Resveratrol modulates the angiogenic response to exercise training in skeletal muscles of aged men

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Angiogenesis is regulated by multiple proangiogenic factors but also by angiostatic factors that modulate the process of capillary growth or regression (11, 21). In skeletal muscle tissue, the most important factor, both for the maintenance of basal capillarization and capillary growth, is VEGF (36, 43, 70). VEGF is primarily found within skeletal muscle fibers but is also present in other cells within muscle tissue, such as endothelial cells and pericytes (24). In skeletal muscle cells, VEGF has been shown to be stored in vesicles and secreted to the interstitium (24), where it acts primarily on VEGF receptor (VEGFR)-2 located on capillary endothelial cells (39). Secretion of VEGF from muscle cells is stimulated by different factors, including muscle contraction (20, 21, 24), and, consequently, the muscle interstitial concentration of VEGF increases markedly during muscle activity (21, 22, 28). Angiostatic compounds thought to be of importance for the modulation of capillary growth in skeletal muscle include thrombospondin (TSP)-1 and tissue inhibitor of metalloproteinase (TIMP)-1. TSP-1 has been proposed to oppose angiogenesis (26, 69), and, in TSP-1 knockout mice, capillarization is higher in skeletal muscle compared with wild-type mice, indicating that TSP-1 has an important angiostatic role (37). The effect of exercise training on protein expression and interstitial levels of TSP-1 is unknown. TIMP-1 inhibits degradation of the extracellular matrix by inhibiting matrix metalloproteinases (MMPs) and may thereby limit capillary growth (3, 62). The expression of TIMP-1 in skeletal muscle is sensitive to muscle activity where acute exercise transiently increases TIMP-1 mRNA levels (23) and endurance training leads to elevated TIMP-1 protein content (21).

Resveratrol, a naturally occurring polyphenol, is an antioxidant that has been proposed to exert antiaging and exercise-mimicking effects by enhancing deacetylase sirtuin (SIRT)-1 and AMP-activated protein kinase (AMPK) in animal models (19, 30, 50) and that also may exert effects on angiogenic pathways (9, 46, 53). SIRT-1 and AMPK regulate the activation and expression of the transcriptional coactivator peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α (27, 29, 54). Thus, resveratrol promotes PGC-1α (30, 52), which, in turn, has been found to be essential for basal and exercise-induced increases in VEGF and for capillary growth in the skeletal muscle of young (6) and aged rodents (33, 34). In addition, SIRT-1 may promote angiogenesis by deacetylating and inhibiting the angiostatic transcription factor forkhead box protein O1 (FoxO1) (47). FoxO1 has been shown to limit angiogenesis in part via upregulation of TSP-1 (38, 56). Resveratrol supplementation would, therefore, through these separate mechanisms, be expected to increase VEGF levels in...
skeletal muscle and promote angiogenesis. The effect of resveratrol on basal and exercise-induced skeletal muscle angiogenesis in humans has not previously been described.

Based on evidence that exercise training increases capillarization and that resveratrol can promote angiogenic signaling, the hypothesis of the present study was that oral supplementation of resveratrol would potentiate the angiogenic response to exercise training. To test this hypothesis, we conducted two double-blind placebo-controlled experiments on healthy aged sedentary men. Twenty-seven subjects were allocated into either a “placebo with exercise training” group or a “resveratrol with exercise training” group for 8 wk to test the effect of resveratrol on exercise-induced angiogenesis. In addition, to elucidate whether resveratrol had an effect on basal levels of angiogenic factors, 16 subjects were allocated into 2 nontraining groups with either placebo or resveratrol supplementation.

EXPERIMENTAL PROCEDURES

Ethical Approval

The present 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.

Subjects and Randomization

Healthy aged (mean: 65 ± 1 yr; range: 60–72 yr) physically inactive (<2 h of moderate-intensity physical activity per week) men were recruited and allocated into a training group (n = 27) to test the effect of resveratrol on exercise-induced angiogenesis and a nontraining group (n = 16) to test the effect of resveratrol on basal angiogenesis (Table 1). Exclusion criteria for the subjects were cardiovascular disease, hypertension, renal dysfunction, insulin resistance, or type 2 diabetes. Basic subject characteristics have previously been described (14, 40). The study was of double-blind placebo-controlled design, and groups were allocated into either placebo or resveratrol supplementation for 8 wk in the following groups: 1) subjects in the training group were allocated into either a combination of exercise training and placebo (n = 13) or exercise training and 250 mg/day trans-resveratrol (n = 14, Fluxome, Stenlose, Denmark) and 2) subjects in the nontraining group were allocated into either placebo (n = 7) or 250 mg/day trans-resveratrol (n = 9) without changes in their sedentary lifestyle. The dose of 250 mg/day trans-resveratrol was chosen as it has been suggested to be a safe dose yet high enough to be effective (68). This dose has been shown to result in plasma resveratrol concentrations of ~0.75 μM (49). The dose of 250 mg/day also resembles recommendations from the commercial industry. For details on the exercise training regime, compliance, etc., we refer the reader to a previous publication (14).

Experimental Design

The experimental days have been previously described in detail (14, 40). In short, before and after the 8-wk intervention period, we measured maximal pulmonary O2 uptake (Oxycon Pro, Viessys Healthcare, Hoechberg, Germany), collected a muscle biopsy from the vastus lateralis muscle with the percutaneous needle biopsy technique (2), and collected muscle dialysates from the vastus lateralis muscle during rest and one-leg knee extensor exercise.

Analysis of VEGF and TSP-1 in Microdialysates

VEGF and TSP-1 concentrations in microdialysates were measured using a Quantikine ELISA kit (R&D Systems, Minneapolis, MN). The estimated relative recoveries of the microdialysis probes used for the collection of dialysate samples at rest and during one-leg knee extensor exercise were 45 ± 2% and 65 ± 3%, respectively.

Immunohistochemistry

Frozen biopsy samples were cut into 8-μm-thick transverse sections in a cryostat, and the sections were fixed by immersion in acetone at −20°C for 30 s followed by incubation for 2 min in 2% formaldehyde at room temperature. Immunohistochemistry was performed as previously described in detail (23). Briefly, sections were rinsed and incubated with a primary antibody against CD-31 (endothelial cell marker, M0822, DakoCytomation, Glostrup, Denmark) and myosin heavy chain (MHC) antibodies against MHC I (M8421, Sigma, St. Louis, MO) and MHC IIa (N2.261, Developmental Studies Hybridoma Bank, University of Iowa). Intermediate fibers were classified based on staining with both MHC I and MHC IIa antibodies. Type IIx fibers were identified based on lack of staining with either antibody. Antibody binding was visualized using an ABCComplex kit with alkaline phosphatase (ABCComplex/AP, DakoCytomation). Capillaries and muscle fibers were visualized at a total magnification of ×400. On average, ~400 fibers were counted per biopsy and 2 individual sections were counted per individual. The mean fiber area was calculated using image-analysis computer software (Tema, version 95).

Quantification of Protein Expression by Western Blot Analysis

Western blot analysis was conducted as previously described (14). The following primary antibodies were used: VEGF (A-20 sc-152, Santa Cruz Biotechnology, Santa Cruz, CA), VEGFR-2 (sc-19530, Santa Cruz Biotechnology), TSP-1 (ab85762, Abcam), TIMP-1 (AF 970, R&D Systems), and FoxO1 (C29H4, Cell Signaling Technology, Leiden, The Netherlands). The secondary antibodies used were goat anti-rabbit or rabbit anti-goat horseradish peroxidase-conjugated antibodies (P-0448 and P-0449, DakoCytomation, 1:5,000). 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, blots were also analyzed for GAPDH (ab9484, Abcam). Statistical analysis showed that GAPDH levels were not affected by the interventions in either group.

Table 1. Overview of allocation of subjects

<table>
<thead>
<tr>
<th>Training Group</th>
<th>Nontraining Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exercise + placebo</td>
<td>Exercise + resveratrol</td>
</tr>
<tr>
<td>n = 13</td>
<td>n = 14</td>
</tr>
<tr>
<td>Age, yr</td>
<td>65.1 ± 0.9</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>26.0 ± 0.5</td>
</tr>
<tr>
<td>Maximal O₂ consumption per kilogram, ml O₂/min⁻¹·kg⁻¹</td>
<td>30.8 ± 1.2</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE. Subjects were allocated into either an exercise training group with or without resveratrol (250 mg/day) treatment or into a nontraining group with or without resveratrol.

H1112 ANGIOGENIC RESPONSE TO EXERCISE AND RESVERATROL

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Statistical Analysis

To test for the effect of training with placebo or resveratrol treatment and the effect of placebo or resveratrol treatment without training, two-way repeated-measures ANOVA was conducted. After a significant time, group, or time × group effect, pairwise differences were identified using Holm-Sidak post hoc analysis. The significance level was set at \( P < 0.05 \), and data are means ± SE; \( n = 13/14 \) and \( n = 7/9 \) (placebo/resveratrol) for training and nontraining groups, respectively, unless otherwise stated. For the microdialysis procedures, there was a malfunction of the probes in two subjects during resting conditions and in one subject during exercise. These samples were excluded from analysis. One muscle biopsy sample was too small to be included in both Western blot analysis and immunohistochemistry and was used for Western blot analysis.

RESULTS

Data on maximal \( O_2 \) uptake, blood pressure, and compliance in the training and nontraining groups have been previously described (14, 40).

Muscle Interstitial VEGF

Training groups. Muscle interstitial VEGF protein concentrations in skeletal muscle at rest were not different between the two training groups before training (\( n = 13/12 \); Fig. 1A). After training, resting interstitial VEGF levels were increased by 24.6% overall (\( P < 0.05 \)). Acute exercise induced an approximately fivefold increase (\( P < 0.001 \)) in interstitial VEGF levels in placebo- and resveratrol-treated groups (\( n = 12/12 \); Fig. 1A). The exercise-induced release of VEGF was not different after compared with before training in either group.

Nontraining groups. In the two nontraining groups, interstitial VEGF protein concentrations at rest and during acute exercise were not different at baseline or after 8 wk of the supplement intervention (\( n = 7/8 \); Fig. 1B).

Muscle Interstitial TSP-1

Training groups. Muscle interstitial TSP-1 concentrations were not different between the two training groups before training, but after training there was a time × group interaction (\( P < 0.05 \); Fig. 2A), and TSP-1 levels were lower in the resveratrol-treated group compared with before training (\( P < 0.05 \)).

Nontraining groups. In the two nontraining groups, interstitial TSP-1 protein concentrations at rest were not different at baseline or after the supplement intervention (\( n = 7/8 \); Fig. 2B).

Skeletal Muscle Fiber Size, Capillarization, and Muscle Fiber Type Distribution

Training groups. Skeletal muscle fiber area, capillary density, and capillary-to-fiber ratio were not different between the two training groups before training. Training increased the capillary-to-fiber ratio overall (\( n = 12/14, P < 0.001 \); Fig. 2A) and in the placebo-treated group (1.5 ± 0.2 vs. 1.8 ± 0.1, \( P < 0.005 \)) but not in the resveratrol-treated group (1.6 ± 0.1 vs. 1.7 ± 0.1). Training increased capillary density overall (\( P < 0.05 \); Fig. 2B). Skeletal muscle fiber area was not different between groups before training and was not affected by either training or training combined with resveratrol intake (Fig. 2C).

There were no differences in MHC I, MHC IIA, MHC IIX, and mixed muscle fiber type distribution between the two training groups before training. MHC I, MHC IIA, and mixed muscle type distribution as well as the fiber distribution were not changed with training or training plus resveratrol, but MHC IIX showed an overall decrease with training (\( P < 0.05 \); Fig. 3D).

Histochemical analysis was only conducted in the training groups.

Expression of Angiogenic Proteins in Skeletal Muscle

VEGF, VEGFR-2, TIMP-1, and TSP-1 protein expression in skeletal muscle tissue were not different between groups at baseline in either the training or nontraining groups (Fig. 4). In the training groups, VEGF protein expression was increased in the placebo-treated group after training (\( P < 0.005 \); Fig. 4A). VEGFR-2 protein levels were increased similarly in the two training groups after training (\( P < 0.001 \); Fig. 4B). The TIMP-1 protein amount was increased in the placebo-treated group after training (\( P < 0.05 \)) and was lower in the resveratrol-treated group compared with the placebo-treated group after training (\( P < 0.05 \); Fig. 4C). TSP-1 protein expression...
was not affected by training with placebo or resveratrol intake (Fig. 4D).

In the nontraining groups, VEGF, VEGFR-2, TIMP-1, and TSP-1 protein expression were unchanged in the placebo-treated group postintervention. In the resveratrol-treated group, TIMP-1 protein levels were lower than in the placebo-treated group postintervention (P < 0.005; Fig. 4C). VEGF, VEGFR-2, and TSP-1 protein levels were not changed in the resveratrol-treated group (Fig. 4, A, B, and D).

FoxO-1 protein expression was not different between groups at baseline and increased overall after training (P < 0.05; Fig. 5) but not after resveratrol alone.

**DISCUSSION**

The present study demonstrates that a period of exercise training in aged men results in a marked angiogenic response, as evidenced by increased capillarization, higher basal muscle interstitial levels of VEGF, and increased protein expression of the angiogenic factors VEGF, VEGFR-2, and TIMP-1 after training. However, resveratrol supplementation combined with training did not potentiate the effect of training, as hypothesized, but led to a somewhat reduced angiogenic response. Specifically, a combination of resveratrol supplementation and training did not result in an increase in capillarization or in VEGF or TIMP-1 protein levels in muscle, suggesting that resveratrol opposed the proangiogenic effect of training. Resveratrol supplementation alone, without parallel training, appeared to have a mildly negative effect on angiogenic factors in muscle.

**Capillary-to-Fiber Ratio and VEGF**

Eight weeks of exercise training evoked a marked (~20%) increase in the capillary-to-fiber ratio in the combined training and placebo group that was of similar magnitude as reported in previous studies (1, 13, 21). The increased capillarization was paralleled by a 35% increase in muscle VEGF protein expression. This finding is in accordance with previous observations of a higher capillary-to-fiber ratio and increased VEGF mRNA levels in skeletal muscles of aged men after 8 wk of aerobic exercise training (13). One of the main hypotheses of the present study was that resveratrol would further enhance training-induced angiogenesis by promoting VEGF expression, as previously described (33, 47). However, in contrast, the group that received resveratrol supplementation in combination with exercise training did not show an increase in the capillary-to-fiber ratio or muscle VEGF protein levels. In addition, in the nontraining trial, VEGF protein expression tended (P = 0.056 by paired t-test) to be lower after 8 wk of resveratrol supplementation, whereas it remained unaltered in the placebo-treated group. Combined, these data suggest that resveratrol does not promote angiogenesis but may rather have a somewhat angiostatic effect. To our knowledge, this is the first report on the effect of resveratrol on basal or training-induced VEGF expression in skeletal muscle in humans. However, in cultured adipocytes, the hypoxia-induced increase in VEGF mRNA expression has been shown to be effectively blocked by resveratrol, supporting a negative effect on VEGF expression (9). In aged mice, resveratrol supplementation alone or combined with exercise training has been shown not to influence either VEGF protein levels or the capillary-to-fiber ratio (52). These findings in mice support a lack of proangiogenic effect of resveratrol.

However, the animal study by Ringholm et al. (52) did not report a negative effect of resveratrol on the training-induced increase in VEGF and capillarization. This finding thus contrasts that of the present human study. The reason for this discrepancy is unclear; however, the literature on resveratrol supplementation in animals and humans indicates that the effects of resveratrol vary greatly depending on species (48, 60).

Studies in cell culture have shown that VEGF is secreted from muscle cells in response to contraction and chemical stimulation (22, 59). Accordingly, during exercise, VEGF is markedly increased in the human muscle interstitium, where it can act on capillary endothelial cells (20, 24), an event believed to be important for the angiogenic process. In the present study, training induced an overall (~25%) increase in basal muscle interstitial VEGF levels in the two training groups with no effect of resveratrol supplementation. An interesting observation was that interstitial VEGF levels were lower (P = 0.04 by paired t-test) in the nontraining group that had received resveratrol for 8 wk. This observation is in line with previous
reports showing that resveratrol is able to reduce VEGF release from human endothelial and retinal cells in culture (10, 63) and that resveratrol lowers hypoxia-induced VEGF release in human adipocytes (9). However, resveratrol did not seem to influence the amount of VEGF secreted during exercise, suggesting that the exercise-induced stimulus overruled the potential effect of resveratrol. As such, interstitial VEGF levels increased approximately fivefold from rest to knee-extensor exercise and the period of exercise training did not influence the magnitude of the increase. This observation of a lack of effect of training on the magnitude of increase in interstitial VEGF is in accordance with previous reports in young and aged subjects (13, 21, 23).

Our findings on the effect of exercise training and resveratrol supplementation on VEGF protein in muscle tissue versus basal VEGF protein in the muscle interstitial fluid did not indicate a relationship between these variables. In previous studies (18, 25), we have shown that in populations with lower levels of muscle VEGF compared with young healthy individuals, the increase in muscle interstitial VEGF levels during exercise are lower. Moreover, in these subjects, training increased both muscle VEGF protein levels and the magnitude of increase in interstitial VEGF with exercise (18, 25). It is, however, unclear whether these findings reflect a direct relationship. The secretion of VEGF from muscle may depend both on the amount of VEGF stored in the muscle and on the actual mechanism of secretion (24). Further studies are needed to elucidate the regulatory mechanisms behind VEGF secretion from muscle.

VEGFR-2 has been shown to be the most important receptor promoting capillary growth in muscle (39). Here, we showed that VEGFR-2 protein levels are higher after exercise training in aged men, whereas resveratrol had no impact on the levels. The effect of exercise training on VEGFR-2 protein expression has not been previously reported, but VEGFR-2 mRNA levels have been shown to be increased after an acute exercise bout in young and aged men (12, 58) and after a period of exercise training in young men (17, 65). The fact that resveratrol did not
affect VEGFR-2 expression but lowered VEGF protein levels confirms previous observations from human umbilical endothelial cells (35).

**Muscle TIMP-1 and TSP-1 Levels**

Two angiostatic proteins believed to modulate the angio-
genic process and prevent excess capillary growth were as-
sessed in the present study: TSP-1 and TIMP-1 (41). Whereas TSP-1 protein expression in muscles remained unaltered with training and resveratrol supplementation, TIMP-1 protein expression was increased after training in the placebo-treated group only. TIMP-1 inhibits degradation of the extracellular matrix and, as such, modulates capillary growth. We have previously found that TIMP-1 mRNA levels, but not protein expression, were increased after exercise training in young men (21). These findings could indicate that greater protein expression of TIMP-1 is detectable after 8 wk but not after 4 wk of exercise training. Resveratrol supplementation inhibited the training-induced increase in muscle TIMP-1 protein. In accordance, the group that received resveratrol supplementation without concomitant training had lower TIMP-1 protein expression compared with the placebo-treated group, indicating that resveratrol interferes not only with exercise-induced changes in TIMP-1 but also with the basal expression of this angioregulatory protein. TIMP-1 exhibits multifunctional ac-
tivities besides inhibition of MMPs (4), such as inhibition of apoptosis (MMP independent) (31). The lower TIMP-1 levels after resveratrol supplementation along with the lack of train-
ing-induced angiogenesis in this group could imply a greater level of apoptosis.

As described above, TSP-1 levels in muscle tissue remained unaltered by training and resveratrol supplementation. However, TSP-1 levels were also determined in the muscle inter-
stitium and, although they were not affected by training alone, the levels decreased when training was combined with resvera-
trol. In light of our other present findings of a somewhat antiangiogenic effect of resveratrol, this was an unexpected observation, since TSP-1 can oppose the angiogenic effect of...
VEGF. Whereas acute exercise has been previously found to induce transient changes in TSP-1 mRNA in humans, muscle protein levels of TSP-1 have been shown to be unaltered after a period of exercise training (21). Moreover, basal muscle interstitial levels of TSP-1 in healthy humans have also been reported to be unaltered after 4 wk of moderate intensity exercise training (23). In peripheral arterial disease patients, basal levels of muscle interstitial TSP-1 protein have been reported to be elevated, most likely reflecting capillary regression (25). This is the first evaluation of how resveratrol affects interstitial TSP-1 in skeletal muscle. One previous investigation of TSP-1 and resveratrol in melanoma-endothelial cell cultures suggested that the antiangiogenic effects of resveratrol is mediated at least in part by TSP-1 (67). We were not able to confirm this in our model, but indications from the nontraining group could suggest that interstitial TSP-1 levels are increased (P = 0.049 by paired t-test) when resveratrol is given as supplement without concomitant training.

A putative mechanism for the negative effect of resveratrol on angiogenesis could be mediated via the angiotrophic transcription factor FoxO1. FoxO1 limits angiogenesis (38) in part by increasing angiostatic TSP-1 (56). FoxO1 protein levels were measured in the present muscle samples, but no effect of resveratrol supplementation on FoxO1 protein expression was observed. In contrast, exercise training did induce an overall increase in FoxO1 protein expression. This observation contrasts a recent finding by Roudier et al. (55) of no change in FoxO1 protein expression in both young and aged subjects after 6 wk of exercise training. The discrepancy is unclear but could be explained by the lower exercise intensity in the study by Roudier et al. (55), although this is speculative.

Perspectives and Limitations

A recent human umbilical vein endothelial cell study (61) found that resveratrol may limit angiogenesis mediated via activation of FoxO1. In the present study, we found no effect of resveratrol on the expression of FoxO1, but studies examining the activation of FoxO1 in response to resveratrol in humans are warranted.

Although not measured in the present study, the level of circulating endothelial progenitor cells could provide a potential mechanism by which resveratrol at different doses affects angiogenesis. Resveratrol has been shown to increase the level of circulating endothelial progenitor cells in rats when supplemented with 10 mg/day but not 50 mg/day (15). Exercise training also increases endothelial progenitor cell numbers (32), but the combined effect of exercise training and resveratrol on endothelial progenitor cells is unknown, as is the effect of doses other than 250 mg/day.

It should be emphasized that it is possible that the concentration of resveratrol is important for its effect on angiogenesis. A previous study (71) has shown that incubation with a low dose of resveratrol (5 μM) increased VEGF and VEGFR-2 protein expression and tube formation in human endothelial cells and vessel numbers in chick chorioallantonic membranes. A higher dose of resveratrol (20 μM), on the other hand, blocked all of these angiogenic responses. The dose of resveratrol (250 mg/day) used in the present study was based on previous literature in humans (49, 64) and recommendations by the producer of resveratrol and was selected to achieve effects while still being safe. We have previously published that resveratrol reduced overall muscle acetylation, indicating increased SIRT-1 activity (40). The total protein content of SIRT-1 was, on the other hand, not changed by resveratrol supplementation (14). Combined, these data on reduced overall acetylation, in combination with the observed effects of resveratrol treatment, suggest that the given dose of resveratrol was effective. However, it cannot be excluded that a different dose of resveratrol may have affected angiogenesis differently than observed here.

Conclusions

The overall conclusion of the present study is that, whereas exercise training effectively induces an angiogenic response and capillary growth in aged sedentary men, resveratrol at the given dose of 250 mg/day has no beneficial effect on angiogenesis and may even lower the training-induced angiogenic response.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES


