Lower Skeletal Muscle Nutritive Blood Flow in Older Women Is Related to eNOS Protein Content

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The relationship between muscle endothelial nitric oxide synthase (eNOS) content and nutritive flow was investigated in nonobese sedentary young (27.7 ± 2.6 years) and older (56.6 ± 2.1 years) women matched for body composition and VO2peak. A muscle biopsy was taken and nutritive blood flow was determined under resting conditions in the vastus lateralis of the quadriceps femoris muscle group. Muscle eNOS protein content correlated with muscle nutritive blood flow (r = .66, p < .05) and body mass index (r = .74, p < .05), but it did not correlate with VO2peak. Muscle eNOS content was 35% lower in young than in older women (266 ± 52 vs 407 ± 53 pg/mg total protein; p < .05). The mean ethanol outflow-to-inflow ratio was higher (indicating lower nutritive flow) in older and young women (.666 ± .042 and .546 ± .043, respectively; p < .05). Resting skeletal nutritive blood flow and muscle eNOS content was lower in older than in young women. A low muscle eNOS protein content may be linked to a low muscle nutritive blood flow in healthy women.

HMPAIREd production of nitric oxide (NO) is associated with hypertension, chronic heart failure, peripheral vascular disease, type 2 diabetes mellitus, and obesity, all of which increase in incidence with advancing age (1–3). These diseases are associated with a common underlying circulatory defect, in that NO-dependent vasodilatation is reduced (1–5). The role of blood flow and nutrient supply in these disease processes and in what may be considered the normal aging process is a current topic of debate (6–10). Dinenno and colleagues (11) and Proctor and colleagues (12) have reported that limb blood flow is lower in older individuals than in young individuals. However, there remain questions as to whether or not nutrient blood flow is reduced in older individuals. Nutritive skeletal muscle blood flow is the relevant flow to study with respect to both microcirculation and metabolism, in that nutritive flow is that portion of limb blood flow that supplies nutrients and hormones to, and removes metabolites from, skeletal muscle. Despite the impaired NO-dependent limb blood flow often observed in older individuals, the content of endothelial nitric oxide synthase (eNOS) has not been determined in healthy older humans. Furthermore, the relationship between eNOS protein content and nutritive blood flow is unknown.

The purpose of this investigation was to determine if nutritive blood flow is lower in older women than in young women and to determine if this reduction is related to differences in eNOS protein content measured in skeletal muscle biopsy samples.

METHODS

Subjects
Seven healthy, sedentary (<20 min/d, 1 d/wk of exercise), young (20–30 years) women and 12 healthy older women who were not on hormone replacement therapy participated in these studies. Biopsies were obtained from a subgroup of these young and older women (n = 7 and n = 6, respectively). Young and older subjects were matched for percentage body fat and peak aerobic capacity. Young female subjects had regular menses for the past year, and they were studied in the late-follicular phase of the menstrual cycle to ensure the presence of high circulating estrogen concentrations. Older women were at least 3 years postmenopausal. Subjects were not taking medications that could affect central or peripheral circulation, did not smoke, were not hypertensive (systolic blood pressure >150 mmHg, diastolic blood pressure >95 mmHg), and did not have diabetes, cardiovascular disease, angina, or any disease state that could affect central or peripheral circulation. Subjects provided informed consent prior to participating in the study according to the University Medical Center Institutional Review Board at East Carolina University.

Protocol
Subjects initially underwent body composition analysis (13) and assessment of peak aerobic capacity. Between days 3 and 8 following these initial assessments, subjects reported to the Human Performance Laboratory at East Carolina University for a blood draw, muscle biopsy, and assessment of resting muscle nutritive blood flow by use of microdialysis. Subjects reported to the laboratory between 3 and 5 hours postprandial. A blood pressure was taken by using auscultation at an antecubital space with the subject in a resting semireclined position. A blood draw was performed from an antecubital vein for subsequent analysis of follicle-stimulating hormone (FSH-IRMA DSL-4700; Diagnostic Laboratories, Webster, TX), estradiol (DSL-4400 Estradiol Radioimmunoassay Kit, Diagnostic Laboratories), and insulin (Micropartical Enzyme Immuno Assay, Abbott.
Laboratories, Abbott Park, IL) to determine circulating levels of these hormones on the day blood flow studies were conducted. A percutaneous muscle biopsy was then obtained for determination of eNOS content. Four microdialysis probes were then percutaneously inserted into the vastus lateralis of the quadriceps femoris muscle group, using a sterile technique, and perfused at 2.0 μl/min with a sterile Ringer’s solution containing 5mM ethanol to monitor nutritive skeletal muscle blood flow. Microdialysis probes were placed a minimum of 3 cm apart. Subjects were placed in a semireclined position and rested for 60 minutes to allow for equilibration of the microdialysis system. Four 10-minute resting dialysate samples were collected over the subsequent 40 minutes following the 60-minute equilibration period to determine resting skeletal muscle nutritive blood flow.

**Muscle Biopsy**

A muscle biopsy was obtained from the vastus lateralis of the quadriceps femoris muscle group. For this, local anesthesia (3.0 ml of 2% lidocaine) was administered subcutaneously above the muscle fascia in the vastus lateralis approximately one third of the distance between the lateral femoral condyle and the iliac crest. A small ~6-mm incision was made, through which a 5-mm-diameter Bergstrom biopsy needle was inserted. A muscle biopsy (~30–50 mg) was clipped and removed from the vastus lateralis. The muscle was frozen under liquid nitrogen and stored for subsequent analysis of eNOS protein content.

**Microdialysis**

Four microdialysis probes were inserted percutaneously into the vastus lateralis with an 18-gauge needle. Anesthesia (0.1 ml of 2% lidocaine) was administered subcutaneously above the muscle fascia to reduce pain the subjects may have felt during insertion of the probes. Microdialysis probes were perfused with Ringer’s solution containing 5mM ethanol and 2.5mM glucose at 2.0 μl/min, using a CMA/102 microinfusion pump (CMA/Microdialysis, Stockholm, Sweden). Dialysis samples were collected in 150-μl polyethylene collection vials and stored at 4°C until analyzed for ethanol within 24 hours by use of a fluorometric-enzymatic assay as previously described (14,15).

**Blood-Flow Determination**

Ethanol (5mM) was included in the microdialysis perfusion medium to monitor nutritive skeletal muscle blood flow in the area of the microdialysis probe (15,16). Ethanol in this low concentration does not affect local metabolism (15). Ethanol concentrations were measured in the perfusate and dialysate solutions, with the results expressed as the ethanol concentration in the outflowing dialysate in relation to the ethanol concentration in the inflowing perfusate:

\[
\text{ethanol outflow/inflow ratio} = \frac{\text{ethanol}_{\text{dialysate}}}{\text{ethanol}_{\text{perfusate}}}. 
\]

The ethanol outflow-to-inflow ratio is inversely related to blood flow in the area of the microdialysis probe (16).

**Peak Oxygen Consumption (VO₂max)**

Peak aerobic capacity was determined on an electronically braked cycle ergometer. The cycle ergometer test was incremental. The cycling test began with a 3-minute stage at 25 W, followed by a 3-minute stage at 50 W, and subsequent 1-minute stages increasing in intensity by 20-W increments. Respiratory gases were analyzed continuously and averaged over 20-second intervals by using a Sensormedics 2900 Metabolic Measurement Cart (Anaheim, CA). The subjects exercised until they could no longer maintain a cadence of 40 revolutions per minute. Achievement of VO₂peak was determined by attainment of two of the following criteria: plateau in oxygen consumption with increased exercise intensity; respiratory exchange ratio >1.1; and heart rate greater than the age-predicted maximal heart rate.

**Muscle eNOS Analysis**

Muscle eNOS was analyzed in whole homogenates by using the Quantikine human eNOS kit (R & D Systems, Minneapolis, MN). This assay uses the quantitative sandwich enzyme immunoassay technique. The homogenate was centrifuged at 3000 × g for 5 minutes. The supernatant was poured off. Cells were washed twice in a saline solution. After each wash, they were centrifuged at 3000 × g for 5 minutes. The supernatant was poured off. Cells were lysed at 2–8°C with the cell lysis buffer. Cells were centrifuged at 3000 × g for 5 minutes. The supernatant was immediately removed and cells were assayed; 100 μl of the assay diluent RD1W was added to each well of the plate, 100 μl of the standard or sample was added to each well, and these were incubated for 2 hours at room temperature on a horizontal orbital microplate shaker. After incubation, each well was aspirated and washed with 400 μl of buffer. This was repeated for a total of three washings. Two hundred microliters of the eNOS conjugate was added to each well and was incubated for 2 hours at room temperature on the shaker. The aspiration–wash procedure was repeated three times. Two hundred microliters of the substrate solution was added to each well and was incubated for 30 minutes at room temperature; 50 μl of stop solution was added to each well. The optical density of each well was then determined within 30 minutes by using a microplate reader at 450 nm.

**Statistical Analyses**

Differences between young and older women with respect to muscle eNOS content, variables of body composition, and aerobic capacity were analyzed by using a nonpaired Student’s t test. A multiple stepwise correlation analysis was performed to determine the relationship among muscle biopsy eNOS content, estradiol concentration, peak aerobic capacity, plasma insulin concentration, and measures of body composition. A stepwise regression analysis was also used to determine the relationship between these variables and skeletal muscle nutritive blood flow. All statistical analyses were performed with SigmaStat software (Jandel Scientific, La Jolla, CA). The level of significance was set at p < .05. Data are presented as mean ± SEM.
Table 1. Subject Characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Young</th>
<th>Older</th>
</tr>
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<tbody>
<tr>
<td>Age (y)</td>
<td>27.7 (2.6)</td>
<td>56.6 (2.1)</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>164.5 (1.5)</td>
<td>163.0 (1.1)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>72.1 (2.3)</td>
<td>73.4 (4.4)</td>
</tr>
<tr>
<td>Body fat (%)</td>
<td>30.4 (2.7)</td>
<td>32.4 (2.2)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.0 (1.2)</td>
<td>28.6 (1.5)</td>
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<tr>
<td>Waist circumference (cm)</td>
<td>83.0 (3.9)</td>
<td>90.6 (5.0)</td>
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<tr>
<td>Hip circumference (cm)</td>
<td>105.7 (2.5)</td>
<td>101.5 (3.0)</td>
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<tr>
<td>Waist-to-hip ratio</td>
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<td>0.9 (0.0)</td>
</tr>
<tr>
<td>Thigh skinfold (cm)</td>
<td>33.9 (3.0)</td>
<td>32.2 (3.8)</td>
</tr>
<tr>
<td>VO₂peak (1/min)</td>
<td>22.8 (1.8)</td>
<td>20.1 (1.4)</td>
</tr>
<tr>
<td>VO₂peak (ml kg⁻¹/min)</td>
<td>22.8 (1.8)</td>
<td>20.1 (1.4)</td>
</tr>
</tbody>
</table>

Notes: BMI = body mass index; VO₂ peak = peak oxygen consumption; parenthetical numbers represent mean ± SE.

RESULTS

Subject Characteristics

Subject characteristics are presented in Table 1. Older women were, on average, 30 years older than the young women. There were no differences between the groups in height, weight, waist circumference, waist-to-hip ratio, body fat percentage, and VO₂peak.

Blood Pressure

Resting systolic blood pressure was 121.5 ± 75 and 119.3 ± 3.1 mmHg in young and older women, respectively (p = NS). Resting diastolic blood pressure was 74.4 ± 2.5 and 72.1 ± 1.4 mmHg in young and older women, respectively (p = NS). There was no significant correlation between systolic or diastolic blood pressure and muscle nutritive flow or muscle eNOS content.

Ethanol Outflow-to-Inflow Ratio (Nutritive Blood Flow)

The ethanol outflow-to-inflow ratio was .546 ± .053 and .666 ± .042 in young and older women, respectively (n = 7, p < .05). Muscle eNOS was the only variable in this study that was an independent predictor of the ethanol outflow-to-inflow ratio, using stepwise multiple regression analysis (r = .66, p < .05; Figure 2 below). There were no other independent predictors of nutritive blood flow among the measured variables of body composition, age, blood pressure, hormonal status, or aerobic capacity.

Muscle eNOS Content

Muscle eNOS content was 407 ± 53 pg/mg total protein in young women and 366 ± 36 pg/mg total protein in older women (p = .05; Figure 1). Body mass index (BMI) and waist circumference were the only variables significantly correlated with muscle eNOS content (BMI, r = .739; waist circumference, r = .722; Figure 3). There were no other independent predictors of muscle eNOS content among the measured variables of body composition, age, blood pressure, hormonal status, or aerobic capacity.

DISCUSSION

To our knowledge, this is the first demonstration that eNOS protein content in muscle homogenates is related to skeletal muscle nutritive blood flow in humans. The present data also demonstrate for the first time that eNOS protein content in muscle biopsy homogenates from older women is lower (35% lower) than that in young women.

Nutritive Blood Flow

The extent to which NO influences nutritive blood flow in humans has not previously been determined. There have been numerous published reports that there are both nutritive and nonnutritive blood flows in skeletal muscle, and that the clearance of intramuscularly injected radio-labeled markers could be affected by vasoactive agents independent of total limb blood flow (17–20). The data presented indicate that eNOS content in homogenates of skeletal muscle is related to nutritive muscle blood flow determined by using microdialysis. Furthermore, eNOS is known to catalyze the formation of NO from arginine. NO has been shown to activate the soluble guanylate cyclase in vascular smooth muscle, resulting in the formation of cyclic guanosine monophosphate (cGMP) and subsequent vasodilatation (21–25). The NO system has been found to be a significant regulator of basal vascular tone, as blockade of NOS can result in a 50% reduction in resting muscle blood flow.
flow (23). NO may also influence capillary permeability (24,25) and capillary basement membrane thickness (26), but it probably does not affect tortuosity or volume fraction independent of the capillary permeability effects (27). The extent to which eNOS content is related to the various individual components of nutritive flow (capillary perfusion, capillary permeability, lymph flow, tortuosity, and volume fraction) cannot be independently determined in the present study, as the microdialysis ethanol outflow-to-inflow data are influenced by changes in the many factors affecting nutritive flow. The muscle eNOS content is, however, apparently related to the sum total of the factors affecting nutritive flow in skeletal muscle.

The nutritive flow under resting conditions in muscle was significantly lower in older women than in young women. The ethanol outflow-to-inflow ratio was higher (indicating lower muscle nutritive blood flow) in older women than in young women, respectively. These data are in agreement with reports that resting limb blood flow is lower in older individuals than in young individuals (11,28). The resting nutritive blood-flow data, in combination with the positive relationship between nutritive blood flow and muscle eNOS content in the present study, suggest that nutritive blood flow may be reduced in states where muscle eNOS content is low.

**Estrogen and Muscle eNOS**

The lower eNOS content in older women was not due to differences in body composition or peak aerobic capacity, as there were no significant differences in these variables between the groups. There was a significantly higher serum estradiol concentration in young women, indicating that the difference in eNOS content may be due to the lower estradiol concentration in women following menopause. Estrogen infusion into the coronary circulation has been shown to improve NO-mediated coronary blood flow in postmenopausal women (29). In studies of forearm blood flow, acute estrogen infusion increased NO-mediated endothelium-dependent vasodilatation as measured by use of plethysmography in postmenopausal women (30), and 8 weeks of estrogen supplementation increased basal NO release (31). Estradiol has also been shown to upregulate eNOS expression in vitro (32–34). It is therefore possible that the lower muscle eNOS content in the older women in the current study was due at least in part to the normal aging process of menopause and resultant loss of estrogen. However, there was no significant correlation between serum estradiol concentration and eNOS content or nutritive

Figure 2. Relationship between muscle endothelial nitric oxide synthase (eNOS) content in skeletal muscle homogenates and nutritive blood flow. Muscle biopsies were obtained from the vastus lateralis of the quadriceps femoris muscle group from young (n = 7) and older women (n = 6) under resting conditions and analyzed for eNOS protein content. Resting nutritive blood flow was then monitored over 40 minutes with four microdialysis probes placed in the vastus lateralis. The mean ethanol outflow-to-inflow ratios obtained over the 40 minutes in each probe were averaged to yield a single outflow-to-inflow ratio for a given participant. Nutritive blood flow and eNOS content: r = .66, p < .05.

Figure 3. Relationship between (A) body mass index (BMI) and (B) waist circumference and muscle endothelial nitric oxide synthase (eNOS) content in skeletal muscle homogenates. Muscle biopsies were obtained from the vastus lateralis of the quadriceps femoris muscle group from young (n = 7) and older women (n = 6) under resting conditions and analyzed for eNOS protein content. BMI (kg/m²) and waist circumference at the minimum waist were also determined and correlated with muscle whole-homogenate eNOS content (BMI vs eNOS, r = .739; waist circumference vs eNOS, r = .722; p < .05).
Blood flow in this study. Older women on hormone replacement therapy were also not included in this study. Therefore, this study does not adequately address the issue of whether estrogen reductions with menopause are responsible for the reduced nutritive flow and eNOS content reported in older women in this study.

Body Composition and Muscle eNOS Content

Although it was not the original focus of the present study, eNOS content was found to correlate with BMI and waist circumference. This is despite the matching of young and older groups for body composition. Relationships have also been reported between waist circumference and/or BMI and markers of the metabolic syndrome of hypertension, hypertriglyceridemia, obesity, and type 2 diabetes mellitus. These diseases are all associated with a common underlying circulatory defect, in that NO-dependent vasodilatation is reduced (1–5). The poor NO-dependent vasodilatation may therefore be due to a lower muscle eNOS in these conditions, although this remains to be documented. The incidence of these conditions, however, does increase with increasing age in women, as does the incidence of increased central adiposity. Although there was a significant correlation between measures of obesity and eNOS content, it appears that obesity is not the cause of the reduced eNOS content in older women because there was no difference in measures of body composition between older and young women. Despite these findings, there may be a link between increased adiposity, reduced eNOS content, and endothelial dysfunction. A multiple stepwise regression analysis demonstrated that BMI and waist circumference were the only independent predictors of muscle eNOS content; furthermore, the muscle eNOS content was the only independent predictor of nutritive blood flow. However, BMI and waist circumference were not correlated with nutritive blood flow. This may be due to the many compensatory factors regulating nutritive blood flow, and it demonstrates that although there may be a connection between body fat and eNOS content, the increased adiposity does not necessarily manifest itself in altered nutritive flow.

Limitations of the Study

Limitations of this study include the use of whole-muscle homogenates for eNOS determination and the lack of data regarding eNOS activity. The eNOS measured in the whole homogenate may have come from either endothelial cells or skeletal muscle fibers (35). Although the NOS in the endothelial cells might be expected to be the more important regulator of blood flow, there is reason to believe that eNOS within the muscle fibers may regulate nutritive flow. The eNOS within the fibers has been shown to predominantly localize near mitochondria, and it has been suggested to regulate mitochondrial respiration (35,36). Furthermore, it has been reported that NO increases glucose uptake (37,38). The regulation of nutritive flow, glucose uptake, and mitochondrial respiration would allow for a coordination of nutritive supply to the muscle cells and nutrient disposal within the cells. The determination of the relative contribution of endothelial cell eNOS and intracellular skeletal muscle eNOS to the positive correlation between eNOS protein content and nutritive blood flow requires further study. Differences in eNOS activity may exist in the groups studied, and the relationship between eNOS activity and nutritive flow also requires further investigation.

Fiber type and capillary density were not measured in this study. Muscle eNOS content has been shown to be unrelated to fiber type (35) but would be expected to be related to capillary density. The relative proportion of type 1 muscle fibers has been reported to increase with age (39,40), and the correspondingly higher capillary density would be expected to result in higher eNOS content in older individuals. However, capillary density has been shown to be either lower (41) or not different (42) in older compared with young individuals. The independent effects of age on capillary density are therefore not clear.

Conclusions

In summary, skeletal muscle nutritive blood flow is lower in older women than in young women. The eNOS content in muscle homogenates obtained from older women is lower than the eNOS content in muscle obtained from young women. There is a significant correlation between muscle eNOS protein content and muscle nutritive blood flow at rest, demonstrating for the first time a relationship between muscle eNOS and nutritive skeletal muscle blood flow. There is also a significant inverse correlation between eNOS content and measures of body composition.

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