Role of PGC-1α in exercise training- and resveratrol-induced prevention of age-associated inflammation

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Abstract

Background/aim—Age-related metabolic diseases are often associated with low-grade inflammation. The aim of the present study was to investigate the role of the transcriptional co-activator PGC-1α in the potential beneficial effects of exercise training and/or resveratrol in the prevention of age-associated low-grade inflammation. To address this, a long-term voluntary exercise training and resveratrol supplementation study was conducted.

Experimental setup—Three month old whole body PGC-1α KO and WT mice were randomly assigned to four groups: untrained chow-fed, untrained chow-fed supplemented with resveratrol, chow-fed voluntarily exercise trained and chow-fed supplemented with resveratrol and voluntarily exercise trained. The intervention lasted 12 months and three month old untrained chow-fed mice served as young controls.

Results—Voluntary exercise training prevented an age-associated increase ($p<0.05$) in systemic IL-6 and adiposity in WT mice. PGC-1α expression was required for a training-induced prevention of an age-associated increase ($p<0.05$) in skeletal muscle TNFα protein. Independently of PGC-1α, both exercise training and resveratrol prevented an age-associated increase ($p<0.05$) in skeletal muscle protein carbonylation.

Conclusion—The present findings highlight that exercise training is a more effective intervention than resveratrol supplementation in reducing age-associated inflammation and that PGC-1α in part is required for the exercise training-induced anti-inflammatory effects.

Keywords

Aging; low-grade inflammation; exercise training; resveratrol; PGC-1α

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1. Introduction

Aging is associated with a broad range of metabolic complications including increased adiposity (Schaap *et al.*, 2012; Wu *et al.*, 2007), loss of muscle mass and strength (Brooks & Faulkner, 1994; Doherty *et al.*, 1993) and oxidative stress (HARMAN, 1956). These unfavorable complications may be caused by aging per se or lifestyle such as decreased physical activity with increasing age.

Many factors are likely involved in the initiation and/or progression of age-related diseases, but reports showing that the majority of lifestyle-related diseases are associated with chronic low-grade inflammation (Handschin & Spiegelman, 2008; Wellen & Hotamisligil, 2005) support the possibility that low-grade inflammation is an important component in the pathogenesis of these diseases (Pedersen *et al.*, 2000; Woods *et al.*, 2012). In addition, low-grade inflammation is observed in elderly subjects even in the absence of chronic diseases (Wei *et al.*, 1992), but whether low-grade inflammation is cause or consequence of age-related metabolic dysfunction is still debated (Woods *et al.*, 2012).

Chronic low-grade inflammation is defined as sustained 2–4 fold elevations in systemic levels of pro-inflammatory cytokines like tumor necrosis factor (TNF)α and interleukin (IL)-6 (Bruunsgaard & Pedersen, 2003; Woods *et al.*, 2012). The immune system is thought to contribute to low-grade inflammation (Woods *et al.*, 2012), but the ability of several tissues like liver, adipose tissue and skeletal muscle (SkM) to express and secrete cytokines (Borge *et al.*, 2009; Frost *et al.*, 2002; Hotamisligil *et al.*, 1993; Pedersen & Febbraio, 2012) raises the possibility that these highly metabolically active tissues also contribute to the systemic levels of inflammatory cytokines during low-grade inflammation. Circulating TNFα is increased in type 2 diabetes patients (Hotamisligil *et al.*, 1995; Plomgaard *et al.*, 2007) and TNFα has been shown to induce insulin resistance in humans (Plomgaard *et al.*, 2005) and rodents (Hotamisligil *et al.*, 1994), indicating that TNFα could be involved in the pathogenesis of type 2 diabetes. Thus, suppression of systemic TNFα may be expected to prevent the development and progression of lifestyle-related diseases.

Exercise training elicits a broad range of adaptations including increased skeletal muscle mass (Frontera *et al.*, 1988) as well as increased skeletal muscle oxidative (Holloszy, 1967) and anti-oxidant capacity (Oh-ishi *et al.*, 1997) and previous studies indicate that exercise training may have anti-inflammatory effects (Starkie *et al.*, 2003; Woods *et al.*, 2012). Intriguingly, the natural anti-oxidant resveratrol (RSV), primarily found in the skin of dark grapes, has been reported to exert effects almost similar to exercise training. Hence, RSV has been shown to possess anti-inflammatory effects in rodents and humans (Olholm *et al.*, 2010; Pearson *et al.*, 2008) as well as to protect rodents from high fat diet-induced obesity and insulin resistance (Baur *et al.*, 2006; Lagouge *et al.*, 2006). Moreover, RSV has been shown to increase longevity in several lower species (Howitz *et al.*, 2003; Wood *et al.*, 2004). Both RSV and exercise training have been shown to activate the energy sensors AMP-activated protein kinase (AMPK) (Baur *et al.*, 2006; Um *et al.*, 2010) and sirtuin (SIRT)1 (Lagouge *et al.*, 2006), which both are believed to converge on the transcriptional co-activator *peroxisome proliferator activated receptor γ co-activator* (PGC)-1α (Canto *et al.*, 2010; Jager *et al.*, 2007).

PGC-1α is known as a master regulator of mitochondrial biogenesis and anti-oxidant defense (Leick *et al.*, 2010; Lin *et al.*, 2002; St-Pierre *et al.*, 2006; Wu *et al.*, 1999). The finding that PGC-1α expression is transiently increased in recovery from a single exercise bout (Baar *et al.*, 2002; Pilegaard *et al.*, 2003) suggests that PGC-1α is a likely mediator of exercise training-induced adaptations in oxidative and anti-oxidant proteins in SkM. Supportive of this, previous findings have highlighted the importance of PGC-1α in exercise training-induced prevention of age-associated reductions in oxidative and anti-oxidant proteins (Leick *et al.*, 2010). Moreover, a positive correlation between physical activity level and SkM PGC-1α mRNA content exists in humans (Alibegovic *et al.*, 2010). Accordingly, an inverse correlation between increasing age and SkM PGC-1α mRNA level (Ling *et al.*, 2004) may suggest that lack of exercise training-induced induction of PGC-1α with aging contributes to age-associated deteriorations in skeletal muscle. Recent studies in mice also indicate that PGC-1α has anti-inflammatory effects (Handschin *et al.*, 2007b; Handschin *et al.*, 2007a; Wenz *et al.*, 2009). However, whether the potential anti-inflammatory effects of exercise training and RSV require PGC-1α and whether combining RSV and exercise training elicits additive effects via PGC-1α are still unresolved.

The aim of the present study was to test the hypothesis that PGC-1α is required for the beneficial effects of long-term exercise training and RSV supplementation in the prevention of age-associated low-grade inflammation. To address this, metabolic and inflammatory markers were determined in adipose tissue, liver, skeletal muscle and blood from whole body PGC-1α knockout (KO) and littermate wild type (WT) mice after long-term voluntary exercise training and/or RSV supplementation.

2. Methods

2.1 Mice

Generation, phenotype and genotyping of whole body PGC-1α KO mice have previously been described in detail (Leick *et al.*, 2008; Lin *et al.*, 2004). Briefly, whole body PGC-1α KO and littermate WT mice were obtained by crossbreeding of heterozygous whole body PGC-1α KO parents. The genotype was assessed on DNA extracted from a small piece of the tail tip by the phenol-chloroform:isoamyl method. DNA fragments were amplified by PCR using specific primers against the WT and the KO alleles (Lin *et al.*, 2004) and subsequently separated on an agarose gel. Whole body PGC-1α KO mice have a CNS-linked neurological disorder resembling Huntingtons disease making them more anxious with sudden movements (Lin *et al.*, 2004). They have reduced oxidative capacity in skeletal muscle (Leick *et al.*, 2008; Leick *et al.*, 2010; Lin *et al.*, 2004), but normal glucose tolerance and do not develop diet-induced obesity (Lin *et al.*, 2004). PGC-1α KO mice run voluntarily less than WT mice when offered a running wheel, (Leick *et al.*, 2008; Leick *et al.*, 2010).

Mice were kept on a 12:12 hour light/dark cycle and had access to water and food ad libitum. The experiments was approved by the "Animal Experiment Inspectorate" in Denmark (#2009/561-1689) and complied with the European convention for the protection of vertebrate animals used for experiments and other scientific purposes (Council of Europe, no. 123, Strasbourg, France, 1985).

2.2 Experimental Setup

Whole body PGC-1α KO and littermate WT female mice were randomly assigned to a young group and four intervention groups with mice housed individually. The intervention groups were divided into: untrained mice receiving rodent chow (Altromin no. 1324, Brogården, Lynge, Denmark) (UT-C), untrained mice receiving chow supplemented with RSV (UT-R), voluntary exercise trained mice having access to a running wheel (Minimitter, Italy) (T-C), and voluntary exercise trained mice having access to a running wheel and receiving chow supplemented with RSV (T-R). The interventions lasted from 3 months to 15 months of age, where the mice were euthanized. The young mice receiving chow and serving as young UT-C were euthanized at 3 months of age. Each of the groups consisted of 8–10 mice. Based on the observation that mice of the PGC-1α KO strain (6 WT mice and 6 PGC-1α KO mice), in our animal facility, on average reach ~18 months of age with no apparent difference between WT and PGC-1α KO mice, we chose to examine 15 month old mice as these mice would be in their last quartile of their lifespan.

Based on previous studies from our group (Leick *et al.*, 2008; Leick *et al.*, 2010) we expected the PGC-1α KO mice to run less voluntarily than WT mice. Running distance and duration was monitored by a regular cycle computer and differences between WT and PGC-1α KO mice were daily adjusted by wheel blocking of WT for shorter periods to ensure similar exercise distance between the different genotypes and interventions. The running distance was on average 5.9±1.9 (WT, T-C), 6.0±2.4 (KO, T-C), 6.0±1.2 (WT, T-R) and 5.8±1.7 (KO, T-R) km per week. Pure RSV (Orchid chemicals, India) was mixed with chow to a concentration of 4 g RSV/kg chow as previously used (Lagouge *et al.*, 2006; Um *et al.*, 2010). The concentration was subsequently confirmed by liquid chromatography-mass spectrometry (Eurofins, Denmark). Body weight and food intake were monitored throughout the experiment. Running wheels were blocked 24 hours before the animals were euthanized. All mice were euthanized by cervical dislocation followed by decapitation to collect trunk blood. Quadriceps muscles, perigonadal visceral adipose tissue (VAT), inguinal subcutaneous adipose tissue (S-AT) and liver were quickly removed, quick frozen in liquid nitrogen and stored at −80° C until analyses. These tissues were chosen based on previous reports showing marked inflammatory responses as well as adaptations to metabolic challenges (Gollisch *et al.*, 2009; Handschin *et al.*, 2007b; Hotamisligil *et al.*, 1993; Wenz *et al.*, 2009).

A separate manuscript covers oxidative adaptations in skeletal muscle from the current experimental setup (Ringholm et al. – In pending review, *Exp Gerontology*).

2.3 Analyses

2.3.1 Echo MRI scanning—Body composition was determined by MRI scanning (EchoMRI, Echo Medical Systems, Houston, TX, USA). A reduced number of animals were measured (n=3–8) due to limited access to the Echo MRI scanner.

2.3.2 Plasma—Plasma cytokines were analyzed using an ultra-sensitive customized MSD multi-spot assay system pre-coated with antibodies against TNFα and IL-6

(MesoScaleDiscovery, Gaithersburg, Maryland, USA) according to manufacturer's protocol. The lower limit of detection (LLOD) was 1.0 pg/ml for TNFα and 4.5 pg/ml for IL-6.

2.3.3 RNA isolation and Reverse Transcription—Total RNA was isolated from crushed quadriceps muscle (20–25 mg), liver (20–25 mg) and V-AT (40–45 mg) by a modified guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski & Sacchi, 1987) as previously described (Pilegaard *et al.*, 2000), except that the tissue was homogenized for 2 min at 30 s^{-1} in a tissue lyser (TissueLyser II; QIAGEN, Germany).

Reverse transcription (RT) was performed using Superscript II RNase H^- and Oligo dT system (Invitrogen, Carlsbad, CA, USA) as previously described (Pilegaard *et al.*, 2000), and the cDNA samples were diluted in nuclease-free H_2O.

2.3.4 Real-time PCR—Real-time PCR was performed using an ABI 7900 sequence-detection system (Applied Biosystems, Foster City, CA, USA) as previously described (Lundby *et al.*, 2005). Primers and Taqman probes were obtained from TAG Copenhagen (Copenhagen, Denmark) (table 1). Real-time PCR was performed in triplicates in a total reaction volume of 10 µl using Universal Mastermix (Applied Biosystems). Cycle threshold was converted to a relative amount by use of a standard curve constructed from a serial dilution of a pooled RT sample run together with the samples. Target gene mRNA content was for each sample normalized to single-stranded cDNA content determined by OliGreen reagent (Molecular Probes, Leiden, The Netherlands) as previously described (Lundby *et al.*, 2005).

2.3.5 Lysate—Crushed quadriceps muscles (~20–25 mg), liver (~20–25 mg) and adipose tissue (~25–35 mg) were homogenized for 2 min at 30 s^{-1} in a tissue lyser (TissueLyser II; QIAGEN) in an ice-cold buffer as previously described (Birk & Wojtaszewski, 2006). Protein content in lysates was measured by the bicinchoninic acid method (Thermo Scientific, Rockford, IL, USA).

2.3.6 SDS-PAGE and western blotting—Protein content and phosphorylation of various proteins were measured in lysates by SDS-PAGE and western blotting as previously described (Birk & Wojtaszewski, 2006). Band intensity was quantified using Carestream IS 4000 MM (Fisher Scientific, ThermoFisher Scientific, Waltman, MA, USA) and Carestream health molecular imaging software. Protein content and phosphorylation were expressed as arbitrary units relative to control samples loaded on each site of each gel and normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein. Commercially available antibodies were used to detect TNFα (#3707), nitric oxide synthase (iNOS) (#2977), nuclear factor of kappa light polypeptide gene enhancer in B-cells (NFκB) inhibitor (IκB)-α (#9242), IκB-β (#9248), p65 (#4764) and GAPDH (#2118) protein as well as p65^{ser536} (#3033), c-Jun N-terminal kinases (JNK) (#9252), JNK$^{Thr183, Tyr185}$ (#9251), p38 mitogen-activated protein kinase (p38) (#9212) and p38$^{Thr180, Tyr182}$ (# 4511) phosphorylation all from Cell Signaling and superoxide dismutase (SOD)2 (#06-984, Millipore), Catalase (#SC-50508) and glutathione peroxidase (GPX)1 (#SC-30147) protein from Santa Cruz Biotechnology inc.

2.3.7 Protein Carbonylation—Protein carbonyl content was determined in quadriceps muscle samples homogenized in phosphate-buffer using an OxiSelect™ ELISA-kit (Cell Biolabs, San Diego, USA) according to manufacturer's protocol. Absorbance was measured at 450 nm and an oxidized/reduced BSA standard curve was generated to determine the concentration of protein carbonyl in each sample.

2.4 Statistics

Results are presented as means ± S.E. Unless otherwise noted, two-way analysis of variance (ANOVA) was applied to test the main effects of genotype and interventions and one-way ANOVA was used to test for differences between the interventions separately within each genotype. If either the equal variance test or the normality test failed, the data were logarithmically transformed before applying the ANOVA test. Student Newman Keuls *post hoc* test was used to locate differences when applicable. The non-parametric Mann-Whitney U test was applied when the equal variance test or the normality test failed even after logarithmically transformation. A p<0.05 was considered significant and a tendency is reported for 0.05 p 0.1.

3. Results

3.1 Body weight, food intake and body composition

Body weights were initially similar within the WT groups and within the PGC-1α KO groups (data not shown). Total body weight increased (p<0.05) ~20% with age in WT and PGC-1α KO mice (Fig. 1a). There was no effect of exercise training and/or RSV on body weight in either WT or PGC-1α KO mice. PGC-1α KO mice weighed ~10% less (p<0.05) than WT mice in all groups. There was no difference in food intake (given pr. gram of mice) between any of the groups (Fig. 1b).

Lean body mass (given as %) decreased (p<0.05) 5–10% with age in all groups (Fig. 1c). There was no effect of exercise training and/or RSV on percentage lean body mass in either WT or PGC-1α KO mice. Fifteen month old untrained and exercise trained PGC-1α KO mice tended to have 3–4% higher (p=0.053 and p=0.051, respectively) percentage lean body mass than WT mice, while no genotype differences were observed in the other groups.

Whole body fat percentage as well as V-AT and S-AT mass increased (p<0.05) 1.6–2.1 fold with age in both WT and PGC-1α KO mice (Fig. 1d, 1e, 1f). While no effect was observed on adiposity by RSV supplementation alone, exercise training decreased (p<0.05) fat percentage ~30% and tended to decrease (p=0.082) V-AT mass in WT mice. Combined exercise training and RSV also decreased (p<0.05) fat percentage (23%) as well as V-AT (37%) and S-AT (27%) mass in WT mice. Fat percentage was ~30% lower (p<0.05) in PGC-1α KO than WT within 15 month old untrained and RSV supplemented mice, and SAT mass was 30 % lower (p<0.05) in PGC-1α KO mice than WT within 15 month old untrained mice. In addition, PGC-1α KO mice had in all groups 25–40% less (p<0.05) V-AT than WT mice.

3.2 Plasma

Selected plasma cytokines were analyzed to evaluate the general systemic inflammatory status associated with the different interventions.

Plasma TNFα—There was an overall 1.7–2.0 fold increase ($p<0.05$) in plasma TNFα with age in WT and PGC-1α KO mice (Fig. 2a). No significant effect of RSV, exercise training alone or exercise training in combination with RSV was observed on plasma TNFα. The plasma TNFα level was 1.7–2.7 fold higher ($p<0.05$) in PGC-1α KO than WT in young and 15 month old untrained mice.

Plasma IL-6—Plasma IL-6 increased 2–2.3 fold ($p<0.05$) with age in WT and PGC-1α KO mice (Fig. 2b). Exercise training alone as well as in combination with RSV reduced ($p<0.05$) the plasma IL-6 level 40–60% in WT and PGC-1α KO mice compared with 15 month old untrained mice. In PGC-1α KO mice, RSV supplementation reduced ($p<0.05$) the plasma IL-6 level ~60% compared with 15 month old untrained mice. In young and 15 month old untrained mice, the plasma IL-6 level was 1.6–1.8 fold higher ($p<0.05$) in PGC-1α KO mice than WT mice.

3.3 Inflammatory mRNA markers in V-AT, liver and SkM

The TNFα and IL-6 mRNA content was determined in V-AT, liver and SkM in order to evaluate the tissue specific inflammatory status. Additionally, the mRNA content of the macrophage specific F4/80 (Khazen *et al.*, 2005) was analyzed as a marker of macrophage infiltration in these tissues.

V-AT—In WT mice, V-AT TNFα mRNA increased ($p<0.05$) 4.4 fold with age, but mice supplemented with RSV, exercise trained as well as exercise trained combined with RSV supplementation did not differ from young control mice in V-AT TNFα mRNA content. In PGC-1α KO mice, RSV increased ($p<0.05$) V-AT TNFα mRNA 1.8 fold compared with 15 month old untrained mice (table 2).

In WT mice, both RSV and exercise training increased ($p<0.05$) V-AT IL-6 mRNA 1.5–1.9 fold compared with 15 month old untrained mice. In PGC-1α KO mice, RSV increased ($p<0.05$) V-AT IL-6 mRNA content 1.5 fold compared with 15 month old untrained mice (table 2).

No differences were observed in V-AT F4/80 mRNA between genotypes or any of the interventions (table 2).

Liver—Liver TNFα mRNA increased 1.6 fold ($p<0.05$) with age only in WT mice. Liver TNFα mRNA increased ($p<0.05$) in PGC-1α KO mice with RSV and combined exercise training and RSV, while no change occurred in WT mice. Fifteen month old PGC-1α KO mice had lower ($p<0.05$) liver TNFα mRNA content than WT, whereas no difference was observed between genotypes in the other groups (table 2).

No differences were observed in liver IL-6 mRNA between genotypes or any of the interventions (table 2.)

No effect of age or any of the interventions was observed in F4/80 mRNA content in the liver. However, 15 month old untrained PGC-1α KO mice had 47% lower (p<0.05) F4/80 mRNA content in the liver than WT mice (table 2).

SkM—TNFα mRNA increased (p<0.05) ~2.1 fold in SkM with age in PGC-1α KO mice. TNFα mRNA content was 1.8 fold higher (p<0.05) in exercise trained WT mice than 15 month old untrained WT mice, whereas no effect was observed with RSV and combined exercise training and RSV.

IL-6 mRNA increased ~2.3 fold (p<0.05) with age in PGC-1α KO mice and tended to increase 1.4 fold (p=0.06) with age in WT mice. Exercise training combined with RSV decreased (p<0.05) SkM IL-6 mRNA content 10–37% in WT and PGC-1α KO mice, while no effect was observed in SkM IL-6 mRNA with RSV alone or exercise training alone (table 2).

SkM F4/80 mRNA content decreased (p<0.05) 50% with age in WT mice, whereas exercise training alone (2.5 fold) or in combination with RSV (1.9 fold) increased (p<0.05) the F4/80 mRNA content in SkM of both WT and KO mice compared with 15 month old untrained mice (table 2). In the RSV group and in the combined exercise trained and RSV group, PGC-1α KO mice had 1.8–2.1 fold higher (p<0.05) F4/80 mRNA level in SkM than WT mice, whereas no differences were present between genotypes in the other groups (table 2).

3.4 TNFα protein in V-AT, liver and SkM

TNFα protein content was further determined in V-AT, liver and SkM. No differences were observed in VAT TNFα protein content between any of the interventions or genotypes (Fig. 3a). An overall tendency (p=0.079) for a 1.2–1.3 fold increase in liver TNFα protein was observed with age in WT and PGC-1α KO mice (Fig. 3b). No effect of exercise training and/or RSV was observed in liver TNFα protein, but these groups did not differ from young mice either. SkM TNFα protein content increased 1.4–1.5 fold (p<0.05) with age in WT and PGC-1α KO mice. Both exercise training alone and exercise training in combination with RSV prevented this age-associated increase in TNFα protein in WT mice, but this response was blunted in PGC-1α KO mice (Fig. 3c). PGC-1α KO mice had 1.5–2.2 fold higher (p<0.05) SkM TNFα protein content than WT mice in RSV, exercise trained as well as combined exercise trained and RSV groups (Fig. 3c).

3.5 Inflammatory signaling in skeletal muscle

The 3 major inflammatory signaling pathways, IKK/NfκB, JNK and p38 were analyzed in SkM in order to investigate the underlying mechanisms behind the observed differences in SkM TNFα protein levels.

No difference was observed in total p65, JNK and p38 protein content between genotypes or interventions (data not shown). In WT mice, IκB-α protein decreased 30% (p<0.05) in SkM with age (Fig. 4a). No differences were observed in SkM IκB-α protein content with exercise training and/or RSV supplementation in any of the genotypes. SkM p65 phosphorylation did not differ between the interventions or the genotypes (Fig. 4b). There

were no differences in JNK (Fig. 4c) or p38 (Fig. 4d) signaling in SkM in any of the interventions or between the genotypes.

3.6 Protein carbonylation and iNOS protein in skeletal muscle

As oxidative stress has been suggested to be a stimulus for inducing inflammation, SkM protein carbonylation (a marker of oxidative stress) was determined. SkM protein carbonylation increased 1.6–2.1 fold ($p < 0.05$) with age in both WT and PGC-1α KO mice (Fig. 5a). RSV, exercise training and combined exercise training and RSV prevented ($p < 0.05$) this age-associated increase in SkM protein carbonylation in both WT and PGC-1α KO mice. Furthermore, young PGC-1α KO mice had 1.8 fold higher ($p < 0.05$) protein carbonylation in SkM than young WT mice (Fig. 5a).

In accordance, the protein content of iNOS increased ($p < 0.05$) ~1.4 fold with age in SkM of WT and PGC-1α KO mice (Fig. 5b). Exercise training and/or RSV supplementation did not change iNOS protein content relative to 15 month old untrained mice, but iNOS protein content was in these groups not different from young mice. Interestingly, PGC-1α KO mice had in all groups 1.2–1.8 fold higher ($p < 0.05$) iNOS protein content than WT mice (Fig. 5b).

3.7 Anti-oxidant enzymes in skeletal muscle

The anti-oxidant enzymes SOD2, catalase and GPX1 were analyzed in SkM to indirectly examine whether the observed indications of oxidative stress in SkM were related to ROS neutralization capacity. Catalase protein content decreased ($p < 0.05$) ~50% with age in SkM of WT mice (Fig. 6b). RSV increased ($p < 0.05$) SkM GPX1 protein 1.8 fold, whereas no effect of RSV was observed in SOD2 or catalase protein. Exercise training increased ($p < 0.05$) GPX1 protein 1.8 fold (Fig. 6c) and tended to increase SOD2 protein ($p = 0.051$) and catalase protein ($p = 0.075$) 1.3–2.2 fold in SkM of WT mice. In WT mice, combined exercise training and RSV increased ($p < 0.05$) catalase protein and GPX1 protein 2.2–2.5 fold compared with 15 month old untrained mice, but with no difference between the exercise trained group and the combined exercise trained and RSVgroup. No effect of RSV, exercise training or combined exercise training and RSV were observed in SOD2, catalase or GPX1 protein in PGC-1α KO mice.

PGC-1α KO mice had in all groups 25–50% lower ($p < 0.05$) SkM SOD2 protein content than WT mice. In contrast, 15 month old untrained PGC-1α KO mice had ~2.0 fold higher ($p < 0.05$) GPX1 and catalase protein content than WT and GPX1 protein increased ($p < 0.05$) 1.6 fold with age in SkM of PGC-1α KO mice.

4. Discussion

The main findings of the present study were that long-term exercise training prevented an age-associated increase in TNFα protein in SkM in a PGC-1α dependent manner. Independently of PGC-1α, long-term exercise training prevented an age-associated increase in systemic IL-6 levels and oxidative stress in SkM. Long-term RSV supplementation also prevented an age-associated increase in systemic IL-6 and oxidative stress in SkM independently of PGC-1α. Taken together, the beneficial effects of long-term RSV

supplementation seemed minor compared with previous reports on short-term RSV supplementation and compared with the present effects of long-term exercise training.

The present increase in adiposity and total body weight with aging is in accordance with previous studies (Guo *et al.*, 1999; Wu *et al.*, 2007). Although a genotype specific difference in body weight and adiposity between PGC-1α KO and WT mice has previously been described (Lin *et al.*, 2004), the current finding that exercise training as well as exercise training combined with RSV supplementation only prevented the age-associated adiposity in WT mice is of notice. The age-associated increase in plasma TNFα and IL-6 with aging in the present study is also in agreement with previous studies (Wei *et al.*, 1992) and supports that increased adiposity is associated with systemic low-grade inflammation. The observed prevention of an age-associated elevation in plasma IL-6 with long-term exercise training is in line with previous studies indicating that exercise training has anti-inflammatory effects (Olholm *et al.*, 2010; Woods *et al.*, 2012). However, it should be noted that the present age-associated low-grade inflammation and improvements with exercise training were not linked to altered glucose tolerance (Ringholm et al., in pending review *Exp Gerontol*), which may suggest that low-grade inflammation precedes glucose intolerance at least in the present setting. A potential role of PGC-1α in the regulation of systemic inflammation has previously been suggested based on studies in both muscle-specific PGC-1α KO mice (Handschin *et al.*, 2007b; Handschin *et al.*, 2007a) and muscle-specific PGC-1α overexpression mice (Olesen *et al.*, 2012; Wenz *et al.*, 2009). The proposal that lack of PGC-1α leads to low-grade inflammation is supported by the present findings that young and aged PGC-1α KO mice had increased plasma TNFα and IL-6 compared with WT mice.

The present findings that aging was associated with increased TNFα mRNA in V-AT and TNFα mRNA and protein in liver as well as increased iNOS and TNFα protein in SkM are in line with previous studies (Lumeng *et al.*, 2011; Wu *et al.*, 2007) and with the present age-associated increase in plasma TNFα. Together, these findings strongly indicate that several tissues are inflamed with aging and potentially contribute to the observed increase in the systemic levels of inflammatory mediators. However, the novel finding that long-term exercise training prevented the age-associated increase in TNFα protein in SkM supports that exercise training specifically elicits anti-inflammatory responses in SkM. Furthermore, the almost similar patterns of plasma TNFα and SkM TNFα protein in response to aging and exercise training indicate that SkM can be an important contributor to the systemic plasma TNFα levels as previously suggested during acute inflammation (Borge *et al.*, 2009; Frost *et al.*, 2002; Olesen *et al.*, 2012). The current demonstration that the reduction in SkM TNFα protein with exercise training was completely blunted in PGC-1α KO mice provides evidence that PGC-1α is required for the exercise training-induced anti-inflammatory effects in SkM. In addition, the novel observation that PGC-1α KO mice had higher iNOS protein levels than WT mice adds to the previously reported anti-inflammatory effect of muscle PGC-1α (Handschin *et al.*, 2007b; Wenz *et al.*, 2009).

To delineate the potential underlying mechanisms behind the observed TNFα protein expression pattern in SkM, several intracellular inflammatory signaling pathways were investigated in SkM. The observed age-associated decrease in SkM IκB-α protein is in accordance with previous findings (Wenz *et al.*, 2009) and may indirectly reflect increased

translocation of NFκB to the nucleus, and thus potentially explain the observed age-associated increase in TNFα protein in WT mice. In contrast, NFκB, JNK or p38 signaling neither explains the observed exercise training-induced nor genotype specific differences in SkM TNFα protein. To our knowledge no previous studies have shown PGC-1α dependent alterations in p38 or JNK signaling, but divergent results have been reported for NFκB signaling (Alvarez-Guardia *et al.*, 2010; Eisele *et al.*, 2013; Olesen *et al.*, 2012; Wenz *et al.*, 2009). Hence, previous studies have both reported that 24 month old muscle-specific PGC-1α overexpression mice had decreased SkM p65 phosphorylation (Wenz *et al.*, 2009) and that young muscle-specific PGC-1α overexpression mice had elevated SkM p65 phosphorylation compared with WT (Olesen *et al.*, 2012). Together with the present findings this may indicate that the interaction between PGC-1α and NFκB depends on the specific experimental setting.

As macrophages are considered an important source of TNFα and iNOS production, the mRNA content of the highly specific murine macrophage marker, F4/80 (Khazen *et al.*, 2005), was determined to evaluate whether differences in macrophage infiltration could explain the observed differences in SkM TNFα and/or iNOS protein content. The finding that SkM F4/80 mRNA content decreased with age and increased with long-term exercise training indicates that macrophages within SkM or in the surrounding capillaries were not responsible for the observed differences in TNFα and iNOS protein in SkM. Although the present data cannot exclude the possibility that infiltrated immune cells have contributed to the observed TNFα and iNOS, these observations imply that SkM fibers may indeed be a considerable source of TNFα (Borge *et al.*, 2009; Frost *et al.*, 2002; Olesen *et al.*, 2012) as well as iNOS production. Taken together, the observed signaling events in SkM in concert with the F4/80 mRNA levels are inadequate to explain the present TNFα and iNOS protein expression patterns. Although speculative this may indicate that the observed TNFα differences are not due to changes in synthesis, but rather degradation/turnover. However, future studies are needed to fully understand the underlying mechanism.

The observed age-associated increase in protein carbonylation in SkM is in accordance with previous studies and supports an association between adiposity, inflammation and oxidative stress (Berg & Scherer, 2005; Hotamisligil *et al.*, 1995). In addition, the increase in protein carbonylation with age may potentially be explained by the observed age-associated decrease in the ROS scavenging protein catalase. Interestingly, exercise training alone and in combination with RSV prevented the age-associated increase in protein carbonylation, which may be due to the observed exercise training and combined exercise training- and RSV-induced increases in anti-oxidant enzymes. These data support that both exercise training and RSV increase the anti-oxidant capacity (Hellsten *et al.*, 1996; Jackson *et al.*, 2011; Leick *et al.*, 2010). However, the observation that RSV supplementation counteracted the age-associated oxidative stress and at the same time only affected GPX1 protein levels may suggest that the RSV-mediated decrease in oxidative stress in part was due to its direct anti-oxidant properties as previously suggested (Howitz *et al.*, 2003; Wood *et al.*, 2004).

Notably, the present finding that young PGC-1α KO mice had increased protein carbonylation in conjunction with reduced SOD2 levels compared with WT mice indicates that PGC-1α is important for the basal ROS handling in young mice (Leick *et al.*, 2010; St-

Pierre *et al.*, 2006). Furthermore these findings support that the increased oxidative stress in these animals in part is due to reduced ROS neutralization capacity as previously reported (St-Pierre *et al.*, 2006). Moreover, while PGC-1α was not required for the observed exercise training and RSV-induced prevention of age-associated oxidative stress, PGC-1α was required for the exercise training-induced increase in SOD2 protein content and in part also for the exercise training- and combined exercise training and RSV-induced increase in catalase and GPX1 protein content. Together these data support that changes in the endogenous anti-oxidant system contribute to the age-associated increase in oxidative stress as well as the exercise training-induced prevention of oxidative stress. However, the precise role of PGC-1α in this is still not clarified.

A key finding of the present study is that long-term RSV supplementation only showed minor effects, which is in contrast to many previous studies in mice (Baur *et al.*, 2006; Lagouge *et al.*, 2006; Um *et al.*, 2010). However, a recent study in mice (Menzies *et al.*, 2013) and two independent human studies in obese men and non-obese women (Poulsen *et al.*, 2012; Yoshino *et al.*, 2012) also failed to show any major impact of RSV supplementation. A study in cultured human primary muscle cells even showed impaired glucose uptake after incubation with RSV (Skrobuk *et al.*, 2012). This underlines the importance of additional studies to fully understand the metabolic effects of RSV. Moreover, the observed additive effect of exercise training and RSV on V-AT and S-AT mass in the present study is modest compared with previous studies in rodents examining the additive effects of exercise training and RSV (Dolinsky *et al.*, 2012; Menzies *et al.*, 2013). The relatively small effects of RSV in the present study do not seem to be dose related. The present dose used corresponds to ~0.7 mg RSV per gram mouse per day, which is similar to previous studies where profound metabolic effects have been observed (Lagouge *et al.*, 2006; Um *et al.*, 2010). The lack of RSV-mediated effects may alternatively be related to the duration of the treatment. Hence, while previous studies have focused on shorter durations (1–4 month), the 12 month treatment in the present study may have had a desensitizing effect. Another likely explanation may be that the 15 month old "control" animals, despite the observed age-associated deteriorations already discussed, were too metabolically "healthy" to obtain metabolic improvements through the RSV treatment. Hence, in contrast to several previous reports on RSV supplementation (Baur *et al.*, 2006; Lagouge *et al.*, 2006; Um *et al.*, 2010), the old mice from the present study did not have impaired glucose tolerance (Ringholm et al., in pending review, Exp. Gerontology) as already mentioned. The novel finding that PGC-1α was not required for the RSV-induced improvements in systemic IL-6 and SkM oxidative stress may indicate that PGC-1α is activated by RSV as suggested (Baur *et al.*, 2006; Lagouge *et al.*, 2006; Timmers *et al.*, 2011), but not mandatory for the metabolic effects of RSV.

In conclusion, the present findings demonstrate that long-term exercise training prevented an age-associated increase in adiposity, systemic low-grade inflammation as well as SkM oxidative stress. In addition, these results show that PGC-1α was required for an exercise training-induced prevention of an age-associated increase in SkM TNFα protein. Long-term RSV supplementation elicited only few effects on SkM oxidative stress and in part on low-grade inflammation and PGC-1α was not required for these effects. Together, the present

findings indicate that regular physical activity is a more powerful intervention to prevent or postpone age-related inflammation than RSV supplementation.

Acknowledgments

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Bullet points

Aging increases adiposity, inflammation and oxidative stress in mice

PGC-1α is required for exercise training-induced reductions in TNFα protein in SkM

Exercise training reduces oxidative stress independently of PGC-1α

Long-term resveratrol suppl. reduces oxidative stress in SkM independently of PGC-1α

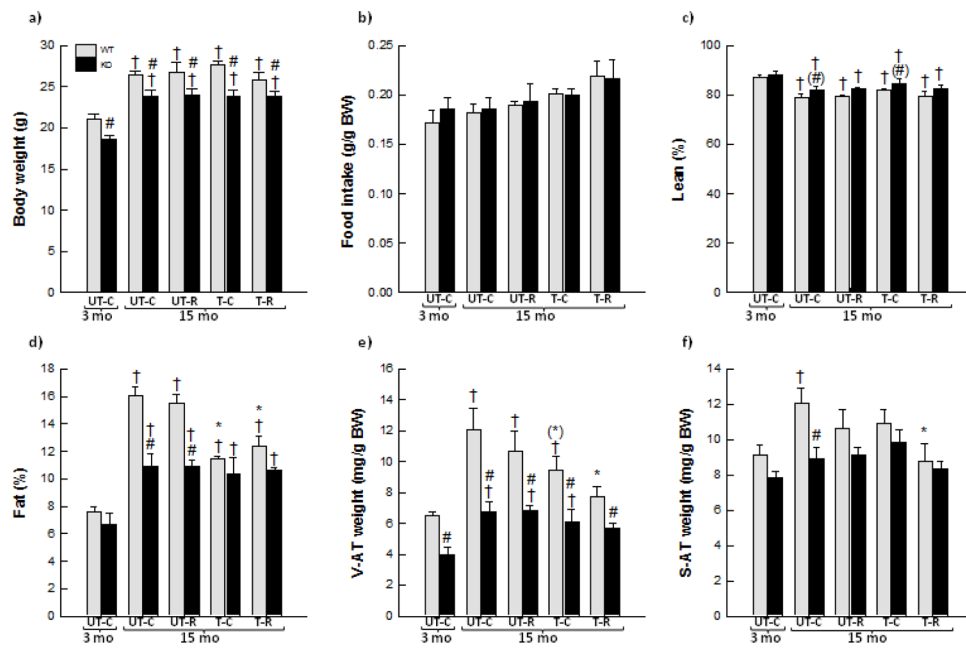Minor effects of long-term resveratrol compared with long-term exercise training

Figure 1.
Body weight (a), food intake (b), lean body mass in % (c), fat % (d), perigonadal visceral adipose tissue (V-AT) weight (e) and inguinal subcutaneous adipose tissue (S-AT) weight from 3 month old (mo) untrained chow mice (UT-C), 15 month old untrained chow mice (UT-C), 15 month old untrained resveratrol (RSV) supplemented mice (UT-R), 15 month old exercise trained (T-C) and 15 month old exercise trained and RSV supplemented (T-R) whole body PGC-1α knockout (KO) and littermate wild type (WT) mice. Values are presented as means ± S.E.; n=8–10 except in (c) and (d) where n=3–8. †: Significantly different from 3 month old UT-C within genotype, p<0.05. *: Significantly different from 15 month old UT-C within genotype, p<0.05. (*): Tends to be significantly different from 15 month old UT-C within genotype, 0.05 p<0.1. #: Significantly different from WT within group, p<0.05. (#) Tends to be significantly different from WT within group, 0.05 p<0.1.
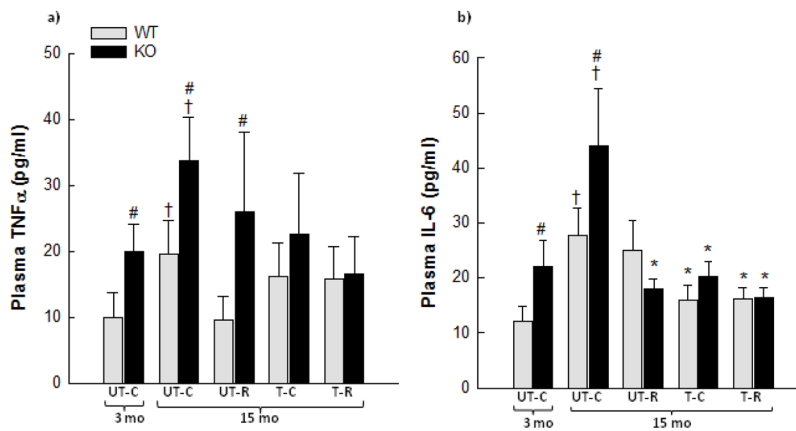
Figure 2.
Plasma tumor necrosis factor (TNF)α (a) and plasma interleukin (IL)-6 (b) from 3 month old (mo) untrained chow mice (UT-C), 15 month old untrained chow mice (UT-C), 15 month old untrained resveratrol (RSV) supplemented mice (UT-R), 15 month old exercise trained (T-C) and 15 month old exercise trained and RSV supplemented (T-R) whole body PGC-1α knockout (KO) and littermate wild type (WT) mice. Values are presented as means ± S.E.; n=8–10. A two-way ANOVA and one-way ANOVA test was applied on logarithmically transformed data when appropriate. †: Significantly different from 3 month old UT-C within genotype, $p<0.05$. *: Significantly different from 15 month old UT-C within genotype, $p<0.05$. #: Significantly different from WT within group, $p<0.05$.
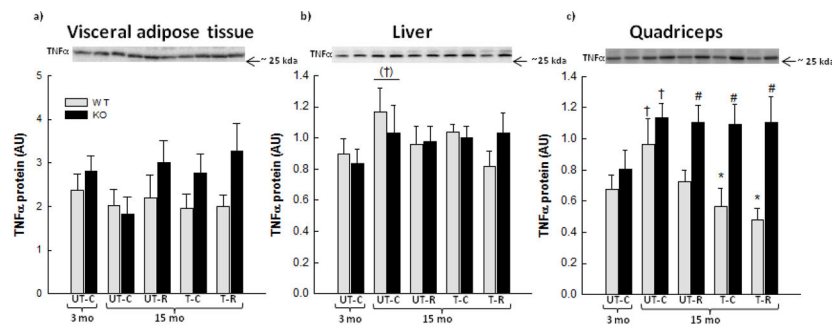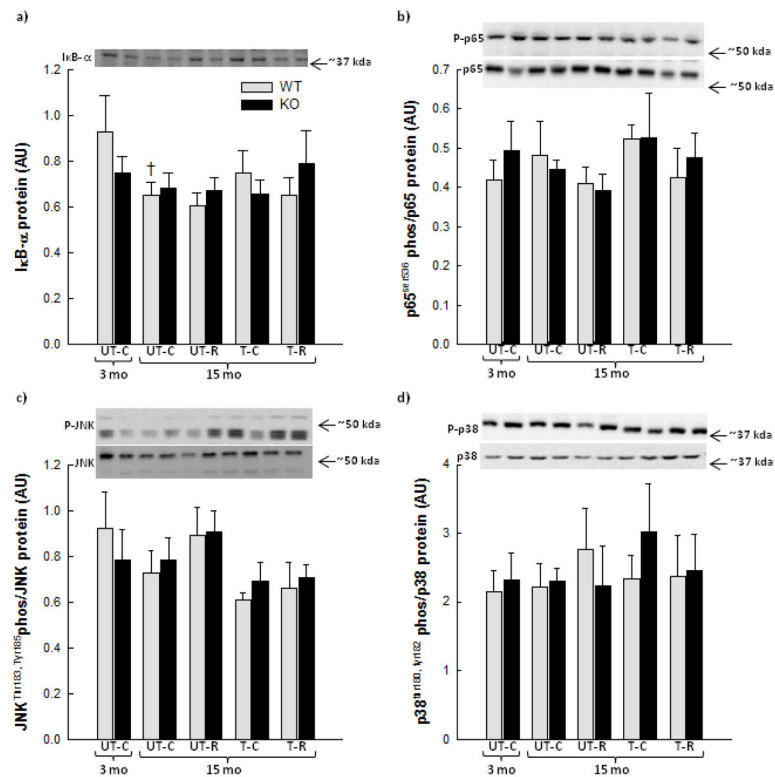
Figure 3.
Tumor necrosis factor (TNF)α protein in perigonadal visceral adipose tissue (V-AT) (a), liver (b) and quadriceps muscle (c) from 3 month old (mo) untrained chow mice (UT-C), 15 month old untrained chow mice (UT-C), 15 month old untrained resveratrol (RSV) supplemented mice (UT-R), 15 month old exercise trained (T-C) and 15 month old exercise trained and RSV supplemented (T-R) whole body PGC-1α knockout (KO) and littermate wild type (WT) mice. Values are presented as means ± S.E.; n=8–10. †: Significantly different from 3 month old UT-C within genotype, $p<0.05$. (†): Tends to be significantly different from 3 month old UT-C, $0.05 \; p<0.1$. *: Significantly different from 15 month old UT-C within genotype, $p<0.05$. #: Significantly different from WT within group, $p<0.05$. A horizontal line indicates a main effect. Representative blots are shown on each figure with samples loaded in the same order as depicted on the graph.

Figure 4.
Nuclear factor of kappa light polypeptide gene enhancer in B-cells (NFκB) inhibitor (IκB)-α protein (a), p65 phosphorylation (phos) (b), c-Jun N-terminal kinase (JNK) phosphorylation (c) and p38 mitogen-activated protein kinase (p38) phosphorylation (d) in quadriceps muscle from 3 month old (mo) untrained chow mice (UT-C), 15 month old untrained chow mice (UT-C), 15 month old untrained resveratrol (RSV) supplemented mice (UT-R), 15 month old exercise trained (T-C) and 15 month old exercise trained and RSV supplemented (T-R) whole body PGC-1α knockout (KO) and littermate wild type (WT) mice. Values are presented as means ± S.E.; n=8–10. †: Significantly different from 3 month old UT-C within genotype, p<0.05. Representative blots are shown on each figure with samples loaded in the same order as depicted on the graph.
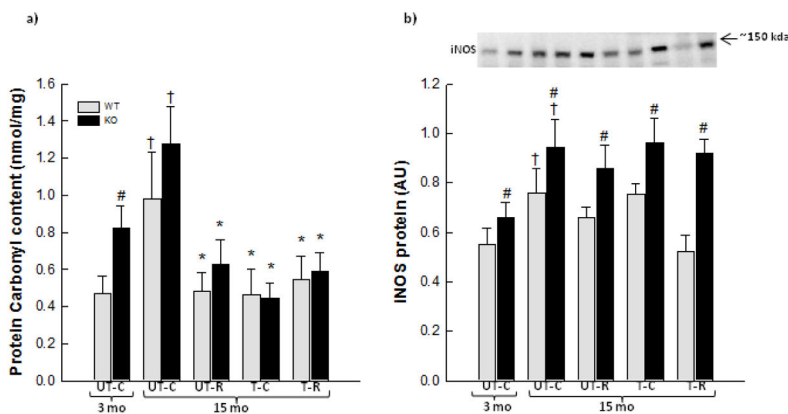
Figure 5.
Protein carbonylation (a) and iNOS protein (b) in quadriceps muscle from 3 month old (mo) untrained chow mice (UT-C), 15 month old untrained chow mice (UT-C), 15 month old untrained resveratrol (RSV) supplemented mice (UT-R), 15 month old exercise trained (T-C) and 15 month old exercise trained and RSV supplemented (T-R) whole body PGC-1α knockout (KO) and littermate wild type (WT) mice. Values are presented as means ± S.E.; n=8–10. †: Significantly different from 3 month old UT-C within genotype, $p<0.05$. *: Significantly different from 15 month old UT-C within genotype, $p<0.05$. #: Significantly different from WT within group, $p<0.05$. Representative blots are shown on each figure with samples loaded in the same order as depicted on the graph.

Figure 6.
Superoxide dismustase (SOD)2 protein (a), catalase protein (b) and glutathione peroxidase (GPX)1 protein (c) in quadriceps muscle from 3 month old (mo) untrained chow mice (UT-C), 15 month old untrained chow mice (UT-C), 15 month old untrained resveratrol (RSV) supplemented mice (UT-R), 15 month old exercise trained (T-C) and 15 month old exercise trained and RSV supplemented (T-R) whole body PGC-1α knockout (KO) and littermate wild type (WT) mice. Values are presented as means ± S.E.; n=8–10. †: Significantly different from 3 month old UT-C within genotype, p<0.05. *: Significantly different from 15 month old UT-C within genotype, p<0.05. (*): Tends to be significantly different from 15 month old UT-C within genotype, 0.05 p<0.1. #: Significantly different from WT within group, p<0.05. Representative blots are shown on each figure with samples loaded in the same order as depicted on the graph.
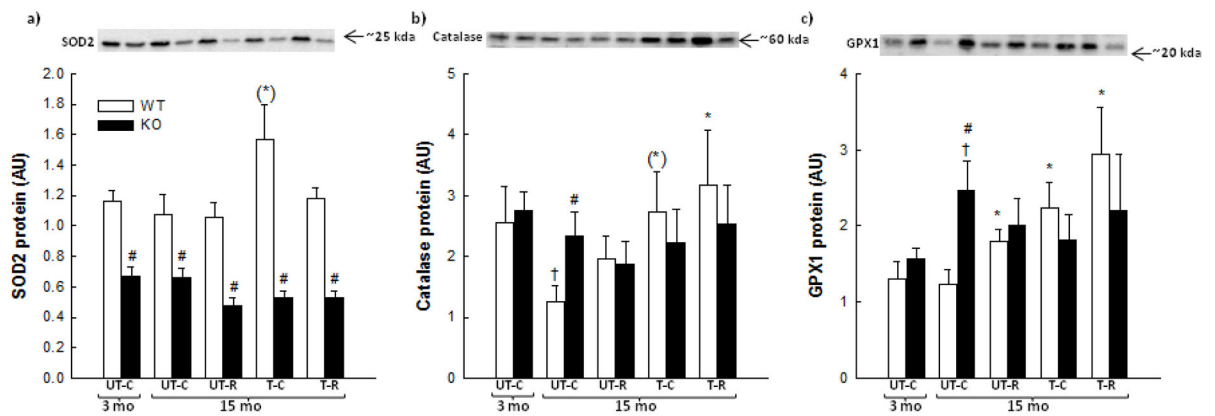
Table 1

Tumor necrosis factor (TNF)α, interleukin (IL)-6 and F4/80 primer and TaqMan probe sequences used for real time PCR.

Gene	Forward primer	Reverse primer	probe
TNFα	5′ ATGGCCCAGACCCTCACA 3′	5′ TTGCTACGACGTGGGCTACA 3′	5′ TCAGATCATCTTCTCAAAATTCGAGTGACAAGC 3′
IL-6	5′ GCTTAATTACACATGTTCTCTGGGAAA′3	5′ CAAGTGCATCATCGTTGTTCATAC′3	5′ ATCAGAATTGCCATTGCACAACTCTTTCTCAT′3
F4/80	5′ GGCTGCCTCCCTGACTTTC 3′	5′ TGCACTGCTTGGCATTGC 3′	5′ TCCTTTTGCAGTTGAAGTTTCCATATCCTTGG 3′
TACE	5′ TGCAAGGCTGGGAAATGC 3′	5′ TTG CACGAGTTGTCAGTGTCAA 3′	5′ AGCAGGAGCTGGAGTCCTGCGC 3′
SOD2	5′ GTGGTGGAGAACCCAAAGGA 3′	5′ AACCTTGGACTCCCACAGACA 3′	5′ AGTTGCTGGAGGCTATCAAGCGTGACTTT 3′
Catalase	5′ CTGGACGTTTTACATCCAGGTCA 3′	5′ TCCTTG TGAGGCCAAACCTT 3′	5′ AGGCAGAAACTTTCCCATTTAATCCATTTGATC 3′
GPX1	5′ GACTGGTGGTGCTCGGTTTC 3′	5′ TTGAGGGAATTCAGAATCTCTTCA 3′	5′ AATCAGTTCGGACACCAGGAGAATGGC 3′

Table 2

mRNA content in visceral adipose tissue, liver and skeletal muscle

Tumor necrosis factor (TNF)α, interleukin (IL)-6 and F4/80 mRNA in perigonadal visceral adipose tissue (V-AT), liver and skeletal muscle (SkM) from 3 months old (mo) untrained chow mice (UT-C), 15 month old untrained chow mice (UT-C), 15 month old untrained resveratrol (RSV) supplemented mice (UT-R), 15 month old exercise trained (T-C) and 15 month old exercise trained and RSV supplemented (T-R) whole body PGC-1α knockout (KO) and littermate wild type (WT) mice. Values are presented as means ± S.E.; n=8–10. Two-way ANOVA and one-way ANOVA tests were applied on logarithmically transformed data when appropriate. Mann-Whitney U nonparametric test was applied when appropriate

	gene	3 mo UT-C		15 mo UT-C		15 mo UT-R		15 mo T-C		15 mo T-R	
		WT	KO	WT	KO	WT	KO	WT	KO	WT	KO
V-AT	TNFα	0.5 ± 0.1	0.8 ± 0.2	$2.3 \pm 0.7^{\dagger}$	$0.7 \pm 0.2^{\#}$	0.9 ± 0.2	$1.3 \pm 0.3^{*}$	1.0 ± 0.3	0.7 ± 0.2	0.9 ± 0.1	1.4 ± 0.4
	IL-6	0.8 ± 0.2	0.6 ± 0.1	0.6 ± 0.1	0.4 ± 0.1	$0.9 \pm 0.1^{*}$	$0.8 \pm 0.1^{*}$	$1.2 \pm 0.1^{*}$	$0.7 \pm 0.2^{\#}$	0.7 ± 0.1	0.5 ± 0.1
	F4/80	0.8 ± 0.2	0.5 ± 0.2	1.2 ± 0.3	0.7 ± 0.3	0.6 ± 0.3	0.7 ± 0.1	1.3 ± 0.2	0.7 ± 0.3	0.7 ± 0.2	0.9 ± 0.3
Liver	TNFα	0.3 ± 0.03	0.3 ± 0.04	$0.6 \pm 0.1^{\dagger}$	$0.3 \pm 0.03^{\#}$	0.5 ± 0.1	$0.5 \pm 0.1^{*}$	0.5 ± 0.1	0.4 ± 0.04	0.5 ± 0.04	$0.5 \pm 0.1^{*}$
	IL-6	0.2 ± 0.02	0.2 ± 0.01	0.2 ± 0.01	0.2 ± 0.02	0.1 ± 0.01	0.2 ± 0.03	0.2 ± 0.02	0.2 ± 0.02	0.2 ± 0.02	0.2 ± 0.03
	F4/80	0.6 ± 0.04	0.5 ± 0.1	0.7 ± 0.1	$0.4 \pm 0.1^{\#}$	0.5 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	0.5 ± 0.03	0.7 ± 0.1
SkM	TNFα	1.1 ± 0.1	0.7 ± 0.1	1.4 ± 0.2	$1.6 \pm 0.3^{\dagger}$	1.2 ± 0.3	1.6 ± 0.2	$2.6 \pm 0.6^{*}$	1.9 ± 0.2	1.9 ± 0.3	2.4 ± 0.4
	IL-6	0.7 ± 0.1	0.5 ± 0.1	$1.0 \pm 0.1^{(\dagger)}$	$1.0 \pm 0.1^{\dagger}$	0.7 ± 0.2	1.0 ± 0.1	$0.7 \pm 0.1^{*}$	$0.7 \pm 0.1^{*}$	$0.9 \pm 0.1^{*}$	$0.7 \pm 0.1^{*}$
	F4/80	2.4 ± 0.3	2.3 ± 0.3	$1.2 \pm 0.2^{\dagger}$	1.6 ± 0.3	0.8 ± 0.2	$1.6 \pm 0.3^{\#}$	$3.7 \pm 1.1^{*}$	$3.0 \pm 0.4^{*}$	$2.0 \pm 0.3^{*}$	$3.5 \pm 0.4^{\#*}$

†Significantly different from 3 month old UT-C within genotype, p<0.05.

$^{(\dagger)}$Tends to be significantly different from 3 month old UT-C within genotype, 0.05 p<0.1.

*Significantly different from 15 month old UT-C within genotype, p<0.05.

$^{\#}$Significantly different from WT within group, p<0.05.