Resveratrol Blunts the Positive Effects of Exercise Training on Cardiovascular Health in Aged Men

Lasse Gliemann[1], Jakob Friis Schmidt[1], Jesper Olesen[2], Rasmus Sjørup Biensø[2], Sebastian Louis Peronard[1], Simon Udsen Grandjean[1], Stefan Peter Mortensen[3], Michael Nyberg[1], Jens Bangsbo[1], Henriette Pilegaard[2] and Ylva Hellsten[1]

[1]Integrated Physiology Group, Department of Nutrition, Exercise and Sports. [2]Centre of Inflammation and Metabolism, Department of Biology; University of Copenhagen, Copenhagen, 2100; Denmark. [3]Centre of Inflammation and Metabolism; Rigshospitalet; Copenhagen, 2100; Denmark.

Corresponding author Lasse Gliemann, Universitetsparken 13, 2. Floor, 2100 Copenhagen Denmark, Phone: 0045 35321608, Fax: 0045 35321600, Email: gliemann@ifi.ku.dk

Key points summary

- In rodents, resveratrol has been shown to enhance training-induced changes in cardiovascular function, exercise performance and the retardation of atherosclerosis. We examined the effect of 8 weeks of exercise training with and without concomitant resveratrol supplementation in aged men.

- Exercise training potently improved blood pressure, blood cholesterol, maximal oxygen uptake and the plasma lipid profile.

- Resveratrol supplementation was found to reduce the positive effect of exercise training on blood pressure, blood cholesterol and maximal oxygen uptake and did not affect the retardation of atherosclerosis.

- Whereas exercise training improved formation of the vasodilator prostacyclin, concomitant resveratrol supplementation caused a shift in vasoactive systems favoring vasoconstriction.

- The present study is the first to demonstrate negative effects of resveratrol on training-induced improvements in cardiovascular health parameters in humans and add to the growing body of evidence questioning the positive effects of resveratrol supplementation in humans.

Abstract

Aging is thought to be associated with decreased vascular function partly due to oxidative stress. Resveratrol is a polyphenol, which, in animal studies has been shown to decrease atherosclerosis, improve cardiovascular health and physical capacity, in part through its effects on Sirtuin 1 signaling and through an improved antioxidant capacity. We tested the hypothesis that resveratrol supplementation enhances training-induced improvements in cardiovascular health parameters in aged men. Twenty-seven healthy physically inactive aged men (age: 65 ± 1 years; BMI: 25.4 ± 0.7 kg/m^2; MAP: 95.8 ± 2.2 mmHg; maximal oxygen uptake: 2488 ± 72 ml O$_2$ min^{-1}) were randomized into 8 weeks of either daily intake of either 250 mg *trans* resveratrol ($n = 14$) or of placebo ($n = 13$) concomitant with high-intensity exercise training. Exercise training lead to a 45% greater ($P < 0.05$) increase in maximal oxygen uptake in the placebo group than in the resveratrol group and to a decrease in MAP in the placebo group only (-4.8 ± 1.7 mmHg; $P < 0.05$). The interstitial level of vasodilator prostacyclin was lower in the resveratrol than in the placebo group after training (980 ± 90 *versus* 1174 ± 121 pg ml^{-1}; $P < 0.02$) and muscle TBX synthase was higher in the resveratrol group after training ($P < 0.05$). Resveratrol administration also abolished the positive effects of exercise on LDL, TC/HDL ratio and triglycerides concentrations in blood ($P < 0.05$). Resveratrol did not potentiate the effect of exercise training on atherosclerosis marker VCAM-1. Sirtuin 1 protein levels were not affected by resveratrol supplementation. These findings indicate that, whereas exercise training effectively improves several cardiovascular health parameters in aged men, concomitant resveratrol supplementation blunts most of these effects.

Abbreviations CAT, catalase; COX, cyclooxygenase; eNOS, endothelial nitric oxide synthase; ET-1, Endothelin 1; ET-A, Endothelin receptor A; ET-B, Endothelin receptor B; GPX, glutathione persoxidase; NOx, nitrite and nitrate; MAP, mean arterial pressure; PGI_2, prostacyclin; ROS, reactive oxygen species; SIRT1, sirtuine 1; SOD, superoxide dismutase; TBX, thromboxane; VCAM-1, vascular cell adhesion molecule.

Introduction

A sedentary lifestyle with a subsequent poor physical fitness level, is one of the major cardiovascular risk factors in otherwise healthy individuals (Perk et al., 2012). Moreover, atherosclerosis, and its clinical sequelae, are well-described consequences of a physically inactive lifestyle and progressing age (Wang & Bennett, 2012). Lowering of blood lipids other than HDL has a major impact on decreasing the atherosclerotic process (Perk et al., 2012) and exercise training has been recognized as an intervention for improving the balance of blood lipids (Lopez-S et al., 1974). The plasma VCAM-1 level is a good indicator of atherosclerosis progression (Peter et al., 1997) and a positive effect of exercise training on plasma VCAM-1 levels has previously been shown (Schumacher *et al.*, 2006; Ranković *et al.*, 2009).

In young healthy subjects, skeletal muscle blood flow and oxygen supply are tightly regulated to match oxygen demand of the muscle (Andersen & Saltin, 1985). The precise vascular control is the result of a complex interplay between sympathetic nervous activity, mechanical factors and vasoactive substances including nitric oxide (NO), prostacyclin (PGI_2) and endothelin-1 (ET-1) (Maeda *et al.*, 1997; Clifford & Hellsten, 2004; Hellsten *et al.*, 2012*b*). With age, vascular function is impaired (Nyberg et al., 2012), resulting in increased resting blood pressure and decreased limb blood flow for a given submaximal workload (Wahren *et al.*, 1974; Proctor *et al.*, 1998; Lawrenson *et al.*, 2003; Kirby *et al.*, 2009; Nyberg *et al.*, 2012; Mortensen *et al.*, 2012*b*). These age-related vascular changes have been shown to be associated with alterations within the NO, PGI_2 and ET-1 systems (Taddei *et al.*, 1995; 2001; Stauffer *et al.*, 2008; Kirby *et al.*, 2009). Specifically, reactive oxygen species (ROS) have been shown to have adverse effects on these systems by interfering with PGI_2 and NO synthesis and by scavenging of NO (Higashi *et al.*, 2002; Schulz *et al.*, 2008;

Schildknecht & Ullrich, 2009). ROS formation occurs continuously and, although deleterious to cell vitality when excessive, ROS also exert beneficial functions such as in molecular signaling (Gomes *et al.*, 2012). This may be why, ROS production and removal is well regulated in young healthy subjects (Gomes *et al.*, 2012). In aged subjects however, ROS levels appear to be less well regulated as production increases and the endogenous antioxidant defense decreases leading to adverse oxidative stress and decreased vascular function (Wei *et al.*, 1998; Finkel & Holbrook, 2000). Known sources of ROS include NAD(P)H oxidase (NOX), uncoupled endothelial NO synthase (eNOS) and mitochondria (Ungvari *et al.*, 2007*a*; Durrant *et al.*, 2009; Yang *et al.*, 2009). NOX is expressed in endothelial-, smooth muscle- and skeletal muscle cells and is the main source of ROS implicated in vascular dysfunction (Schulz et al., 2008) and exercise-induced oxidative stress (Cave et al., 2006). ROS removal is accomplished by antioxidants and antioxidant enzymes, including superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT) (Powers & Lennon, 1999). The endogenous antioxidant defense system is highly important in the prevention of excessive ROS levels, tissue inflammation and consequent vascular dysfunction (Förstermann, 2008). Exercise training effectively enhances blood antioxidant capacity (Miyazaki et al., 2001) and improves vascular function by reducing oxidative stress (Taddei *et al.*, 2000; Eskurza *et al.*, 2004) but the antioxidant defense may also be boosted by exogenous antioxidant supplementation (Anderson et al., 1997; Cao et al., 1998; Hellsten et al., 2007). Thus, as exercise is associated with enhanced production of ROS (Davies et al., 1982; Hellsten et al., 2007), antioxidant supplementation in combination with physical training may be particularly relevant for aged individuals where ROS levels are likely to be less well regulated (Bailey et al., 2010).

The antioxidant resveratrol is a naturally occurring polyphenol (Baur & Sinclair, 2006). In rodents resveratrol supplementation has been shown to decrease cardiovascular risk factors, including blood lipids (Murase et al., 2009) and VCAM-1 (Matos et al., 2012), to improve cardiovascular function, physical capacity (Taubert & Berkels, 2003; Lagouge *et al.*, 2006) and to decrease inflammation in the vasculature of aged animals leading to improved vascular function (Pearson et al., 2008). Specifically, the positive effect of resveratrol on training response and aerobic capacity in rats has been shown to be mediated via Sirtuin 1 (SIRT1) (Lagouge *et al.*, 2006; Price *et al.*, 2012; Hart *et al.*, 2013). In humans resveratrol has been shown to improve metabolic function of in obese men mediated via SIRT1 (Timmers *et al.*, 2011) but recent reports contradicts this finding (Skrobuk *et al.*, 2012; Yoshino *et al.*, 2012). Although the effects of resveratrol on cardiovascular health have been examined in rats, few studies have been conducted on humans and to date no study has examined the combined effect of exercise training and resveratrol on vascular function in aged humans.

Based on the knowledge that; a) aging is associated with increased oxidative stress; b) exercise is associated with an increased ROS production and; c) resveratrol improves cardiovascular parameters by decreasing ROS and via SIRT 1 mediated signaling, the following hypothesis was tested in the present study: Oral resveratrol supplementation enhances the positive cardiovascular adaptations to exercise training in aged subjects by increasing SIRT1 mediated signaling and by promoting the endogenous antioxidant system. To elucidate this we conducted a double blind exercise training study including 27 aged healthy men randomized into two groups. Both groups underwent 8 weeks of high-intensity exercise training and one group received resveratrol and the other group placebo supplementation.

Experimental procedures

Ethical approval

The study was approved by the Ethics Committee of Copenhagen and Frederiksberg communities (H-2-2011-079) and was conducted in accordance with the latest guidelines of the *Declaration of Helsinki*. Written informed consent was obtained from all subjects before enrollment in the study.

Subjects

Twenty-seven healthy aged (60-72 years) physically inactive (less than 2 hours of moderate intensity physical activity per week) men were recruited. All subjects were non-smokers and underwent a medical examination. None had been diagnosed with cardiovascular disease, hypertension, renal dysfunction, insulin resistance or type 2 diabetes and all subjects had normal ECG. Two subjects were diagnosed with hypercholesterolemia regulated by their own physician (medication was maintained during the experimental period) whereas the other participants had normal cholesterol levels.

Randomization

The study was of a randomized double-blind placebo-controlled design. Subjects were allocated into either a combination of exercise training and placebo (n = 13) or exercise training and 250 mg day^{-1} *trans*-resveratrol (Fluxome Inc., Stenlose, Denmark, n = 14) based on maximal oxygen consumption, BMI, blood glucose and cholesterol. The interventions lasted for 8 weeks. The subjects received tablets every two weeks and were instructed to take their daily tablet at the same time every

morning. Subjects noted time of consumption for each tablet and any discomforts that might appear throughout the intervention period.

Exercise training regime

All subjects performed 8 weeks of supervised high intensity interval training (cycle ergometer) twice a week and full body circuit training (Crossfit) once a week. Intensity of the training sessions was controlled with TEAM2 WearLink+ (Polar, Kempele, Finland) heart rate monitors. In addition subjects conducted a timed 5 km walk once a week.

Pre testing

Before the two main experimental days the subjects visited the laboratory where body composition was determined with a whole-body dual-energy X-ray absorptiometry scanning (DEXA; Prodigy, GE healthcare, Chalfont St. Giles, UK). In addition femoral arterial blood flow was measured at rest, after 30 seconds of passive leg movement and during one-leg knee-extensor exercise (at 10 and 30 W) with ultrasound Doppler (Logic E9, GE Healthcare, Pittsburgh, PA, USA) equipped with a linear probe operating an imaging frequency of 9MHz and Doppler frequency of 4.2-5.0 MHz (Nyberg et al., 2012). Due to technical issues passive flow was only measured after training in the two groups. After 30 minutes rest, subjects performed an incremental bicycle ergometer test to determine maximal pulmonary oxygen uptake (l min^{-1}, Oxycon Pro, Viasys Healthcare, Hoechberg, Germany). For determination of performance related to functional performance, subjects conducted the following series of tests on a separate day: i) a timed "Up & Go" test (Podsiadlo & Richardson, 1991), as a test of functional mobility, ii) a 30 second "Chari-stand" test

(Jones *et al.*, 1999), as a test of lower body strength, iii) "The New Danish Steptest" (Isaksen & Pedersen, 2006), as a test of maximal functional capacity and iv) "Unipedal stance test" (Springer *et al.*, 2007), as a test of balance.

Experimental days

On the first experimental day, subjects arrived at the laboratory after an overnight fast. Blood samples were collected and a muscle biopsy was obtained from m. vastus lateralis with percutaneous needle biopsy technique (Bergstrom, 1975).

On the second experimental day, subjects were seated in a one-leg knee-extensor ergometer and performed a ten-minute exercise bout (10 W) to become accustomed to the movement. After local anaesthesia (Lidocaine, 20 mg mL^{-1}), four custom-made microdialysis probes with at 4 cm membrane (960 kD cut-off) were inserted into the m. vastus lateralis of the experimental leg. After insertion of the probes, the subjects performed another ten-minute knee-extensor exercise bout (10 W) to minimize the tissue response to insertion trauma (Nordsborg *et al.*, 2003). The probes were perfused with Ringer acetate buffer at a rate of 5 μl min^{-1} and to determine the relative exchange over the membrane, a small amount (2.7nM) of [2-^{3}H] adenosine (<0.1 μCi ml^{-1}) was added to the perfusate, to allow for calculation of probe recovery. After 45 minutes of rest microdialysate was collected for 3 x 20 minutes, while subjects were resting. After 1.5 hour of supine rest blood pressure was measured three consecutive times with an automatic sphygmomanometer (M7, OMRON, Vernon Hills, IL) on the left and right upper arm. Subjects then performed 45 minutes of knee-extensor exercise (10 W) and dialysate were collected for 2 x 20 minutes, excluding the first 5 minutes to account for probe delay. Immediately after collection, samples were weighed, 5μl of the dialysate were allocated into 3 ml Ultima Gold (Perkin Elmer)

scintillation liquid and the remaining dialysate was frozen at -80° C. The probe recovery (PR) was calculated as [PR = (dpm$_{infusate}$ - dpm$_{dialysate}$ / dpm$_{infusate}$)], where dpm denotes disintegrations per minute (Scheller & Kolb, 1991; Jansson *et al.*, 1994). The [2-^{3}H] ATP activity of the dialysate was measured on a liquid scintillation counter (Tri-Carb 2910 TR; Perkin Elmer). Probes with abnormal perfusion rate (> ±10%) were excluded.

All tests and experimental days were performed before and after the 8-week intervention period.

Quantification of protein expression by western blot

Freeze-dried tissue samples were dissected free of connective tissue, visible fat and blood under stereomicroscope with an ambient temperature of ~18°C and relative humidity below 30%. The tissue samples were homogenized in lysis buffer and Western blot analysis was preformed as previously described (Høier *et al.*, 2011) with the exception that the membrane image was digitalized on a ChemiDoc MP system (Bio-Rad, Hercules, CA, USA). Equal amounts of total protein were loaded for each sample in accordance to the antibody optimization (detailed antibody information is available in supplemental table S1). Samples from each group were distributed evenly across the gel and all samples from one subject were loaded on the same gel. To control for loading differences, the blots were also analyzed for glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Analysis of NOx, Endothelin-1 and VCAM-1 in plasma

The stable metabolites of NO in plasma, nitrite and nitrate, were measured using a fluorometric EIA kit (Cayman Chemical Co., Ann Harbor, MI, USA). Endothelin-1 and VCAM-1 in plasma were measured with Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA).

Analysis of 6-keto prostaglandin $F_1\alpha$ in microdialysate

The stable metabolite of PGI_2, 6-keto prostaglandin $F_1\alpha$ in microdialysate was measured using a fluorometric assay kit (Cayman Chemical Co., Ann Harbor, MI, USA).

Statistical analysis

To test the effect of training- and training plus resveratrol, a two-way repeated measures ANOVA was conducted. After a significant F-test, pairwise differences were identified using the Student-Newman-Keuls post hoc procedure. The significance level was set at $P < 0.05$. Data are means ± SEM and n = 13/14 for the placebo/resveratrol group respectively, unless otherwise stated. The occasional missing data were either due to sample limitations or technical issues.

Results

Compliance with the interventions

Both groups exhibited high training compliance and completed an average 1.9 ± 0.1 and 1.0 ± 0.1 spinning and Crossfit sessions per week. Training intensity was equally high in both groups (67 % of training time above 70% HR_{max} and 14 % of training time above 90 % HR_{max}). Based on self-reports all subjects took their daily tablet and the ingested doses of resveratrol were well tolerated by the subjects.

Functional capacity

Maximal oxygen uptake was not different between the groups before training but 8 weeks of exercise training increased maximal pulmonary oxygen uptake more (P < 0.05) in the placebo group than in the resveratrol group (a 443 ± 38 and a 308 ± 46 ml O_2 min^{-1} increase in the placebo- and resveratrol group, respectively; P = 0.03; table 1). After training maximal oxygen uptake was higher in the placebo group than in the resveratrol group (P < 0.05; figure 1). Performances in the timed Up & Go test, 30-second Chair-stand test, Step test and Unipedal stance test were not different between groups before training. After training performance in the Up & Go test was increased in the placebo group only (P < 0.001) while performances in the Chair-stand test, Step test and 5k-walk test were increased in both groups (P < 0.001). Balance was unaffected by the interventions in both groups (table S2).

Body composition

Training did not change BMI but total body fat was decreased similarly in both groups after training relative to baseline (P < 0.05; table 1).

Cardio-vascular parameters

Mean arterial blood pressure was not different between the placebo group and resveratrol group before training but was 5 mmHg lower after than before training in the placebo group only (P < 0.005; n = 13/13, table 1). Blood glucose, total cholesterol (TC) and HDL concentrations were not different between groups and were not changed with training. The TC/HDL ratio and concentrations of LDL and triglycerides were not different between groups before training, but were lower in the

placebo group only after training (P < 0.05; n = 13/13, table 1). VCAM-1 levels were similar between groups before training and decreased significantly in both groups with training (P < 0.05; table 1).

Training did not change exercise hyperemia in either group and there was no difference between groups at rest, after 30 seconds passive work (data were only collected for rest and passive work post intervention) or at either 10W or 30W workloads before or after the training period (n = 13/12, figure 2).

Protein expression in skeletal muscle

eNOS levels were not different between groups before training and were not affected by training. Phosphorylation of eNOS at serine residue 1177 (eNOS-P^{Ser1177}; phosphorylation at this residue increases enzyme activity) was not different between groups before training, but was decreased by 11% in the placebo group with training (P < 0.01; n = 11/13; figure 3A). nNOS protein amount was not different between groups before or after training with no effect of resveratrol. There was a significant overall increase in nNOS protein expression with training (P < 0.02; figure 3A) independent of groups. Muscle PGI$_2$-synthase protein expression was similar in the two groups before and after training and the PGI$_2$-synthase protein levels increased similarly 31 % and 43 % (P < 0.05; n = 12/14; figure 3B) with training in the placebo and resveratrol group, respectively. COX-1 and COX-2 protein expression in muscle homogenates were not different between groups before or after training. There was a significant overall increase in COX-1 protein expression with training (P < 0.05; figure 3B) with no difference between groups. COX-2 increased similarly in both groups with training (P < 0.001; n = 12/14; figure 3B). TBX synthase was not

different between the groups before training but increased 61% with training in the resveratrol group only ($P < 0.01$; figure 3B).

Endothelin-1 levels in muscle lysate were similar in the two groups prior to training and did not change with training (figure 4). Muscle endothelin receptor type A levels were not different between the placebo and the resveratrol group before training and were increased similarly with training ($P < 0.02$; n = 12/13; figure 4). Endothelin receptor type B protein content was not different between groups before training but increased significantly in the resveratrol group with training ($P < 0.05$; n = 12/13; figure 4). Muscle SIRT1 protein content was similar in the two groups before the training intervention and did not change with training in either group (figure 5). Content of NOX in muscle lysate was similar in the placebo and resveratrol groups before training and increased 55 % and 71 %, in the placebo and resveratrol group, respectively ($P < 0.001$; n = 12/14; figure 6) with training. CAT, GPX-1 and SOD1 protein amounts were not different between groups either before or after training. GPX-1 protein content showed a significant overall increase with training ($P < 0.05$; figure 6). SOD2 protein expression was not different between groups before training and increased by 30% and 50% in the placebo- and the resveratrol group, respectively, with training ($P < 0.05$; figure 6).

Interstitial prostacyclin

PGI_2 in dialysate from m. vastus lateralis at rest was not different between the placebo group and the resveratrol group before training (1233 ± 198 *vs.* 1143 ± 122 pg ml^{-1}), but was higher in the placebo group than in the resveratrol group after training (1174 ± 121 *vs.* 980 ± 90 pg ml^{-1}; $P < 0.02$; n = 13/12). Before training PGI_2 was unchanged during exercise in the placebo group (1397 ± 172 pg ml^{-1}) and increased significantly

in the resveratrol group (2242 ± 525 pg ml^{-1}; $P < 0.02$). After training PGI$_2$ increased during exercise in the placebo group (1921 ± 323 pg ml^{-1}; $P < 0.02$; figure 7) but not significantly in the resveratrol group (1510 ± 179 pg ml^{-1})

Plasma NOx and Endothelin-1. Plasma NOx levels were similar in the placebo- and the resveratrol group before- (34.5 ± 3.0 *vs.* 34.7 ± 2.7 µmol min^{-1}; n = 12/14) and after training (33.9 ± 2.3 *vs.* 34.5 ± 2.7 µmol min^{-1}). The placebo group had significantly higher plasma Endothelin-1 levels than the resveratrol group before training ($P < 0.01$; figure 4), but not after training.

Discussion

The major findings of the present study were that 8 weeks of exercise training induced a number of beneficial cardiovascular effects in aged men, but in contrast to our hypothesis, parallel supplementation with resveratrol reduced several of these improvements. Specifically, resveratrol supplementation combined with training abolished the reduction in blood pressure and in blood lipids, altered the balance between prostanoid vasodilators and vasoconstrictors and led to a significantly lower increase in the training induced increase in maximal oxygen uptake. The observed effects were not related to a change in SIRT1 protein expression as the SIRT 1 level was not altered with either exercise training or training combined with resveratrol supplementation.

Based on previous animal studies (Murase *et al.*, 2009; Dolinsky *et al.*, 2012; Menzies *et al.*, 2013; Hart *et al.*, 2013), we hypothesized that supplementation with resveratrol would potentiate the positive effects of exercise training on cardiovascular risk factors.

However, although exercise training alone was effective in decreasing the plasma LDL cholesterol level, the TC/HDL ratio, the triglyceride concentration and the VCAM-1 level, resveratrol supplementation did not potentiate the effect of exercise training on these cardiovascular risk factors. Moreover, resveratrol supplementation abolished the effect of exercise training on blood lipids and had no effect on plasma VCAM-1 levels. Regulation of lipoproteins has recently been shown to be mediated by AMPK and PGC-1α (Greene et al., 2012) and recent evidence suggest that resveratrol inhibits, rather than activates AMPK and PGC-1α in humans (Skrobuk et al., 2012; Yoshino et al., 2012), which could explain the negative effect of resveratrol on the training induced improvements in blood lipids.

In the present study exercise training alone lowered mean arterial blood pressure whereas the group receiving resveratrol in the training period experienced no reduction in blood pressure. Decreased vascular function and increased blood pressure in aging and various cardiovascular diseases are associated with alterations in several factors including an imbalance in vasodilator and vasoconstrictor systems (Taddei *et al.*, 1993; Spratt *et al.*, 2001; Kirby *et al.*, 2009). One of the vasodilator systems that have been proposed to be a major factor in improvements in cardiovascular function with exercise training is NO and eNOS (Hambrecht et al., 2003). In this study, eNOS content and the concentration of NO metabolites in plasma remained unaltered with training. However, in accordance with our previous observations (Hansen *et al.*, 2011; Hellsten *et al.*, 2012*a*) the prostanoid system was improved by training as evidenced by an increase in the protein content of COX and PGI$_2$ synthase in the muscle tissue and the muscle interstitial concentration of PGI$_2$. The mechanism underlying the lack of blood pressure reduction in the group receiving resveratrol supplementation in

parallel with training is not clear, however, the training induced increase in interstitial PGI_2, observed in the placebo group, was absent in the resveratrol group and there was a parallel increase in the protein content of TBX synthase in muscle in the resveratrol group which was not present in the placebo group. As TBX synthase and PGI_2 synthase both compete for substrates in the arachidonic acid pathway, the increase in TBX synthase may suggest a shift in the balance between prostanoid vasodilator versus vasoconstrictor formation in the resveratrol group. This shift in balance could be one explanation for the abolished training induced reduction in blood pressure in the resveratrol group.

Another factor that could have affected blood pressure changes was the levels of ET-1. Plasma ET-1 levels tended ($P = 0.06$) to be reduced after training in the placebo group, and may, combined with the apparent increase in PGI_2 formation, have contributed to the reduced blood pressure after training. Furthermore, the observed training induced increase in ET-A receptors in the present study could be associated with the ameliorations in glucose handling generally seen after a training period (Goodyear & Kahn, 1998; Shemyakin *et al.*, 2011). Resveratrol supplementation had no further effect on ET-1 levels in plasma, ET-1 or ET-A receptor concentration in the skeletal muscle but it was found that the ET-B receptor protein content in the muscle increased only in the resveratrol group. This increase could promote either vasodilation or constriction depending of the cellular site of the receptor (Miyauchi & Masaki, 1999).

As an indicator of vascular function, we measured femoral arterial blood flow in response to exercise. The magnitude of flow during exercise was similar to that observed previously in our group for aged individuals (Nyberg *et al.*, 2012) however,

no differences in flow between groups or with exercise training were observed. Vascular function was also assessed in the two groups by measurements of leg blood flow in response to passive movement of the lower leg. Passive movement leads to an increase in leg blood flow which has been shown to be highly dependent on the formation of NO (Mortensen *et al.*, 2012*a*). In both the placebo and resveratrol groups, the increase in flow with passive movement was limited relative to what has been observed in young healthy individuals (Mortensen *et al.*, 2012*a*) and there was no difference between groups. This finding is in accordance with our previous observation of an age-related decline in passive flow response (Mortensen *et al.*, 2012*a*). Due to technical issues passive flow was only measured after training in the two groups, thus a training effect was not possible to assess.

The current intervention with high-intensity cycling training in combination with intense cross-fit training was shown to be highly effective in improving maximal oxygen uptake, as evidenced by an extensive increase in maximal oxygen uptake (19%) in the placebo group. However, the observation that resveratrol supplementation combined with exercise training induced a 45% lower increase in maximal oxygen uptake than training with placebo was unexpected. Recent studies on rodents have shown that resveratrol supplementation during a period of exercise training potentiates the increase in maximal oxygen uptake compared with exercise training alone (Dolinsky *et al.*, 2012; Menzies *et al.*, 2013; Hart *et al.*, 2013). The effect of resveratrol has been related to increased mitochondrial biogenesis mediated via SIRT1 activation of AMPK and PGC-1α. The opposite finding in the current study may be explained by species differences in the effect of resveratrol on these pathways. Accordingly resveratrol has recently been shown to blunt (Yoshino et al.,

2012) or even inhibit (Skrobuk et al., 2012) AMPK and PGC-1α in human tissue. Discrepancy between animal and human response have previous been shown and when investigating models of inflammatory diseases the translational potential of murine models to the human species have been shown to be particularly poor (Seok et al., 2013). In the present study we did not observe any increase in protein expression of SIRT1 in either the placebo or the resveratrol group supporting the findings that neither exercise nor resveratrol are stimulators of SIRT1 protein content in aged humans. In comparing the current negative effect of resveratrol on maximal oxygen uptake to previous rodent studies it is also important to emphasize that, in contrast to what is suggested in rodents (Lagouge *et al.*, 2006; Menzies *et al.*, 2013; Hart *et al.*, 2013), in humans, aerobic power is generally not thought to be associated with limitations in mitochondrial respiration capacity (Boushel et al., 2011) but rather limited by central cardiovascular components such as ventricular size, myocardial contractility and blood volume (Saltin *et al.*, 1976; Ehsani *et al.*, 1991). Thus, the lower increase in maximal oxygen uptake with training in the resveratrol group was unlikely to be related to mitochondrial biogenesis. Future studies should address whether the negative effect of resveratrol is due to adverse effects on central cardiovascular aspects such as blood volume and/or myocardial adaptations in humans.

Another possible explanation for the lower increase in maximal oxygen uptake in the resveratrol group may be related to the described role of ROS in signaling processes and in mediating part of the response and adaptations to exercise training (Jackson, 2011). The increase in maximal oxygen uptake with endurance training has been shown to be mediated by ROS (Baar, 2004; Radak et al., 2008) and thus, abrogating ROS by increasing the antioxidant defense level may retard exercise induced increase

in maximal oxygen uptake. This theory has in part been shown in training studies with oral administration of antioxidant vitamin C + E in young healthy men (Ristow et al., 2009) and with oral administration of statins in subjects with metabolic syndrome (Mikus et al., 2013). Thus, it could be speculated that at least some of the negative effects of resveratrol supplementation on training induced adaptations in the current study may have been related to the antioxidant properties of this compound.

An additional finding in the present study was that exercise training increases the ROS synthesizing NOX protein content in aged men without an effect of resveratrol supplementation. This up-regulation could also be related to ROS mediated adaptive responses to exercise training (Silveira *et al.*, 2003; Gomes *et al.*, 2012). NOX has also been proposed to stimulate Ca^{2+} release and uptake, respectively, potentially increasing muscle contractility (Xia *et al.*, 2003; Hidalgo *et al.*, 2006). The observation that resveratrol supplementation had no effect on the training induced increase in NOX is in contrast to the observation that incubation of endothelial cells with resveratrol down regulates NOX (Orallo et al., 2002). The discrepancy may suggest that the response in NOX in cultured endothelial cells differs from an *in vivo* situation.

Based on the available literature, one of the hypotheses of the present study was that resveratrol supplementation combined with exercise training would increase the antioxidant defense and thereby lower excessive ROS and improve bioavailability of the potent vasodilator NO in aged individuals. However, we found that high-intensity exercise training in aged men increased the antioxidant enzyme SOD2 and decreased GPX-1 protein content whereas CAT and SOD1 protein content were unaltered in skeletal muscle and that resveratrol supplementation had no additional effect. The present observation that resveratrol supplementation, with concomitant exercise

training was unable to induce any changes in the antioxidant system is in contrast to the previous findings from animal models where resveratrol has been shown to up regulate CAT, GPX-1 and SOD1 protein expression in skeletal muscle (Ungvari *et al.*, 2007*b*; Spanier *et al.*, 2009). To our knowledge, no previous study has measured antioxidant enzymes in skeletal muscle of aged subjects in response to exercise training and data from young healthy subjects are scarce and inconclusive (Hellsten *et al.*, 1996; Tiidus *et al.*, 1996; Devries *et al.*, 2008). Present results suggest that resveratrol supplementation has no additive effect on endogenous antioxidant systems.

In the present study a daily dose of 250 mg of trans-resveratrol was used. Previous studies on physically inactive humans using varying concentrations of resveratrol (10 to 2.000 mg day^{-1}) shows diverging effect of resveratrol supplementation (Brasnyó *et al.*, 2011; Timmers *et al.*, 2011; Yoshino *et al.*, 2012; Poulsen *et al.*, 2012; Crandall *et al.*, 2012). Given that several effects were observed in present study, albeit mainly negative, the used dose appears to have been effective. The duration of the supplementation period was 8 weeks, which is substantially longer than previously used in most human studies where the longest supplementation period has been 30 days.

In conclusion, we demonstrate that high-intensity exercise training potently improves a number of parameters related to vascular function and cardiovascular health in aged men, but that concomitant oral resveratrol supplementation blunts several of these positive effects of exercise training. Specifically, resveratrol had adverse effects on improvements in maximal oxygen uptake, on blood pressure reduction and on the lowering of blood lipids induced by exercise training. The finding rejects the

hypothesis that resveratrol improves cardiovascular health by enhanced SIRT 1 dependent signaling and improved antioxidant defense. Furthermore, our data suggest that training enhances the capacity for ROS formation via increased levels of NOX and that removal of ROS via resveratrol treatment may limit training induced adaptations. It may therefore also be questioned whether in general the level of ROS formation in aged men indeed is detrimental to cardiovascular health as previously proposed.

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Table 1

Table 1. Subject characteristics

	Placebo n = 13		Resveratrol n = 14	
	Pre	*Post*	*Pre*	*Post*
Age (years)	65 ± 1	-	65 ± 1	-
Weight (kg)	84.2 ± 2.2	83.0 ± 2.0*	79.8 ± 3.8	79.5 ± 3.7
Body mass index (kg/m^2)	26.0 ± 0.5	25.7 ± 0.5	25.4 ± 0.7	25.3 ± 0.7
Body Fat (%)	25.8 ± 1.5	24.4 ± 1.4*	27.6 ± 1.9	26.1 ± 1.8*
MAP (mmHg)	95.3 ± 2.2	90.8 ± 1.8*	96.3 ± 3.0	93.7 ± 2.7
HR$_{rest}$ (BPM)	63 ± 2	58 ± 2*	63 ± 1	61 ± 2
HR$_{max}$ (BPM)	168 ± 4	168 ± 3	167 ± 5	164 ± 4
VO$_2$max (ml O$_2$ min^{-1})	2582 ± 84	3026 ± 88*	2402 ± 113	2710 ± 112*#
Δ VO$_2$max (ml O$_2$ min^{-1})	-	444 ± 38	-	308 ± 46 #
VO$_2$max kg^{-1} (ml O$_2$ min^{-1} kg^{-1})	30.8 ± 1.2	36.6 ± 1.1*	30.1 ± 1.0	34.1 ± 0.9*#
Glucose (mmol l^{-1})	5.3 ± 0.1	5.2 ± 0.1	5.4 ± 0.2	5.4 ± 0.2
Total cholesterol (mmol l^{-1})	5.1 ± 0.2	4.9 ± 0.3	5.6 ± 0.2	5.6 ± 0.3
HDL (mmol l^{-1})	1.4 ± 0.1	1.5 ± 0.1	1.4 ± 0.1	1.4 ± 0.1
LDL (mmol l^{-1})	3.3 ± 0.2	3.0 ± 0.2*	3.6 ± 0.2	3.4 ± 0.2
TC/HDL ratio	3.9 ± 0.3	3.6 ± 0.3*	4.3 ± 0.3	4.1 ± 0.3
Triglycerides (mmol l^{-1})	1.3 ± 0.2	1.0 ± 0.2*	1.4 ± 0.2	1.5 ± 0.2
VCAM-1 (ng ml^{-1})	519 ± 45	450 ± 21*	554 ± 29	456 ± 26*

Data are presented as mean ± SEM. * denotes significantly different from pre, # denotes significantly different from placebo (P < 0.05).

Figure legends

Figure 1. **Absolute changes in maximal oxygen uptake from baseline to after a period of exercise training with (■) or without (O) resveratrol supplementation in aged men.** Left panel: Individual delta values in maximal oxygen uptake (VO$_2$max) arranged from low change to high change. Right panel: Group mean delta values in maximal oxygen uptake. O placebo group; ■ resveratrol group. Mean data are presented as mean ± SEM. # denotes significantly different from placebo (P < 0.05).

Figure 2. **Leg blood flow in response to exercise and passive movement in aged men after 8 weeks of either a placebo or resveratrol supplementation in combination with endurance training.** Femoral arterial blood flow was measured at rest and after 30 seconds of passive leg extension movement and during active leg

extensions performed at a work load of 10 W and 30 W. Blood flow during exercise was determined both before and after training. Rest and passive leg movement flows were only measured post intervention. Data are presented as mean ± SEM.

Figure 3. Panel A: **Protein expression and phosphorylation status of nitric oxide synthase before and after a period of exercise training and either placebo or resveratrol supplementation in aged men.** Protein expression of total endothelial Nitric Oxide Synthase (eNOS), phosphorylated eNOS (eNOS^{Ser1177}), ratio of eNOS^{Ser1177} to total eNOS, and neural NOS (nNOS) in homogenates from m. vastus lateralis. Data are presented as mean ± SEM. * denotes significantly different from pre, $ denotes significant effect of time ($P < 0.05$). Panel B: **Protein expression of the prostaglandin system enzymes.** Protein expression of cyclooxygenase 1 (COX-1), cyclooxygenase 2 (COX-2), prostacyclin synthase (PGI$_2$) and thromboxane synthase (TBX) in homogenates from m. vastus lateralis. Data are presented as mean ± SEM. * denotes significantly different from pre, $ denotes significant effect of time ($P < 0.05$).

Figure 4. **Muscle endothelin-1 protein expression muscle protein amount of endothelin receptors and plasma levels of endothelin.** Protein expression of endothelin 1 (ET-1), endothelin receptor A (ET-A) and endothelin receptor B (ET-B) in homogenates from m. vastus lateralis and fasting plasma levels of ET-1 before and after a period of exercise training and either placebo or resveratrol supplementation in aged men. Data are presented as mean ± SEM. * denotes significantly different from pre, # denotes significantly different from placebo ($P < 0.05$).

Figure 5. **Protein expression of SIRT1 before and after a period of exercise training and either placebo or resveratrol supplementation in aged men.** Protein expression of Sirtuin 1 (SIRT1) in homogenates from m. vastus lateralis. Data are presented as mean ± SEM.

Figure 6. **Protein expression of endogenous antioxidant enzymes before and after a period of exercise training and either placebo or resveratrol supplementation in aged men.** Protein expression of catalase (CAT), gluthation peroxidase 1 (GPX1), superoxide dismutase 1 (SOD1), superoxide dismutase 2 (SOD2) and NAD(P)H oxidase (NOX) in homogenates from m. vastus lateralis. Data are presented as mean ± SEM. * denotes significantly different from pre, $ denotes significant effect of time ($P < 0.05$).

Figure 7. **Changes in skeletal muscle interstitial prostacyclin levels before and after a period of exercise training and either placebo or resveratrol supplementation in aged men.** Relative changes from pre to post in interstitial 6-keto prostaglandin F1α in m. vastus lateralis at rest and during exercise. Muscle interstitial fluid was collected through microdialysis probes positioned in the m.v. lateralis muscle. Data are presented as mean ± SEM. * denotes significant changes from pre to post ($P < 0.05$).

Table 1. **Subject characteristics.** Data are presented as mean ± SEM. * denotes significantly different from pre, # denotes significantly different from placebo ($P < 0.05$).

Supplemental table 1.

Table S1. Western Blot Details

Antibody	Manufacture	Catalog nr.	Sample heating	Protein loading amount	Gel type	Electrophoresis run time	Predicted molecular weight	Blotting time	Blocking agent	Antibody concentration	Secondary antibody	Secondary antibody concentration
CAT	abcam	ab1877	96˚	5 µg	10%	90 min	59 kD	120min	5% Milk	1:5000	Rabbit	1:5.000
COX-1	abcam	ab53766	96˚	12 µg	10%	80 min	69 kD	90min	3% BSA	1:500	Rabbit	1:5.000
COX-2	abcam	ab52237	96˚	12 µg	10%	80 min	69 kD	90min	2% Milk	1:500	Rabbit	1:5.000
Endothelin receptor A	Nordicbiosite	D424	96˚	12 µg	10%	90 min	50 kD	90min	2% Milk	1:3000	Rabbit	1:5.000
Endothelin receptor B	Santa Cruz	sc-21197	NH	20 µg	10%	90 min	50 kD	90min	5% Milk	1:200	Goat	1:5.000
Endothelin-1	abcam	ab88093	96˚	12 µg	16.5%	150 min	24 kD	90min	3% BSA	1:1000	Mouse	1:5.000
eNOS	BD Transduction	612665	96˚	16 µg	10%	90 min	140 kD	120min	2% Milk	1:200	Mouse	1:5.000
eNOS P-Ser[1177]	Calbiochem	574597	NH	20 µg	10%	90 min	140 kD	120min	3% BSA	1:50	Mouse	1:5.000
GAPDH	abcam	ab22604	-	-	-	-	36 kD	90min	5% Milk	1:16.000	Mouse	1:5.000
GPX1	abcam	ab22604	96˚	16 µg	16.5%	120 min	22 kD	120min	5% Milk	1:1000	Rabbit	1:5.000
NADPH oxidase [p67]	BD Transduction	610912	NH	16 µg	10%	90 min	67 kD	90min	3% BSA	1:500	Mouse	1:5.000
nNOS	BD Transduction	610309	NH	10 µg	10%	90 min	155 kD	120min	5% Milk	1:10 000	Mouse	1:5.000
PGI$_2$ synthase	Santa Cruz	sc-20933	NH	10 µg	10%	90 min	52 kD	90min	2% Milk	1:200	Rabbit	1:5.000
SOD1	Calbiochem	574597	NH	1 µg	16.5%	120 min	18 kD	120min	3% BSA	1:1000	Sheep	1:5.000
SOD2	Calbiochem	AB10346	96˚	1 µg	16.5%	120 min	25 kD	120min	2% Milk	1:5000	Rabbit	1:10.000
TBX	abcam	ab39362	96˚	10 µg	16.5%	90 min	54 kD	120min	5% Milk	1:250	Rabbit	1:5.000

Supplemental table 2.

Table S2. Performance Test

	Placebo n = 13		Resveratrol n = 14	
	Pre	*Post*	*Pre*	*Post*
Step test (est. ml O_2 min^{-1} kg^{-1})	28.3 ± 0.9	31.9 ± 1.6*	29.3 ± 1.0	36.0 ± 1.2*#
Chair stand (Count per 30s)	15.7 ± 0.9	22.7 ± 1.5*	17.9 ± 1.0	24.4 ± 1.4*
Up & Go (s)	3.6 ± 0.1	3.1 ± 0.1*	3.5 ± 0.1	3.3 ± 0.1
Balance open (s)	35.3 ± 3.7	38.4 ±3.2	34.5 ± 3.9	38.8 ± 3.7
Balance closed (s)	7.2 ± 1.5	6.9 ± 0.9	8.2 ± 2.8	8.2 ± 1.8
5k-walk (min)	54 ± 2	49 ± 2*	56 ± 2	49 ± 3*

Data are presented as mean ± SEM. * denotes significantly different from pre, # denotes significantly different from placebo ($P < 0.05$).