Örebro University
School of Health and Medical Sciences
Master program: Sports Physiology & Medicine, 120 credits
Degree Project, 45 credits, Spring 2012

The effect of resistance training on molecular mechanisms responsible for muscle protein breakdown in healthy old men

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Abstract

Background- Resistance training has been shown to be effective in restoring the strength deficit in the elderly and improve quality of life. Prolonged inflammatory reactions, inadequate repair of skeletal muscle, as well as maladaptation to injury also take place in the presence of low grade systemic inflammation in old people which leads to a higher incidence in injuries and a delay in muscle regeneration. The objectives of the present study were A) to investigate the transcriptional responses of IKKB, HSP27, TNF-α, TNFRs, and IL-1β to one single bout of resistance exercise and to one year of RT in old subjects, B) to evaluate the effect of age on transcriptional responses of IKKB, HSP27, TNF-α, TNFRs, and IL-1β to a single bout of exercise, and C) to examine the relationship between change in mRNA levels of HSP27 with change in mRNA levels of IKKB and TNF-α.

Material & methods- The study comprised of 15 healthy old (69±4 years) and 6 young men (23±3 years). Old subjects were randomized into a training group (n=8) and a control group (n=6). Muscle tissue from vastus lateralis was assayed for mRNA expression of IKKB, HSP27, TNF-α, TNFR1, TNFR2, and IL-1β.

Results- 6 h and 24 h after exercise significant elevations compared to baseline were seen for cytokine and HSP27 mRNA levels while significant reductions compared to baseline were found at 6 h, 48 h, and 72 h for IKKB mRNA levels in the untrained experimental old group. The exercise-induced responses of mRNA levels of TNF-α followed the same patterns, while a significantly reduced magnitude of change was observed for mRNA levels of IL-1β post resistance training. The pattern of the exercise-induced response of IKKB mRNA levels was significantly altered post one year compared to before one year resistance training in the old training group. One year of regular resistance training in old subjects induces significant reductions in basal mRNA levels of TNF-α, TNFR2, and IKKB. Changes in mRNA levels of HSP27 were significantly related to changes in TNF-α after 6 h pre and post one year RT in old subjects.

Conclusion- The results indicate that through resistance training, levels of pro-inflammatory cytokines can decrease and an increase in power and strength is achieved leading to a better quality of life in the elderly. This is the first study indicating a re-gained balance in the NFkB response to exercise in the old population.

Key words: sarcopenia, resistance training, cytokines, NFkB, HSP 27

Abbreviations

C	Control group
FADD	Fas-associated death domain protein
HSPs	Heat shock proteins
IkB	Inhibitor of kappa B
IKKB	Inhibitor of nuclear factor kappa B kinase beta subunit
IL-1β	Interleukin-1 beta
mRNA	Messenger ribonucleic acid
NFkB	Nuclear factor kappa-light-chain-enhancer of activated B cells
OC	Old control
OE	Old experimental group
PAPw	Peak anaerobic power watts
PPO	Peak power output
RIP	Receptor interacting protein
RM	Repetition maximum
ROS	Reactive oxygen species
RT	Resistance training
SJ	Squat jump
TNFR	Tumor necrosis factor receptor
TNF-α	Tumor necrosis factor alpha
TRADD	Tumor necrosis factor receptor-associated death domain protein
TRAF	Tumor necrosis factor-receptor associated factor
YS	Young subjects

RT	Resistance training
S	Strength
E	Endurance
HYP	Hypertrophy

Introduction

Age-related skeletal muscle atrophy

Facilitating movement, strength, and respiration is dependent on the maintenance of skeletal muscle mass. With an increasing age, a degenerative loss of muscle mass takes place and to date, there are not many solutions available for treatment of this loss termed sarcopenia (Lenk et al., 2010). Humans lose approximately 20-40% of their skeletal muscle mass between the age of 20 to 80 years and the loss is a result of multifactorial processes (Carmeli et al., 2002; Buford et al., 2009). Age-related skeletal muscle atrophy leads to a reduction in strength and a decline in power output which is the main reason for an increased risk of falls and an inability to maintain a quality of life and perform regular tasks of daily living (Buford et al., 2009; Lenk et al., 2010; Phillips & Leeuwenburgh, 2005). From the molecular point of view, a transcriptional upregulation of key mediators controlling atrophy has been shown to take place in aged skeletal muscle (Glass, 2005). The gene transcription takes place as a result of external stimuli transmitted to intracellular factors closely regulated by signaling pathways linked to skeletal muscle protein break-down (Ferrington et al., 2005; Ogawa et al., 2010; Lenk et al., 2010); however, the interaction between the intracellular and extracellular processes that take place in atrophic signaling are not fully understood and it is difficult to conclude the precise signaling process that takes place in protein degradation due to the vast number of different stimuli that can induce this phenomenon (Buford et al., 2010).

Recently, it has been implicated that a low grade systemic inflammation is a major cause for significant decreases in muscle strength, power output, and function, and usually occurs with aging, disuse, and/or disease (Buford et al., 2009; Buford et al., 2010; Ogawa et al., 2010; Schaap et al., 2009; Phillips & Leeuwenburgh, 2005). Low grade systemic inflammation in the old population is a phenomenon known as “inflammaging” (Fagiolo et al., 1993) and is characterized by a change in the immune system leading to higher systemic concentrations and an elevated basal production of pro-inflammatory cytokines (Ferrucci et al., 2005). Prolonged inflammatory reactions, inadequate repair of skeletal muscle, as well as maladaptation to injury also take place in the presence of low grade systemic inflammation in old people which leads to a higher incidence in injuries and a delay in muscle regeneration (Brooks & Faulkner, 1988; Rader & Faulkner, 2006; Peake et al., 2010; Kayani et al., 2008).

Pro-inflammatory cytokines

Tumor necrosis factor alpha (TNF-α) is a pro-inflammatory cytokine serving multiple functions and tissue wasting (cachexia) is one of its characteristic actions (Lenk et al., 2010; Moldoveanu, 2001). TNF-α is produced by various cell types including macrophages, lymphocytes, and fibroblasts, and is secreted as a result of inflammation, infection, and environmental stresses (Moldoveanu, 2001). Tumor necrosis factor receptor 1 and 2 are expressed by most cells and there are both membrane bound and soluble receptors for TNF-α (Naudé et al., 2011). The receptors which are part of the TNF receptor super-family are thus mediators of most of the cellular responses of TNF that are called forth. It is speculated that TNFR1 is mainly responsible for cell apoptosis. However, the mechanism by which TNFR2 act is not as well documented (Liu, 2005), and studies have reported signaling between the two membrane-bound TNF receptors though ligand passing which implies that TNFR2 is responsible for channeling of TNF-α to TNFR1 (Tartaglia et al., 1993; Saperstein et al., 2009).

The TNFRs act through a common pathway, namely the nuclear factor kappa-light-chain-enhancer of activated B cells (NFkb)/Rel-pathway. A balance in the signaling process mediated by the receptors may therefore optimize the initiation of the apoptotic process (Pincheira et al., 2008). Upon binding of TNF-α to its receptors a complex chain of events occur and as a result Caspases, transcription factors activation Protein-1 and NFkB are activated (Pincheira et al., 2008; Liu, 2005). TNFR1 possesses a death domain leading to interactions between other death domain proteins. The TNFR-associated death domain protein (TRADD) and the Fas-associated death domain protein (FADD) act together to activate Caspase 8 to in turn initiate other Caspases (3,6,7) leading to induced apoptosis (Pincheira et al., 2008). The TNF receptor associated factor 2 (TRAF2) and the receptor interacting protein (RIP) interact with TRADD and have been shown to be responsible for the activation of NFkB. Binding of TNF-α to TNFR2 leads to collaboration between TRAF1, TRAF2, the cellular inhibitor of apoptosis 1, (cIAP1) and 2 (cIAP2) (Liu, 2005).

Another pro-inflammatory cytokine, interleukin-1 β (IL-1β) is part of the interleukin-1 family and is produced by activated macrophages. IL-1β is another important mediator in the inflammatory response and signals through the type 1 IL-1 receptor (Moldoveanu et al., 2001). TRAF 6, which was initially discovered to couple TNFRs to signaling pathways is also a signal transducer to IL-1

and is responsible for coupling the receptor complex to downstream kinases which subsequently activates NFkB (Bradley & Pober, 2001).

NFkB/Rel-pathway

NFkB/Rel is a pleiotropic transcription regulator activated by various extra- and intracellular stimuli. The most critical activators are the inflammatory cytokines TNF-α, IL-1β (Cai et al., 2004; Meng & Yu, 2010), and reactive oxygen species (ROS) (Bar-Shai et al., 2008; Meng & Yu, 2010). It is involved in one of the most prominent signaling cascades in skeletal muscle atrophy and regulates the expression of various genes involved in response to cellular growth control, immune, and inflammatory responses (Beg & Baldwin, 1994; Buford et al., 2010). The NFkB/Rel complex is comprised of the p50 (NFKB1)/p65 (RelA) heterodimer. Previously it was thought that this heterodimer was solely responsible for transcription of target genes; however, three additional mammalian NFkB/Rel proteins have been identified: c-Rel, NFkB2 (p52/p100), and RelB; although their role in NFkB activity remains largely unknown (Beg & Baldwin, 1994; Zandi et al., 1997; Barkett & Gilmore, 1999; Buford et al., 2010). Currently, there are two pathways by which NFkB is known to be activated initiating muscle atrophy, the canonical (classical) and the non-canonical (alternative) pathway (Karin & Ben-Neriah, 2000; Buford et al., 2010). The canonical pathway is known to mainly be involved in pathology-mediated atrophy and involves the p65/p50 heterodimer-complex (Bar-Shai et al., 2005) while the non-canonical pathway is involved in disuse-mediated atrophy and acts through the p50/p50 homodimer (Hunter et al., 2002).

Considerable knowledge has been gained in the activation process of NFkB, especially cytokine-induced NFkB activation by TNF and IL-1 through the canonical pathway (Karin & Ben-Neriah, 2000). The TNF/IL-1 induced NFkB activation mainly depends on the phosphorylation and degradation of inhibitor of kappa B (IkB) proteins which the inhibitor of nuclear factor kappa B kinase complex (IKK) is responsible for (DiDonato et al., 1997; Karin & Ben-Neriah, 2000). The kinase mainly responsible for a rapid activation of NFkB induced by pro-inflammatory cytokines is the inhibitor of nuclear factor kappa B kinase beta subunit (IKKB) (Häcker & Karin, 2006). IKKB phosphorylates IkB, and upon phosphorylation, ubiquitination and proteasomal degradation of the inhibitor takes place. As a result, NFkB is translocated into the nucleus where it activates many genes containing NFkB binding sites (Scmid & Birbach, 2008). At the level of IKKB, converging of

many signaling pathways occur, especially of those stimuli induced by TNF-α and IL-1β (Karin & Ben-Neriah, 2000; Scmid & Birbach, 2008; DiDonato et al., 2007).

IKKB mainly activates the canonical pathway and activation of NFkb has been shown to induce muscle wasting *in vitro*. Concomitantly, inhibition of IKKB/NFkB has the adverse effect in which muscle atrophy is reversed (Cai et al., 2004). Moreover, compared to young adults, higher basal levels of IKKB has been demonstrated in old people (Buford et al., 2010) and it has been shown that the canonic pathway is predominant in muscles from old mice compared to young ones where the non-canonic pathway is predominant (Bar-Shai et al., 2005).

Heat shock proteins

Heat shock proteins (HSPs) have diverse roles including chaperone activity, thermo-tolerance, protein folding, protein transport, cell development regulation, and cell differentiation (Jakob et al., 1992; Liu & Steinacker, 2001; Kayani et al., 2008). Overexpression of HSPs is essential for the survival of cells subjected to stresses that would be lethal in their absence (Garrido et al., 2003). The HSPs are named according to their molecular weight whereas the large HSPs (especially HSP70) are the most studied ones (Mymrikov et al., 2011). Recently the small heat shock protein, HSP25/27 (HSP25 is the mouse homologue), has come to be more importantly known as an apoptosis inhibitory protein (Park et al., 2003; Dodd et al., 2009) where it directly or indirectly participates in the regulation of apoptosis (Mymrikov et al., 2011). Upregulation of the cytoprotective marker HSP27 may ameliorate age-related skeletal muscle atrophy induced by inflammatory signaling (Lawler & Hindle, 2011), and it seems TNF-α contributes to a significant rate of phosphorylation of HSP27 (Huey et al., 2007). It is known that elevated levels of HSP27 inhibits IKKB in non-muscle cells (Park et al., 2003); moreover, recent work by Dodd et al. (2009) confirmed that HSP27 inhibits IKKB induced NFkB activation also in skeletal muscle *in vitro* (Dodd et al., 2009).

Cytokines & exercise in aged skeletal muscle

There is growing evidence that lower physical activity in older people is linked to a higher prevalence of inflammatory markers such as TNF-α which has been shown to be associated to reduced muscle mass and more importantly muscle strength. While the inflammatory response and exercise-induced apoptosis is a normal regulatory process, excess and prolonged production of pro-inflammatory cytokines in response to exercise is detrimental and is more frequent in old than young skeletal muscle (Phaneuf & Leeuwenburgh, 2001; Bautmans et al., 2004; Thalacker-Mercer et al.,

2009). The cytokine response to exercise in elderly remains poorly understood and most of the data derive from studies of acute exercise (Hamada et al., 2005). Injured muscle after exercise causes monocytes to infiltrate the skeletal muscle for repair whereas neutrophils are accumulated. This accumulation is correlated with an up-regulation in IL-1β, a cytokine preventing protein synthesis in skeletal muscle (Malm et al., 2002). Initial observations suggest that old skeletal muscle display an inability to up-regulate IL-1β transcript levels in response to acute exercise (Jozsi et al., 2000). However, more recent studies have shown the mRNA expression of TNF-α and IL-1β to be significantly elevated in response to exercise in young (Buford et al., 2009; Huey et al., 2007; Hirose et al., 2004; Liao et al., 2010) and old (Hamada et al., 2005; van der Poel et al., 2011) human skeletal muscle whereas serum levels of inflammatory cytokines failed to be up-regulated (Buford et al., 2009; Peake et al., 2005; Nienam et al., 2004; Bautmans et al. 2004).

Resistance training (RT) is a safe and effective training method to implement in elderly to preserve muscle mass, strength, and function. It can have multiple effects, such as serving as an anti-inflammatory agent (Buford et al., 2009) and can act on several signaling pathways counteracting skeletal muscle wasting (Lenk et al., 2010; Peake et al., 2010). Human skeletal muscle has been thought to act mainly as an effector organ responding to stimuli such as nerve impulses. Recently it has been discovered that skeletal muscle possesses the ability to secrete cytokines (Plomgaard et al., 2005) which is why an implementation of resistance training (RT) can change muscle morphology and function acting on pathways such as NFkB (Buford et al., 2009, 2010; Bautmans et al., 2004). Long-term effects of resistance training (RT) have been shown to decrease and/or prevent inflammation and decrease basal levels of inflammatory mRNA expression in elderly (Greiwe et al., 2001; Lenk et al., 2010). Moreover, signaling of TNF-α via NFkB promotes apoptosis mainly in type II muscle fibers (Phillips & Leewenburgh, 2006) which explains the relevance of RT in elderly to counteract age-related skeletal muscle atrophy.

NFkB plays a pivotal role in the inflammatory response (Buford et al., 2010) in acute as well as chronic inflammation (Schmid & Birbach, 2008). Previous work has demonstrated an inability to adequately activate NFkB pathway in response to acute exercise in old mice (Vasilaki et al. 2003, 2006; Thalacker-Mercer et al., 2009). Moreover, the rapid production of HSPs after exercise is one of the major critical adaptive responses in skeletal muscle (Kayani et al., 2008) and acts as a cellular

protective mechanism against ROS (McArdle et al., 2002) and excess production of pro-inflammatory cytokines (Bar-Shai et al., 2005). Previous work has demonstrated an up-regulation of HSP expression after both acute exercise (McArdle et al., 2002; Morton et al., 2006, 2009) and chronic training (Ferrington et al., 2005); however, the adaptive response of HSP27 to exercise in skeletal muscle is to date not well understood (Liu & Steinacker, 2001; Folkesson et al., 2008) and HSP27 production in old human muscle has not been investigated; however, muscles of old rodents has shown limited response of HSP25 to acute exercise (Vasilaki et al., 2003; 2006; Kayani et al., 2008; Thompson et al., 2003).

Most studies examining the relationship between inflammatory cytokines and exercise have focused on endurance training and not on RT. The few studies who have studied the effects of RT on inflammatory markers in elderly have investigated short-term training effects. To date, studies implementing chronic training interventions in healthy elderly are lacking (Mourkioti & Rosenthal, 2008; Buford et al., 2010) and knowledge regarding the role of inflammatory cytokines, NFkB-pathway, and HSP27 in human skeletal muscle and the way by which it acts in response to exercise training in the old population is limited. No study has investigated the effect of training on HSP27 in old human skeletal muscle. Furthermore, the relationship between HSP27 and IKKB, and HSP27 and TNF-α in human is unknown. Therefore, the aims of the present study were A) to investigate the transcriptional responses of IKKB, HSP27, TNF-α, TNFRs, and IL-1β to one single bout of resistance exercise and to one year of RT in old subjects, B) to evaluate the effect of age on transcriptional responses of IKKB, HSP27, TNF-α, TNFRs, and IL-1β to a single bout of exercise, and C) to examine the relationship between change in mRNA levels of HSP27 with change in mRNA levels of IKKB and TNF-α.

Material & methods

Subjects & Ethics

The study comprised of 15 healthy old and 6 young adult male volunteers from Jyväskylä, Finland. No previous experience in regular resistance training was reported by any of the subjects. Health status was examined by a physician and exclusion criteria included overweight (body mass index > 28), diabetes, cardiovascular or pulmonary diseases, or other diseases preventing the subjects from

participating in the RT-intervention and/or strength testing. The old subjects were randomized into two groups; one old experimental group (OE) (n=8; mean±s.d: 68±2 years, 81.2±10.0 kg, 173.6±4.7 cm, 27.4±6.0% body fat) and one old control group (OC) (n=7; mean±s.d: 69±4 years, 83.6±8.2 kg, 179.0±6.1 cm, 30.2±4.7% body fat). Young subjects (YS) (n=6; mean±s.d: 23±3 years, 79.8±6.9 kg, 176.9±4.6 cm, 20.5±4.9% body fat) were predisposed as a comparison group to OS. Both the OE and YS performed an acute bout of resistance exercise and participated in the one year RT-intervention. The OC did not perform any resistance exercise nor take part in the one year RT-intervention. The health status and physical activity level of the subjects were evaluated by a questionnaire and a medical control. The subjects were informed of the experimental protocol, potential risks and benefits, and procedures. The subjects signed a written consent form prior to inclusion in the study. An ethical application was sent to the ethical committee and ethical approval was received by the ethics committees of the University of Jyväskylä and the Central Finland Health Care District.

Resistance training-protocol

The RT-protocol lasted for two 26-week periods (12 months) controlled by qualified exercise instructors and was performed in a commercial gym. The first period (month 1-6) consisted of whole body exercises 2 times/week to then increase to 3 times/week in period 2 (month 7-12) where the training was divided into upper- and lower body sessions.

Exercises were performed for the main muscle groups of the body. Main lower body exercises consisted of leg press, squat, and knee extension/flexion. For other main muscle groups of the body four to five exercises were performed (e.g. bench press, lateral pulldown, and elbow flexion/extension). The loads assigned to each individual were verified through the 10RM testing. Both periods of the RT-protocol was split into three training periods (~8 weeks/period) with different aims: muscle strength endurance (S+E) (40-60% of 1RM; 3 x 15-30 RM sets/exercise; ~1-2 min rest between sets), hypertrophy (HYP) for an increase in total muscle mass (60-80% of 1RM; 3 x 6-12 RM sets/exercise; ~2 min rest between sets), and to optimize strength gains while still focusing on hypertrophy (S+HYP) (70-90% of 1RM; 3 x 5-8 RM sets/exercise; ~3 min rest between sets). Depending on the subjects' strength gains throughout the training period the overall load was adjusted accordingly. Each training session lasted for approximately 60-90 min in length including warm-up and cool-down.

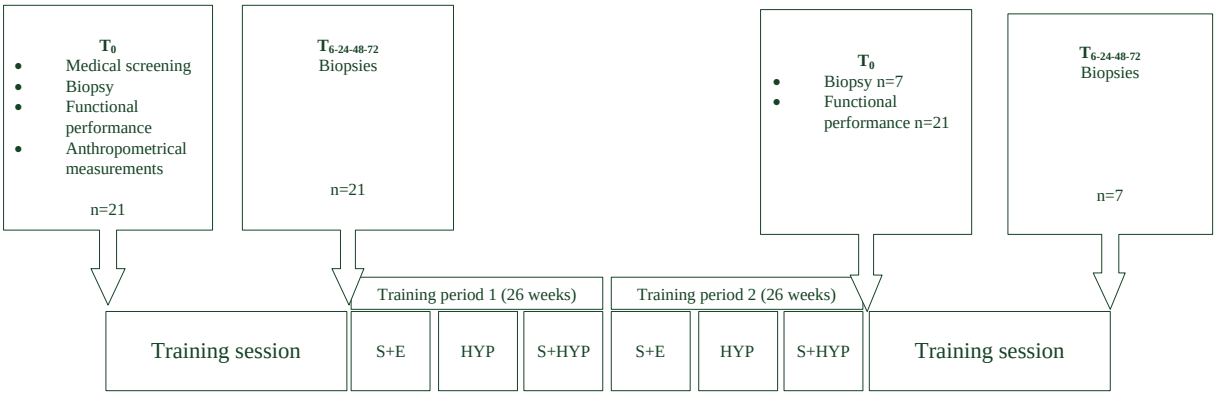


Figure 1. Overview of the exercise protocol and major evaluation moments (S+E: strength and endurance training, HYP: hypertrophy training, S+HYP: strength and hypertrophy training).

One repetition maximum

Maximal bilateral concentric strength (1 RM) of the leg (hip and knee) extensors was measured through a horizontal leg press exercise with a David 210 dynamometer (David Fitness and Medical, Outokumpu, Finland). The starting position was 110° hip-angle and a 70° knee-angle. On verbal command the subject initiated the movement by a full extension of hips and knees to reach 180°. The load was progressively increased after each trial by 2.5-5 kg until failure to reach the requirement of full extension. Between trials ~2 min rest was given. Determination of 1 RM was set at the trial with maximal load being encompassed by the subject passing the criteria of full hip and knee extension. This was accomplished within 3-5 trials.

Squat Jump

In order to assess maximal dynamic explosive force production of the leg extensors a squat jump (SJ) was performed. The SJ which is performed without a preliminary countermovement was performed on a contact mat. The starting position was a 90° knee angle, feet hip-width apart, and hands resting on the sides of the hip. In order to make sure the subject reached the correct knee angle, subjects were instructed to descend until the back of the thighs touched a rubber chord which was placed at a height corresponding to 90° knee angle of the subject. The hands were held on the hips during the entire test procedure and the subjects were encouraged to land with extended knees/feet to minimize differences in technique. Upon verbal instructions the subject initiated the jump by jumping vertically with maximal effort. Subjects were given three trials whereas the best

jump height was used for calculations. Maximum vertical jump height was calculated by determining the rise of the body's center of gravity as follows: h = gt^2/8, where h = jump height, g = 9.81 m x s−2, and t = flight time. Subsequently, Sayers equation (Sayers et al. 1999) was implemented to estimate peak power output (PPO) from the vertical jump through the equation: PAPw (Watts) = 60.7 x jump height (cm) + 45.3 x body mass (kg) – 2055. The equation calculates peak anaerobic power output (PAPw) and provides a greater accuracy than other estimation formulas for determination of peak power developed by Harman et al. and Lewis equations according to a cross-validation of jump power equations (Sayers et al., 1999).

Muscle biopsy procedure

Muscle biopsy samples were obtained from the middle portion of the *vastus lateralis* muscle using the percutaneous needle biopsy technique. After cleansing with an antiseptic solution local anaesthetics (2 ml lidocaine-adrenaline, 1%) was administered. Approximately 100 mg of muscle tissue was removed and cleaned of any visible adipose and/or connective tissue, and blood. The muscle sample was then snap frozen in liquid nitrogen (-180°C). The samples were stored in -80°C for subsequent mRNA analysis. There were 5 time points at which the biopsies were obtained for YS and OE: before, 6 h, 24 h, 48 h, and 72 h after a single bout of resistance exercise consisting of a 5 x 10RM leg presses with 2 minutes of recovery between the sets. For the OE these were obtained both before and after the 12-month training protocol. Biopsy samples were obtained from OC at three time points (before, 6 h, and 24 h after the exercise bout performed by OE and YS). Due to drop outs among the old subjects, not all biopsy samples were obtained; therefore the number of subjects included for mRNA analyses include OE, n=7, OC, n=6, and YS, n=6. The values of the control group pre exercise were also used as post comparisons.

RNA extraction and reverse transcription

Approximately 35-50 mg of muscle tissue was used for purification and extraction of total RNA. RNA extraction was performed using RNeasy Fibrous Tissue Mini Kit (Qiagen) according to the manufacturer's instructions. Following RNA extraction the concentration and purity was determined using a spectrophotometer (nanoDrop 2000, Thermoscientific). Samples with a nucleic acid concentration containing less than 20 µg/ml were re-extracted. The final concentration was determined by a calculation based on yield of RNA and the weight of the sample.

Reverse transcription was utilized by SuperScript II RT from Invitrogen. For each sample 0.75 µg RNA was reverse transcribed with a reverse transcription reaction mixture (250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$), 100 mM DTT, and a dNTP mixture according to the manufacturer's instructions. Superscript and RNase Out were then added to the master mixture. The tubes were incubated as following: 3 min at +70°C, 10 min at +25°C, 50 min at 42°C, and 4 min at 4°C. Samples were kept in -20 degrees C for long term storage.

Quantitative real-time PCR

Real-time PCR was performed using 7900 Fast Real-Time PCR System with TaqMan® Fast Universal PCR Master Mix (2X) (Applied Biosystems, Foster City, California) and gene-specific primers. The primers for IKKB (catalog number Hs00233287_m1), HSP27 (catalog number Hs03044127_g1), TNF-α (catalog number Hs01113624_g1), TNFR1 (catalog number Hs01042313_m1), TNFR2 (catalog number Hs00961749_m1), IL-1β (catalog number Hs01555410_m1), and RPLPO (catalog number Hs02992885_s1) were purchased from Applied Biosystems (Foster City, California). Samples and standard curve dilutions were loaded in duplicates for each run. Moreover, one sample was chosen as an internal control and loaded in each run. The amplification was run for 40 cycles (stage 1: 20 sec, 95°C; stage 2: (1 sec, 95°C and 20 sec, 60°C)).

The quantification of the PCR products was generated using the comparative critical threshold ($C_{T)}$ method where the relative expression level of the target gene was normalized by subtracting the corresponding reference gene C_T values calculated as $2^{-\Delta\Delta Ct}$ (C_T is the threshold PCR cycle at which fluorescence is detected above baseline) (Schmittgen, 2000; Schmittgen & Livak, 2008). The reference gene RPLPO (large ribosomal protein) was chosen after showing a stable expression after performing preliminary tests on RPLPO and B2M (beta-2 microglobulin). Moreover, RPLPO has previously been shown to be one of the most stable and equally expressed genes in skeletal muscle (Stern-Straeter et al., 2009). Prior to quantification, similar PCR efficiency of the standard curve was established between the slope of the target gene and reference gene through the equation: $E= (10^{-1/slope}-1) \times 100$. Two samples per well confirmed the quality of the experiment. The SDS 2.4 software (Applied Biosystems, Foster City, California) was used to analyze results.

Statistical analyses

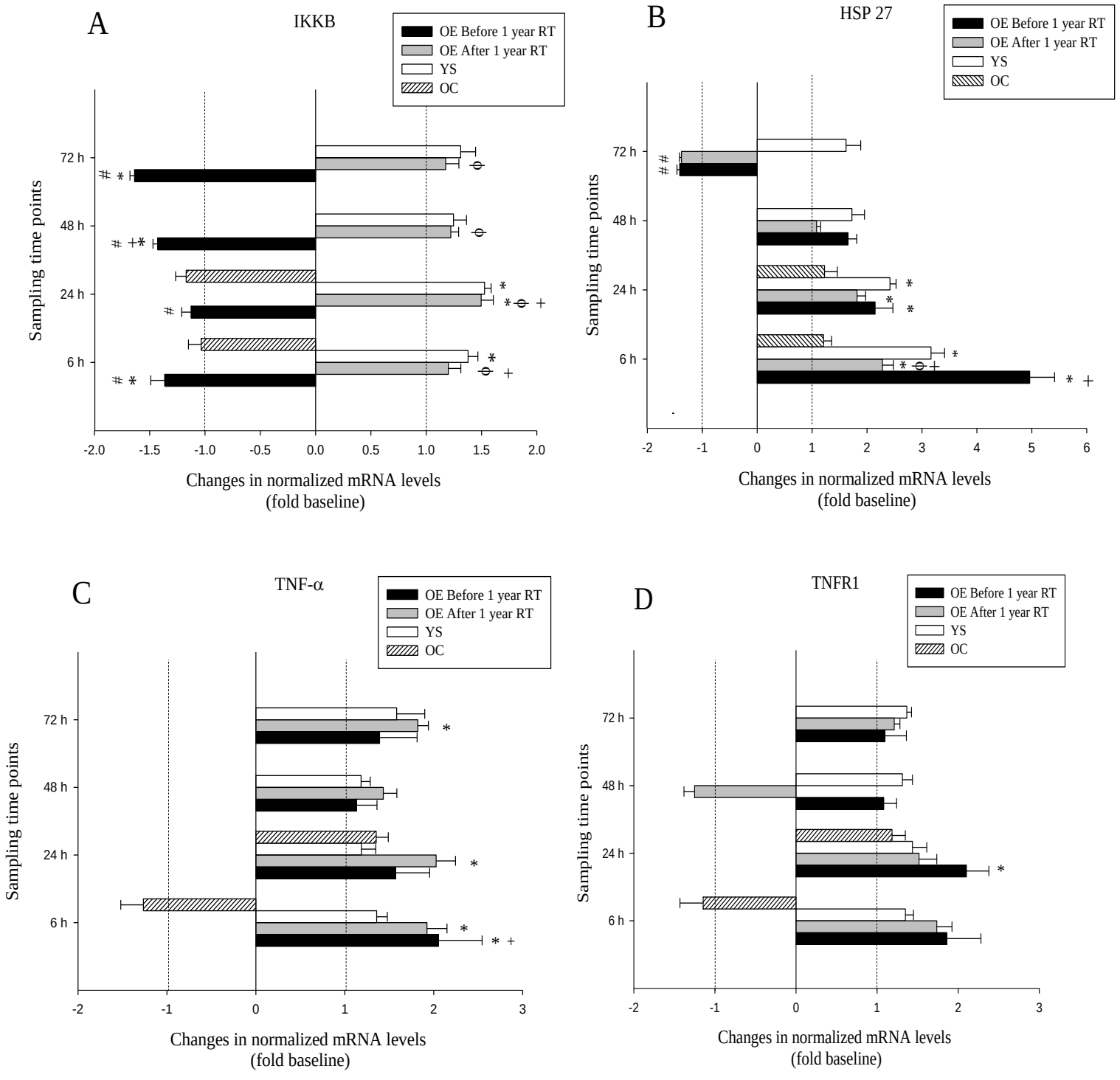
The normalized mRNA expression data of IKKB, HSP27, TNF-α, TNFR1, TNFR2 and IL-1β, and functional test data were analyzed using repeated-measures analysis of variance (RM-ANOVA) for within-subject comparisons and one-way ANOVA was used for between-group analyses where data were analyzed as fold change. Student Newman Keuls post-hoc test was used to identify differences between time points within subjects. Non-normally distributed data were log transformed so the data were normally distributed before analysis. Correlations between changes (baseline to 6 h) in normalized mRNA expression of HSP27, IKKB, and TNF-α were performed using Pearson's Product Moment Correlation test. Fold change was calculated for mRNA expression as the ratio of the final value to the initial value. A fold change value less than one was replaced by the negative of its inverse. Percent change was calculated for functional tests by subtracting the final value by the initial value divided by the initial value. Significance level was set at $p<0.05$ and tendency threshold set at $p<0.1$. Statistical analyses were performed using SigmaPlot 11.0 (SYSTAT software Inc., San Jose, California). Results are presented as mean ± SEM.

Results

Acute response to exercise on mRNA levels of IKKB, HSP27, TNF-α, TNFR, and IL-1β in untrained subjects.

No significant elevations of mRNA levels of IKKB, HSP27, TNF-α, TNFR, and IL-1β were seen in the OC at any time point. The levels of IKKB mRNA content in OE were significantly decreased compared to baseline after an acute bout of exercise at 6 h, 48 h, and 72 h. Conversely, YS showed a significant increase in mRNA levels of IKKB at 6 h and 24 h compared to baseline. The pattern of response in mRNA levels of IKKB was significantly different in OE compared to YS at all time points (figure 2A). Expression of HSP27 mRNA levels were significantly higher versus baseline at 6 h and at 24 h, and a tendency for an increase versus baseline could be seen at 48 h ($p=0.055$) in OE. At 72 h mRNA levels of HSP27 were decreased versus baseline (1.4 fold), and the response was significantly different compared to YS. YS showed a significant increase in mRNA levels of HSP27 at 6 h and 24 h compared to baseline as depicted in figure 2B. TNF-α and TNFR2 mRNA levels were significantly elevated at 6 h compared to baseline, and for TNFR1 mRNA levels were significantly elevated at 24 h compared to baseline in OE. The magnitude of increase for mRNA expression of TNFR1 in OE had a tendency ($p=0.079$) to be greater at 24 h compared to YS. YS

showed no significant elevations compared to baseline at any time point for TNF-α and TNFR1; however, significant elevations could be found at 6 h and 24 h for TNFR2 (figure 2C-E). At 6 h and at 24 h, OE showed significantly elevated mRNA levels of IL-1β versus baseline and a significantly greater magnitude of increase compared to YS. There were no significant increases in mRNA levels of IL-1β in YS at any time point versus baseline (figure 2F).



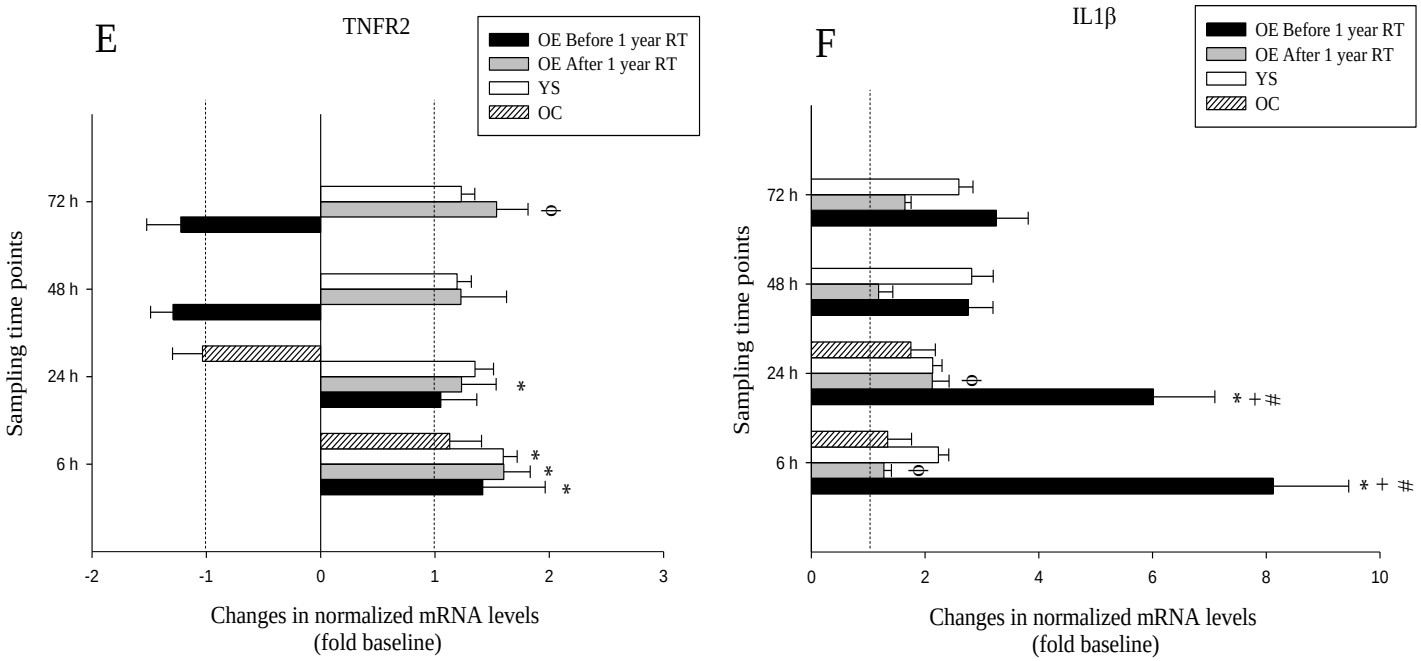


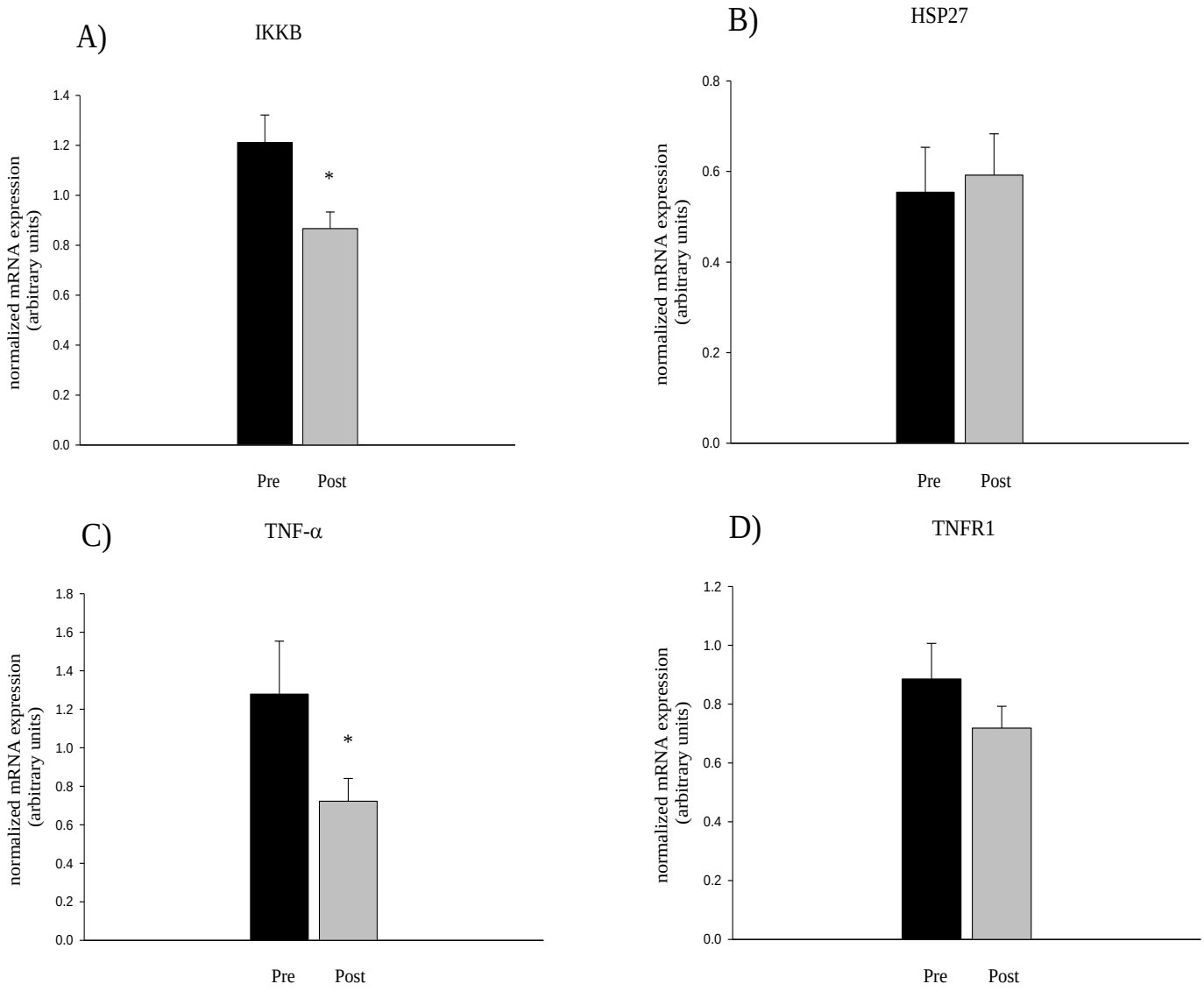
Figure 2. Changes (fold baseline) in normalized mRNA levels of IKKB (A), HSP27 (B), TNFα (C), TNFR1 (D), TNFR2 (E), and IL-1β (F) at different time points after a single bout of exercise in YS and before and after one year RT in OE. *p<0.05 vs. baseline after a single bout of exercise before and after one year RT, $^{\Phi}$p<0.05 vs. before one year RT, $^{\#}$p<0.05 vs. YS, $^{+}$p<0.05 vs. OC.

Long-term effects of one year RT on the acute response to exercise on mRNA levels of IKKB, HSP27, TNF-α, TNFR, and IL-1β.

The effects of a one year RT-protocol on IKKB, HSP27, and cytokine mRNA levels are presented in figure 2 and 3. After one year of RT, mRNA levels of IKKB in OE revealed a significant increase at 24 h versus baseline. In contrast to findings prior to the RT-protocol, there were no changes versus baseline at 6 h, 48 h, and 72 h. Post exercise changes in mRNA levels of IKKB observed after one year RT revealed that the magnitude of changes were significantly different at all time points compared to those observed post exercise prior to one year RT. Interesting results reveal that OE post exercise responses after training exhibited similar fold increases versus baseline as YS at 6 h, 24 h, 48 h, and 72 h as depicted in figure 2A (OS: 1.2, 1.5, 1.2, and 1.2 fold; YS: 1.3, 1.5, 1.2, and 1.3 fold). HSP27 mRNA levels in OE after training were significantly elevated at 6 h and 24 h. At 72 h, the magnitude of change for mRNA expression of HSP27 was significantly different compared to YS. After one year RT, mRNA levels of HSP27 revealed a decreased amplitude of increase compared to mRNA levels prior to RT versus baseline at 6 h (figure 2B). Post exercise changes for mRNA levels of TNF-α after one year training in OE revealed a significant increase at time points 6

h, 24 h, and 72 h versus baseline. There were no significant post training-induced alterations at any time point (figure 2C). No significant modifications could be seen at any time point for mRNA levels of TNFR1 or TNFR2 in OE versus baseline after one year RT. However, post training modifications for mRNA levels of TNFR2 revealed a significant difference in the magnitude of change compared to before one year RT at 72 h (figure 2D-E). For mRNA levels of IL-1β no significant alterations could be seen versus baseline at any time point in OE post training. In contrast to findings before one year RT, there were no significant increases versus baseline at any time point post training. The amplitude of the increase of IL-1β mRNA levels at 6 h and 24 h post exercise was significantly reduced compared to pre training (figure 2F).

Results for alterations in basal mRNA levels are shown in figure 3. Significantly lower mRNA levels for IKKB, TNF-α, and TNFR2 were found in OE versus pre training (figure 3A, 3C, and 3E). Conversely, HSP27 mRNA levels seemed to be relatively unaffected at baseline versus before one year RT (1.06 fold increase) (figure 3B).



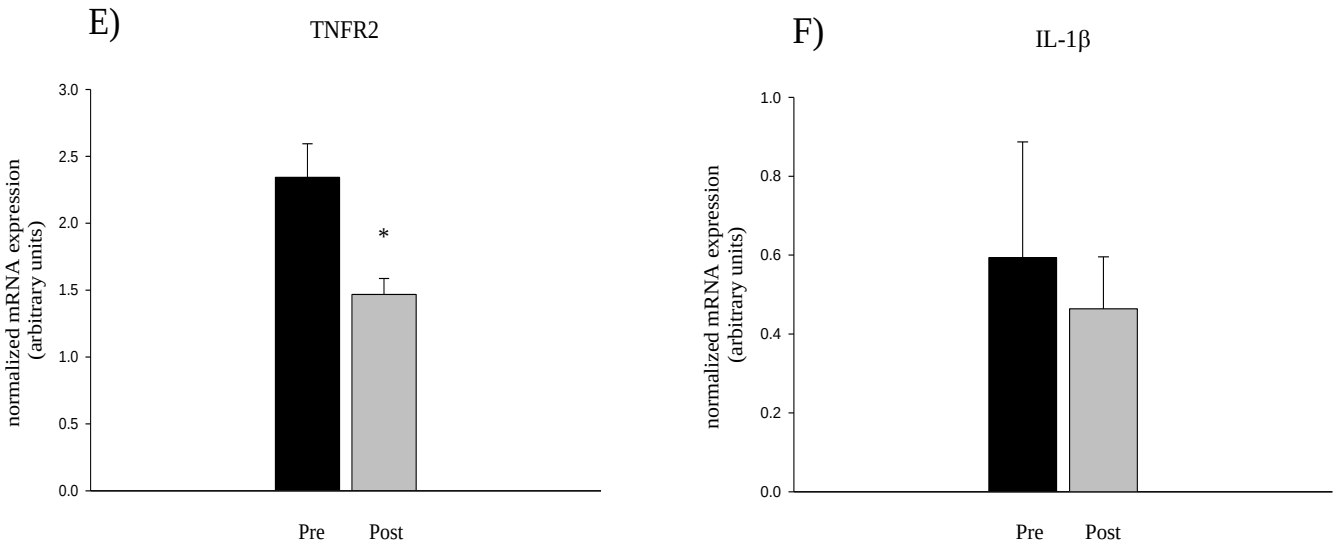


Figure 3. Baseline values before and after one year RT in OE on normalized mRNA levels of IKKB (A), HSP27 (B), TNF-α (C), TNFR1 (D), TNFR2 (E), and IL-1β (F). *****$p<0.05$ vs. before one year RT.

Relationship between changes in mRNA levels of HSP27 and mRNA levels of IKKB and TNF-α

A strong significant positive correlation existed between changes in mRNA levels of HSP27 and changes in mRNA levels of TNF-α at 6 h (fold baseline) in OS both pre ($r=0.81$; $p<0.05$) and post one year RT ($r=0.70$; $p<0.05$). In YS a moderately strong positive correlation was seen between changes in mRNA levels of HSP27 and TNF-α although not found to be statistically significant ($r=0.60$). No significant correlations between changes in mRNA levels of HSP27 and IKKB were found in OS pre RT ($r=0.39$), OS post RT ($r=0.56$), or YS ($r=-0.04$).

Changes in muscle strength and PPO

OE showed a significant improvement in 1 RM after one year of RT, from 126.6±8.8 kg to 153.1±6.7 kg (21.0±9.4%). Significant improvements in 1 RM were also observed for YS, from 177.1±29.9 kg to 199.5±33.0 kg (7.3±6.9%). Results from SJ show a significant improvement in estimated PPO/kg body mass in OE compared to pre training, from 33.5±0.5 W/kg to 36.4±0.5 W/kg (8.6±2.7%); however, no significant changes could be observed for PPO/kg for YS before one year RT (43.3±0.9 W/kg) compared to results from pre training (44.3±1.3 W/kg) resulting in an

increase in PPO of only 2.5±2.7%. No significant modifications could be observed in 1 RM or SJ for the OC pre versus post training.

Discussion

The present study demonstrated that mRNA levels of IKKB, which is the main mediator regulating the critical step in the activation of NFkB (DiDonato et al., 1997; Karin & Ben-Neriah, 2000), was significantly reduced in response to exercise in untrained elderly. A significant distorted response compared to young untrained subjects was also found. This is in line with previous reports that have shown marked differences between skeletal muscle of young and old rats in the NFkB activation pattern (Bar-Shai et al., 2005). Findings by Vasilaki et al. (2003, 2006) demonstrated a failure of NFkB to be fully activated in old skeletal muscle of mice following a bout of isometric contractions. The present study demonstrate novel interesting findings revealing an altered pattern of response of mRNA levels of IKKB post training compared to before one year RT. This indicates that exercise-induced adaptations take place for mRNA levels of IKKB and resembles IKKB mRNA responses of young untrained subjects after acute exercise. These findings together with the findings of the exercise-induced response of the pro-inflammatory cytokine IL-1β revealing a decrease in amplitude post training support the hypothesis that a suppressed activation of NFkB in OE in response to exercise may be in part due to over-expression of TRAF which is initially activated by vast elevations in pro-inflammatory cytokines (Bradley & Pober, 2001). However, the exact cause for a malfunction in up-regulation of NFkB in human skeletal muscle remains to be further elucidated. Another main finding of the present study was that basal mRNA levels of the critical NFkB-activator IKKB were significantly reduced post one year RT in OE. This is in line with findings by Buford et al. (2010) who demonstrated a reduced mRNA content of IKKB in young untrained subjects and old active subjects compared to old untrained subjects although findings were not statistically significant.

The exercise-induced responses of HSP27 mRNA levels were significantly increased at 6 h and at 24 h both before and after training in OE. In contradiction to these findings, Morton et al. (2008), found no stress response of HSP27 (biopsies taken 48 h and 7 days post exercise) in trained subjects or in untrained young male (biopsies taken 24 h, 48 h, 72 h, and 7 days post exercise) after a non-damaging running exercise (2006). Vasilaki et al. (2006) also demonstrated a lack of mRNA

upregulation of HSP25 in old rodent tibialis anterior muscle in response to isometric contractions. It is unclear why the mRNA levels of HSP27 were markedly decreased 72 h post exercise but it seems a rapid increase in mRNA expression occurs with a progressive decrement with time. This may also explain the lack of findings in the stress response of HSP27 in the work by Morton et al. (2006, 2008) and Vasilaki et al. (2006) where biopsies were taken at later time points. The type of exercise may also explain discrepancies between findings. A study by Folkesson et al. (2008) found rapid HSP27 protein relocation in human vastus lateralis with RT (leg extension exercise) but not with endurance training (one-leg ergometer cycling) when examined through immunohistochemistry after acute exercise. Another study by Thompson et al. (2003) eliciting the maximal eccentric component in both the biceps brachii, through maximal voluntary contractions in a biceps curl exercise apparatus, and vastus lateralis, through downhill running in an acute bout of exercise found a significant increase in mRNA and protein levels of HSP27 in the biceps brachii, but not in the vastus lateralis. This further supports the suggestion that high-force contractions are necessary to elicit a significant amplification of HSP27 mRNA levels; thus HSP27 may be both muscle and exercise specific. Another hypothesis is that HSP27 may be phosphorylated at an early stage which can be supported by findings by Huey et al. (2007) Huey and co-authors found evidence that HSP25 shifts to the insoluble fraction abruptly after functional overload in rodents. They also suggested that muscle specific differences in TNF-α concentration are associated with differences in the response of HSP25.

Interesting findings reveal that the amplitude of increase of HSP27 mRNA expression was significantly decreased at 6 h after compared to before the RT-intervention and started to resemble those responses of untrained young subjects at the same time point. However, a decrease versus baseline was found both before and after one year RT at 72 h which was not seen in untrained young subjects. Basal HSP27 mRNA expression was not markedly altered in response to a one year RT-protocol in OE. In accordance with the present study, Morton et al. (2008) found resting HSP27 levels to be unaffected by training as no difference was found between aerobically trained and untrained young men before a non-damaging running exercise protocol. On the other hand, basal levels of larger HSPs have been found to be altered after training (Morton et al., 2008; Thompson et al., 2003; Lawler et al., 2006). From the present findings and by work from others, it seems basal HSP27 is unaffected by training. Considering previous findings claiming complete abolishment of NFkB-activation in conditions of over-expressed HSP27 (Dodd et al., 2009), an almost non-existent

alteration in basal HSP27 mRNA levels found in the present study provides no protection against increased levels of basal IKKB and circulating inflammatory cytokines found before one year RT entailing detrimental consequences since the cytoprotective function is not activated.

Examining the acute response to exercise in the present study revealed significant elevations mainly seen at 6 hours after exercise versus baseline for mRNA levels of the inflammatory cytokines in untrained elderly. In accordance with this study, Hamada et al. (2005) also found a significant up-regulation of TNF-α mRNA levels in old untrained male skeletal muscle after an acute bout of downhill running. Findings by van der Paul et al. (2011) also demonstrated an up-regulation in mRNA levels of TNF-α in old rodent skeletal muscle although at 72 h following acute exercise. The same author found a reduced amplitude of increase in response to exercise in mRNA levels of TNF-α in young compared to old rodent skeletal muscle which is in line with the present findings. However, discrepancies exist between present findings and previous studies when examining TNF-α mRNA levels in young skeletal muscle, which lacked to show any significant up-regulations compared to baseline after acute exercise (Steensberg et al., 2002; Liao et al., 2010; Nienam et al., 2004; Hamada et al., 2005). Buford et al. (2009) examining cytokine levels of TNF-α and IL-1β after acute RT in post-menopausal women also found elevations in mRNA levels, although at 3 hours post exercise. In the literature, serum levels of inflammatory markers have mainly been measured with various outcomes. Elevations (Koçtürk et al., 2008; Liao et al., 2010) as well as no increases (Steensberg et al., 2002; Bautmans et al., 2004; Peake et al., 2005; Hirose et al., 2004; Buford et al., 2009; Thalacker-Mercer et al., 2009) have been reported following an acute bout of exercise. Peake et al. (2005) examined serum levels of TNF-α and soluble TNFR1 after an acute submaximal and maximal RT-bout in untrained young men. Soluble TNFR1 was elevated after 1 h, 3 h, and after 1 day while TNF-α remained unaltered. These findings support the proposal that examining the local expression gives a better indication on cytokine effects rather than analyzing plasma levels.

Acute exercise elicited significant increases for mRNA levels of TNF-α versus baseline at 6 h before one year RT while after one year RT significant increases were found at 6 h, 24 h, and 72 h. The present study failed to demonstrate a reduced amplitude of increase in mRNA levels of TNF-α in trained old subjects compared to before one year RT and compared to untrained young subjects. Untrained young subjects displayed a reduced amplitude of increase of mRNA levels of TNF-α

versus baseline compared to untrained and trained elderly which gives an indication that the response to acute exercise of TNF-α is elevated with age. Woods et al. (2000) have stated that the magnitude of the cytokine response to exercise seems to be decreased with age and inflammatory processes that are induced during exercise are amplified with an increased intensity under normal circumstances; however, the present findings indicate that this might not be the case for pro-inflammatory cytokines. A possible explanation for the unaltered pattern of inflammatory response post one year RT may be the progressive increase in intensity of the present RT-program. Moreover, it has been implied that eccentric contractions are necessary for an up-regulation of TNF-α (Armstrong et al., 1983; Fielding et al., 1991). The resistance training protocol used in the present study involved the eccentric muscle contraction component which induces greater muscle damage resulting in the two-fold increase of TNF-α versus baseline at 6 h both before and after one year RT. However, the intensity of the resistance training protocol may not have been sufficient to induce a significant elevation in young skeletal muscle in the present study. The overall (absolute) expression for OE post training was lower than pre training values and the fact that basal TNF-α levels were significantly lower compared to before one year RT also plays a role in these findings.

The significant up-regulations of the exercise-induced responses seen for mRNA levels of TNFRs before one year RT were not seen in trained elderly. Results from the present study reveal that mRNA levels of TNFR2 in trained old subjects started to display similar fold changes versus baseline as untrained young subjects. No significant alterations could be found after versus before one year RT in old skeletal muscle for mRNA levels of TNFRs. Unlike basal mRNA levels of TNFR2, TNFR1 mRNA levels were not significantly down-regulated after one year RT. Ligand passing between the two TNF receptors was studied by Tartaglia et al. (1993) in cell lines where it was stated that TNFR2 can significantly reduce TNF-α concentration through regulating the rate of TNF-α association with TNFR1. Another study conducted by Saperstein et al. (2009) found that TNFR2 channeled TNF-α to TNFR1. The same study also demonstrated that TNF-α binds more easily to TNFR2 and when both receptors were available on the same cell; the binding rate of TNF-α with TNFR1 was greatly up-regulated. Therefore, it seems that TNFR2 has an indirect but crucial role in cell killing and that the significant reduction in TNFR2 is crucial in the attenuation-process of sarcopenia.

The IL-1β mRNA exercise-response in untrained elderly showed significant elevations at 6 h and 24 h versus baseline. The magnitude of increase was also significantly elevated compared to untrained young subjects. However, the exercise-induced response of IL-1β transcripts after one year RT had a significant reduction in the increase in amplitude compared to responses before one year RT. The gene expression of IL-1β at baseline was not significantly altered post one year RT. In line with the findings of the present study, Hamada et al. (2006) also reported a greater exercise-induced response of IL-1β mRNA levels in aged male human skeletal muscle. A greater exercise-induced response of IL-1β mRNA levels was also demonstrated by van der Poel et al. (2011) but in aged *rodent* skeletal muscle compared to young rodent skeletal muscle. Jozsi et al. (2000) found that the exercise-induced response of IL-1β mRNA levels of untrained young human skeletal muscle was similar to the baseline IL-1β gene expression in untrained old human skeletal muscle. This finding was also noted in the present study. Jozsi et al. (2000) did however not detect any response of IL-1β mRNA levels in vastus lateralis in untrained old subjects while this response could be found in untrained young subjects. The exercise protocol used was a low-intensity RT session; therefore, higher intensity training may be required to elicit IL-1β responses in aged skeletal muscle. Thus, the magnitude of response of IL-1β seems to decrease with training and is not in agreement with the statement mentioned previously by Woods et al., 2000 that the cytokine response to training seems to decrease with age. Rather, excess and prolonged production of IL-1β in response to exercise with aging seems to take place and with training, the magnitude of responses are decreased potentially through an increase in the radical scavenger enzyme activity preventing excess ROS production to trigger inflammatory responses (Meng & Yu, 2010). It could also be that IL-1β is not triggered by eccentric contractions as is the case with TNF-α (Armstrong et al., 1983; Fielding et al., 1991).

The present study demonstrates that one year of regular resistance training induces significant reductions in basal cytokine mRNA levels of TNF-α, and TNFR2. IL-1β and TNFR1 were slightly decreased post one year RT (1.3 fold). These results support the hypothesis that chronic training can reduce circulating pro-inflammatory cytokines in elderly (Greiwe et al., 2001; Schaap et al., 2009; Ogawa et al., 2010; Meng & Yu, 2010; Lenk et al., 2010; Phillips & Leeuwenburgh, 2005).

The control group included in the present study showed no significant alterations between time points in mRNA levels or functional test results. However, slight increases in mRNA levels of TNF-α, TNFR1, and IL-1β were observed at time points 6 h and 24 h which can be supported by findings

by Malm et al. (2000) stating that an inflammatory response can take place as a result of the muscle biopsy itself. However, these findings do not explain the changes observed in the experimental group.

Both before and after one year RT, the exercise-induced changes in HSP27 mRNA expression were significantly related to the change seen in TNF-α mRNA concentrations in OE at 6 h which was the peak mRNA expression for both markers. A study by Huey et al. (2007) demonstrated similar findings where a rapid increase in HSP25 together with an increase in TNF-α occurred in C_2C_{12} myotubes; therefore, there seems to be a selective up-regulation of the protective system (HSP27) depending on the level of disruptions to the cytoskeleton machinery possibly induced by TNF-α (Huey et al., 2007). In contrast, HSP27 mRNA modifications were not concurrent with IKKB mRNA changes in OE or in YS. Findings by Dodd et al. (2009) demonstrate that HSP25 inhibits IKKB and subsequent NFkB-activation in resting disused skeletal muscle when examining hind-limb immobilization in rats. The increase in NFkB-activity is prevalent in both skeletal muscle disuse (non-canonic pathway) and in skeletal muscle atrophy (canonical pathway) while the level of HSP25/27 is decreased (Dodd et al., 2009; Garrido et al., 2003; Park et al., 2003). It can also be speculated that HSP27-induced NFkB-inhibition does not take place in response to exercise since the present findings reveal a vast increase in HSP27 as well as TNF-α at 6 h in untrained elderly. This occurs concomitantly with an improper IKKB-response previously discussed. These are the first findings regarding the relation between HSP25 and IKKB in human skeletal muscle and their interaction after a training protocol implemented in old men.

The present study demonstrated that one year of RT resulted in a significant increase in muscular strength and PPO in the elderly participants suggesting functional beneficial outcomes from a RT-implementation. YS also displayed a significant increase in strength; however, no significant increases were observed in PPO. It would be interesting for future studies to evaluate the rate of force development in elderly after a long-term training intervention since a reduction in capacity of old skeletal muscle to generate high forces in short time has been observed (Bautmans et al., 2004).

This is the first study examining both chronic and acute effects of a long-term RT-intervention on mRNA levels of molecular mechanisms responsible for muscle protein breakdown in aged skeletal muscle. In the present study muscle biopsies are obtained at five time points both before and after one year of training which strengthens the reliability of the study since cytokine levels can differ

dramatically from one time point to another after an acute bout of exercise (Louis et al., 2007). Another strength of the present study is the inclusion of an old control group not performing any exercise or training with biopsies obtained at three of the first same time points as OE and YS (0 h, 6 h, and 24 h). Younger men were also included in the study adding significant value to the assessment of the response of inflammatory cytokines, NFkB, and HSP27 across life-span to exercise. Moreover, positive effects and adaptations that are gained as a result of RT are due to local and not systemic changes which should be considered in future studies. One limitation acknowledged of the present study is that protein levels were not measured since post-transcriptional changes may or may not occur. Another limitation is that the results are only applicable to men as women were not included in the study. Future research with larger cohorts including healthy old subjects is required for the assessment in the response to a RT-intervention of cytokines and their receptors. Furthermore, measurements of phosphorylated IkB, nuclear contents of NFkB subunits, and protein levels should be included to get new insight into the mechanisms by which NFkB is activated after a long term RT-intervention.

Conclusion

With aging a significant reduction in strength, power output, and an inability to completely recover from exercise-induced muscle damage occur which can dramatically reduce quality of life whereas a low grade systemic inflammation seems to be the cause. RT offers a safe method easy to implement in the elderly and contributes to an increase in muscle strength and muscle mass. Furthermore, reductions in pro-inflammatory cytokines, both basal and in response to exercise are major health benefits gained from a RT-implementation in the elderly. These basal decrements are necessary to stop or delay the progression of sarcopenia since the cytoprotective mechanism HSP27 seems to be unaffected by training. Moreover, many diseases also result from a disruption of the NFkB and/or IKKB function. This is the first study indicating a re-gained balance in the NFkB response to exercise in the old population. Other interesting novel findings indicate that the exercise-induced responses of old men started to resemble those of young male adults after one year of training. However, the IKKB-induced NFkB pathway activation does not exist in isolation and there are many other mechanisms connecting their activity to other cell-signaling networks; therefore, further investigation is required regarding the crosstalk determining the consequences of NFkB-activation and ultimately cell fate.

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