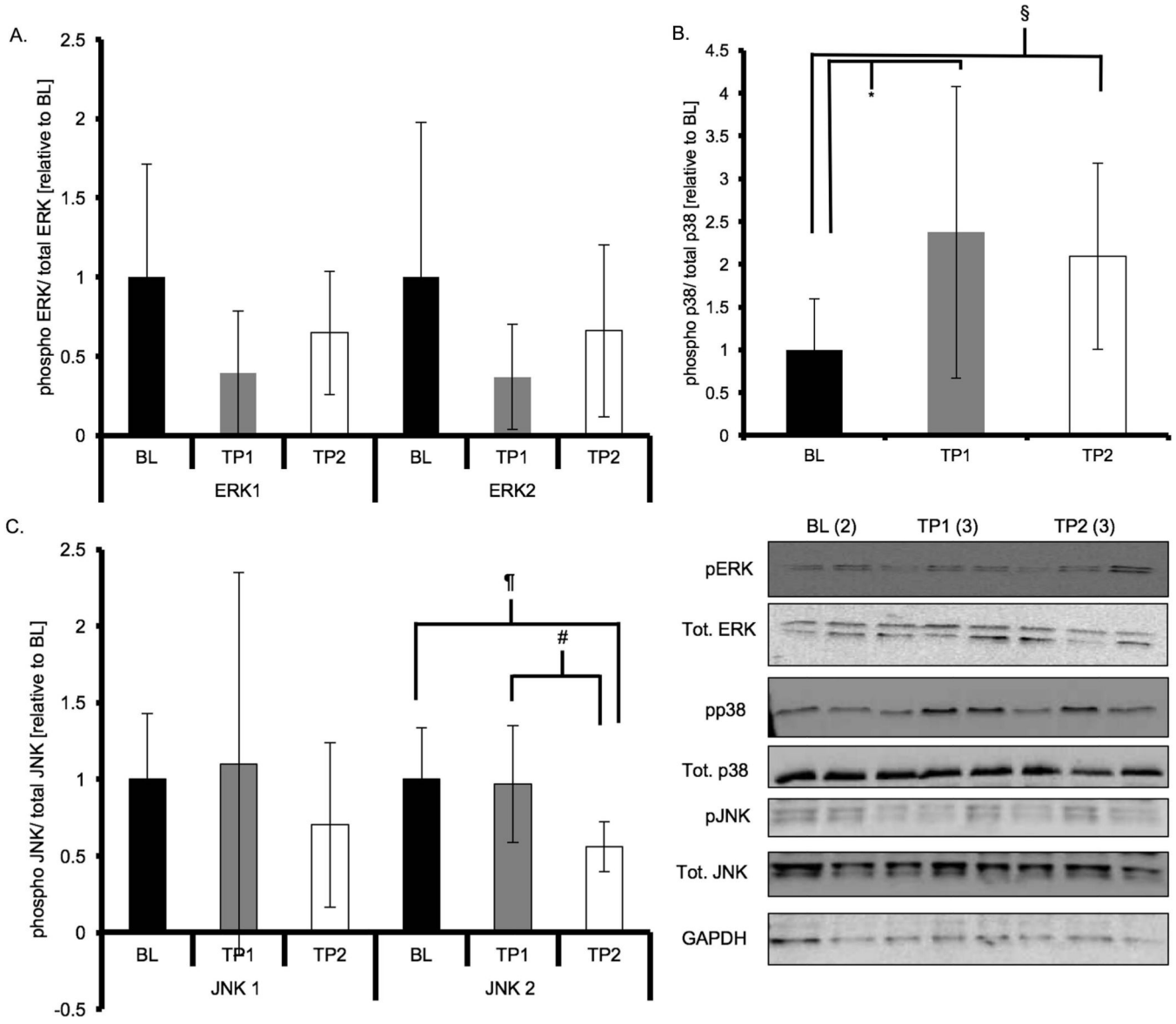

Figure 5. p38 MAPK phosphorylation in cardiac muscle is increased following acute treadmill running

Results of densitometric analysis and representative immunoblots of MAPKs (total and phosphorylated) and GAPDH are shown; GADPH was a loading reference. Phosphorylated to total protein ratios were derived and expressed relative to BL. Data are presented as means ± S.D. BL: baseline; TP1: immediately after the exercise bout; TP2: one-hour after the exercise bout. * TP1 significantly different than BL ($p < 0.05$). # TP1 significantly different than TP2 ($p < 0.05$). § TP2 tended to be different than BL ($p < 0.1$).

Table 1

Treadmill acclimation procedures.

Session	Time (min)	Belt speed (m*min^{-1})	Shock pad	Days
1	0 – 5	0	No shock	1–2
	5 –15	2	No shock	
2	0 – 2	0	No shock	4–5
	2 – 5	2	No shock	
	5 – 10	5	Light	
	10 – 15	10	Light	
3	0 – 2	0	No shock	7
	2 – 5	5	Light	
	5 – 10	15	Light	
	10 – 15	20	Moderate	
4	0 – 2	0	No shock	8; 10
	2 – 5	5	Light	
	5 – 10	15	Moderate	
	10 – 15	20 – 24	High	

Light shock (1.02 mA; 2.7 pulses*sec^{-1}); Moderate shock (2.04 mA; 2.7 pulses*sec^{-1}); High shock (3.06 mA; 2.7 pulses*sec^{-1}). Intensity in milliamperes (0.34 to 3.4mA) and repetition rate at 200msec pulses at a rate of 0.3 to 3 pulses per second. m*min^{-1} = meters per minute.

Table 2

Primer sequences for gene expression analysis.

Gene name	Primer sequence
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) F	5'- GTG TCC GTC GTG GAT CTG 3’
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) R	5'- CCT GCT TCA CCA CCT TCT TG 3’
18S F	5' GAT CCA TTG GAG GGC AAG TCT 3'
18S R	5' CCA AGA TCC AAC TAC GAG CTT TTT 3'
Telomere repeat binding factor 1 (TRF1) F	5'-CAT GGA CTA CAC AGA CTT AC-3'
Telomere repeat binding factor 1 (TRF1) R	5'-ATC TGG CCT ATC CTT AGA CG-3'
Telomere repeat binding factor 2 (TRF2) F	5'-TGT CTG TCG CGC ATT GAA GA-3'
Telomere repeat binding factor 2 (TRF2) R	5'-GCT GGA AGA CCT CAA TAG GAA-3'
Protection of telomeres 1a (POT1a) F	5'-CCC TGA ATC TAC TCA AGG AAG-3'
Protection of telomeres 1a (POT1a) R	5'-GAA GCG AAC AAT GTC TCC AA-3'
Protection of telomeres 1b (POT1b) F	5'- CTT TAA GCC TCC GGC CTT AAG CAA AG-3'
Protection of telomeres 1b (POT1b) R	5'- CTT GGA CAT GAT TAT CAG CAA CGA CA-3'
mouse telomerase reverse transcriptase (mTert) F	5'- GCC TTG AGC ACA ATG ACC -3'
mouse telomerase reverse transcriptase (mTert) R	5'- ATA TCC GTG GTG GCA CAA AT -3'

Table 3

Body weight and running speeds for the experimental groups.

	BL (n = 4)	**TP1 (n = 8)**	**TP2 (n = 8)**
Body mass (g)	19.7 ± 1.8	18.3 ± 2.8	17.4 ± 2.3
Peak treadmill running speed ($m*min^{-1}$)	-	36.8 ± 14.4	32.6 ± 6.5
Average running speed during acute treadmill bout ($m*min^{-1}$)	-	24.8 ± 11.0	21.0 ± 4.0

Data are presented as means ± standard deviation. BL: Baseline; TP1: immediately after acute treadmill running bout; TP2: 1-hour after acute treadmill running bout. g = grams. $m*min^{-1}$ = meters per minute.