Skeletal muscle capillary density and microvascular function are compromised with aging and type 2 diabetes

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Groen BB, Hamer HM, Snijders T, van Kranenburg J, Frijns D, Vink H, van Loon LJ. Skeletal muscle capillary density and microvascular function are compromised with aging and type 2 diabetes. J Appl Physiol 116: 998–1005, 2014. First published February 27, 2014; doi:10.1152/japplphysiol.00919.2013.—Adequate muscle perfusion is essential for muscle mass maintenance, as it determines the rapid postprandial delivery of amino acids, nutrients, and growth factors to the muscle, thereby stimulating muscle protein synthesis (46). An impaired responsiveness of the skeletal muscle microvasculature to postprandial insulin release may be attributed to impairments in muscle microvascular structure and endothelial function, which may include a decline in capillary density (29) and structural changes of the endothelial wall (7, 9). In accordance, several studies have shown that aging has been associated with a lower capillary density of skeletal muscle tissue (42). Furthermore, Solomon and colleagues (45) recently demonstrated reduced skeletal muscle capillary density and nitric oxide bioavailability across the glucose tolerance continuum in older obese adults.

Besides changes in microvascular structure, there are also data to indicate that the endothelial wall function is compromised in senescent tissue (7, 34). In the search for novel targets to assess microvascular function, the recognition of the potential role of the endothelial glycocalyx layer in mediating vascular function offers new and exciting opportunities. Nearly 45 years ago, Luft et al. (31) introduced the endothelial glycocalyx layer as a dynamic intraluminal layer that consists of endothelial cell-derived proteoglycans, glycoproteins, and adsorbed plasma proteins. This network determines vascular permeability, transduces shear stress to the endothelium, and modulates interaction of leukocytes and thrombocytes with the vascular wall (54). Prior work has shown that glycocalyx permeability assessed in the sublingual cavity is representative of generic impairments in endothelial function in various other tissues in a large variety of subject populations (14a, 33, 44, 47, 51). As such, endothelial glycocalyx thickness in the oral cavity has been shown to correlate with impairments in endothelial function in the liver (47), brain (33), and kidney (44). Diabetes mellitus is associated with both microvascular and macrovascular diseases affecting numerous organs, including skeletal muscle, skin, heart, brain, and kidneys (3). Diabetes-associated vascular alterations include anatomic, structural, and functional changes leading to multiorgan dysfunction (11). Besides vascular alterations, it has also been reported that elderly type 2 diabetes patients show a more pronounced decline in skeletal muscle mass when compared with age-matched normoglycemic controls (38). Hence, it has been proposed that the loss of skeletal muscle mass represents not only a cause, but also a consequence of type 2 diabetes (27). Therefore, we hypothesize that the vascular density and the endothelial glycocalyx layer are even more compromised in the older population with type 2 diabetes.

Aging is associated with the loss of skeletal muscle mass and strength, resulting in functional impairments and an increased risk of developing chronic metabolic diseases (22, 24). Recent work suggests that with aging, skeletal muscle tissue becomes less sensitive to the main anabolic stimuli, i.e., food intake and physical activity (23, 39, 53). The proposed blunted muscle protein synthetic response, also known as “anabolic resistance,” has been suggested to represent a key factor responsible for the progressive loss of skeletal muscle mass with aging (10). Although the cause of this proposed anabolic resistance remains to be established, there is an emerging body of evidence suggesting that the resistance of senescent muscle to the postprandial insulin-mediated stimulation of muscle perfusion plays a key role.

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The present study investigates the impact of both aging and type 2 diabetes on skeletal muscle capillary density as well as microvascular function. Therefore, we selected 15 healthy young men (age: 24 ± 1 yr) and 15 older men (age: 70 ± 2 yr) males, as well as 15 age-matched older type 2 diabetic patients (age: 70 ± 1 yr). An oral glucose tolerance test was applied to assess whole body glucose tolerance and insulin sensitivity, and muscle biopsies were collected to assess skeletal muscle fiber type specific capillary density. Sidestream darkfield (SDF) imaging was used to assess capillary density and endothelial glycocalyx layer characteristics as a marker for endothelial microvascular function.

METHODS

Subjects. Fifteen healthy young men, 15 healthy older men, and 15 age- and body mass index (BMI)-matched type 2 diabetes patients were selected to participate in this study. Exclusion criteria were impaired renal or liver function, obesity (BMI > 30 kg/m²), glyco-sylated hemoglobin (HbA1c) content > 10.0%, cardiac disease, hyper-tension, diabetes complications, and exogenous insulin therapy. All type 2 diabetic patients were prescribed with oral blood glucose-lowering medication [metformin (n = 8), sulfonylurea derivatives (n = 2), metformin in combination with sulfonylurea (n = 4), or metformin in combination with a DPP-4 inhibitor (n = 1)].

Ethics statement. After explanation of the study protocol and potential risks and benefits, all subjects provided written informed consent before participating in the study. This study was approved by the Medical Ethical Committee of the Academic Hospital Maastricht and conform the to the principles outlined in the Declaration of Helsinki for use of human subjects and tissue.

Experimental protocol. Baseline characteristics [age, weight, height, blood pressure, leg volume, physical activity level (PAL) index (1)] were determined in all subjects prior to inclusion in the study protocol. Thereafter, various measurements were performed in two visits: a CT scan to assess muscle cross-sectional area 15 cm above the patella from the dominant leg, an oral glucose tolerance test, sidestream darkfield (SDF) imaging of the oral cavity, and a muscle biopsy collection procedure.

Oral glucose tolerance test. Two days prior to the oral glucose tolerance test (OGTT), subjects were instructed to refrain from intense physical activity, alcohol, and coffee. In addition, type 2 diabetes patients discontinued antidiabetic medication 2 days prior to the OGTT. The subjects resumed the use of their prescribed medication after the test. All subjects consumed a standardized dinner the evening prior to the test containing 0.18 MJ/kg body wt, consisting of 55 energy percent (En%) carbohydrate, 15 En% protein, and 30 En% fat. After 2200, the subjects were allowed only water ad libitum until the beginning of the experiments. Following an overnight fast, the subjects arrived at the laboratory at 0800 in the morning of the test day by car or public transportation. A catheter (Baxter BV, Utrecht, the Netherlands) was inserted into an antecubital vein for venous blood sampling. After insertion of the catheter, subjects were asked to stay in a supine position for the remainder of the test. After collection of a basal blood sample, subjects ingested a standard glucose drink [82.5 g dextrose monohydrate (AVEBE, Veendam, The Netherlands) in 250 ml water]. Subsequent blood samples were collected at t = 30, 60, 90, and 120 min. Aliquots of plasma were frozen in liquid nitrogen and stored at −80°C until analyses. Plasma glucose concentrations were determined by radioimmunoassay [Human Insulin RIA (HI-14K), Millipore, MA]. To determine HbA1C content, a 3-ml blood sample was collected in EDTA-containing tube and analyzed by high-performance liquid chromatography (Bio-Rad Diamat, Munich, Germany). Venous plasma glucose and insulin concentrations obtained during the OGTT were used to assess pancreatic β-cell function and insulin sensitivity. These parameters were assessed using the updated homeostasis model assessment HOMA, and the oral glucose insulin sensitivity (OGIS) index, respectively (28).

SDF imaging. Visualization of the sublingual microvascular system was performed with sidestream dark-field (SDF) imaging and has been described in detail elsewhere (33, 51). Perfused blood vessels with a diameter ranging from 1 to 50 μm can be visualized, thereby covering the average capillary diameter that ranges around 10 μm. Covered by a disposable cap, the probe of the camera can be placed on tissue surface. For this purpose SDF measurements were taken in the oral cavity underneath the tongue, since this area is easily accessible and is considered as a valid derivative to assess microvascular function in other tissues (14a, 33, 44). By placing the probe under the tongue, images of the sublingual microcirculation are obtained (30). The SDF recordings using the hand-held SDF camera (MicroScan Microscope System; Micro Vision Medical, Microscan B.V., Amsterdam, The Netherlands) were made prior to the OGTT. Recordings of the sublingual microcirculation were taken at 3 different sites under the tongue per time point, and all recordings were taken by the same researcher.

Using the images from the SDF camera, microcirculatory parameters were measured. The recordings were analyzed with dedicated software (Glycocheck ICU, Glycocheck B.V., Maastricht, The Netherlands) to determine the number of capillaries per diameter class per square millimeter. Permeability of the endothelial glycocalyx wall, also referred to as the perfused boundary region (PBR), was determined as described previously (16) and illustrated in Fig. 1. In each subject, Glycocheck ICU analyzing software automatically measured the red blood cell (RBC) column in at least 3,000 vessel segments. For each measurement segment, 840 radial intensity profiles were obtained for measurement of RBC column width, and PBR was calculated as distance of median (P50) RBC column width to the (estimated) outer edge of the RBC-perfused lumen (Fig. 1). Vessel segments were classified in 1-μm-wide diameter classes, and median PBR values were determined for each diameter class before calculating the average PBR over the 5- to 25-μm diameter range.

Immunohistochemical analyses. A muscle tissue biopsy was obtained from all subjects to determine muscle fiber characteristics and vascularization. A percutaneous needle biopsy (50–80 mg) was taken from the middle region of the vastus lateralis muscle, 15 cm above the patella and approximately 2 cm away from the fascia. Muscle biopsies were carefully freed from any visible fat and blood, after which the tissue was embedded in Tissue-Tek and rapidly frozen in liquid nitrogen-cooled isopentane (Sakura Finetek Europe). Muscle biopsies were stored at −80°C for subsequent immunohistochemical analysis.

From all muscle biopsies, 5-μm-thick cryosections were cut at −20°C. Samples of a young, an elderly, and a type 2 diabetes patient were mounted together on uncoated glass slides. Care was taken to properly align the samples for cross-sectional fiber analyses. Serial cross-sections were stained simultaneously for muscle fiber type and capillaries. First used antibodies are directed against myosin heavy chain (MHC)-1 (A4.840, dilution 1:25; Developmental Studies Hybridsoma Bank, Iowa City, IA), laminin (polyclonal rabbit anti-laminin, dilution 1:50; Sigma, Zwijndrecht, the Netherlands), and CD31 (dilution 1:50; DAKO, Glostrup, Denmark). The latter antibody was used for capillary determination in human muscle tissue (25). Appropriate secondary antibodies were applied: goat anti-mouse IgM AlexaFluor488, goat anti-rabbit IgG AlexaFluor568 (dilution 1:500 and 1:200, respectively; Molecular Probes, Invitrogen, Breda, the Netherlands) and biotin + avidin-D Texas Red (dilution 1:133 and 1:200, respectively; Vector Laboratories, Burlingame, CA).

Staining procedures were as follows: after 5 min fixation in acetone, the slides were air dried and incubated for 45 min at room temperature with CD31 primary antibodies directed against Platelet Endothelial Cell Adhesion Molecule-1 diluted in 0.05% Tween-
phosphate-buffered saline (PBS). For optimizing the staining result, slides were first incubated for 60 min with biotinylated horse anti-mouse IgG (dilution 1:150; Vector Laboratories), diluted in 0.05% Tween-PBS. Slides were then washed (3 × 5 min PBS). Thereafter, slides were incubated for 60 min at room temperature with avidin-D Texas Red diluted in 0.05% Tween-PBS. After another washing step with PBS, slides were incubated for 45 min with the primary antibodies directed against laminin and MHC-I diluted in 0.05% Tween-PBS. Subsequently, slides were incubated for 30 min at room temperature with the appropriate secondary antibodies in 0.05% Tween-PBS. After a final washing step, all slides were mounted with cover glasses using Mowiol (Calbiochem, Amsterdam, The Netherlands). Staining procedures resulted in laminin stained in blue, MHC-I in green, and CD31 in red (Fig. 2). All images were digitally captured, using fluorescence microscopy with a Nikon E800 fluorescence microscope (Nikon Instruments Europe, Badhoevedorp, The Netherlands) coupled to a Basler A113 C progressive scan color CCD camera with a Bayer color filter. Epi-fluorescence signal was recorded using a Texas Red excitation filter (540–580 nm) for CD31, a DAPI UV excitation filter (340–380 nm) for laminin, and a fluorescein isothiocyanate excitation filter (465–495 nm) for MHC-I. From the slides, images were captured at a 120× magnification. Laminin was used to determine the basement membrane, and all fibers within each image were identified as Type I or Type II fiber. Image processing and quantitative analyses were done using Lucia 4.81 software package (Nikon). Capillaries were quantified manually on each fiber to estimate the following indexes: the number of capillaries around a fiber [capillary contacts (CC)], fiber area (FA), perimeter (P), and the capillary-to-fiber ratio on an individual-fiber basis (CF/R), as described elsewhere (21). To examine the potential for blood-tissue exchange, the capillary-to-fiber perimeter exchange (CFPE) index (20) was calculated. All image recordings and analyses were performed by an investigator blinded to participant coding.

Statistics. Data are presented as means ± SE, and statistical analyses were performed using SPSS version 21 (SPSS, Chicago, IL). Differences between groups were determined by one-way analysis of variance (ANOVA). When a significant difference was found, pairwise comparisons by a Bonferroni post hoc test were used to assess differences. Fiber type-specific results were analyzed by a two-way repeated-measures ANOVA, with fiber type (type I vs II) as within-subject factor and group (young vs. older vs. type 2 diabetic patients) as between-subject factor. When a significant difference was found, pairwise comparisons by a Bonferroni post hoc test were used to assess differences. Correlations between different parameters were calculated using Pearson’s product-moment correlations. For all comparisons significance was recognized at P < 0.05.

RESULTS

Subjects. Subjects’ characteristics of the three groups (young, older, and type 2 diabetic patients) are shown in Table 1. All subjects completed the study protocol, and no adverse events were reported. Young subjects were significantly different from older and type 2 diabetic patients in age, BMI, HbA1c, 2 h glucose (OGTT), area under the curve for plasma glucose and insulin concentrations during OGTT, OGIS, and systolic and diastolic blood pressure. Significant differences between older and type 2 diabetic patients were seen in HbA1c, basal plasma glucose levels, 2 h glucose (OGTT), area under the curve for OGTT, OGIS, and HOMA-IR. Leg volume and physical activity levels did not differ between groups.

Muscle characteristics. Cross-sectional area of the quadriceps upper leg muscles was determined with a single slice CT scan from both legs. Cross-sectional area (Table 2) was significantly lower in the older (−16%) and type 2 diabetic patients (−20%) when compared with the young (P < 0.05
and $P < 0.01$, respectively). Fiber area was analyzed in the muscle biopsy stainings from the vastus lateralis muscle (Fig. 2). One-way ANOVA showed significant differences in mixed muscle fiber area between groups, with fiber area being significantly lower in the type 2 diabetic patients when compared with the young ($P = 0.007$). No significant differences were observed between muscle fiber types (effect of fiber type $P = 0.410$). Both type I and type II muscle fiber size in the type 2 diabetic patients were significantly smaller when compared with the young ($P = 0.009$). Furthermore, a strong tendency for a fiber type × group interaction ($P = 0.071$) was observed implying some level of fiber type specificity in the observed differences in muscle fiber type size between groups.

**Microvascular density in muscle biopsy stainings.** Muscle characteristics for microvascular density were analyzed in microvascular biopsies from the vastus lateralis muscle. Morphometric results (i.e., capillary contacts, individual capillary-to-fiber ratio, and CFPE index) are presented in Table 3. All data are provided for mixed muscle, as well as for type I and type II muscle fibers separately. In the mixed muscle tissue, both the older and type 2 diabetic patients show fewer capillary contacts and a lower individual capillary-to-fiber ratio compared with the young ($P < 0.05$ for the older and $P < 0.01$ for the type 2 diabetic patients for both parameters, respectively). No differences were observed for CFPE indexes between groups. With respect to fiber type specific capillarization parameters in the muscle biopsies, no fiber type × group interactions were observed. With respect to fiber type, all groups show fewer capillary contacts and lower individual capillary-to-fiber ratio and CFPE indexes in type II compared with type I muscle fibers. Looking at fiber type-specific differences, only capillary contacts and individual capillary-to-fiber ratio differed between the older and type 2 diabetic patients compared with the young.

**Microvascular density in SDF imaging.** Vessels with a diameter ranging from 1 to 50 μm were detected in the oral cavity using SDF imaging. Microvascular density was determined by calculating the total number of capillaries per square millimeter. Microvascular density was similar between groups. The relative number of small capillaries per square millimeter, expressed as the percentage small capillaries in relation to the total number, is shown in Fig. 3. Figure 3, inset, shows the percentage capillaries with a diameter between 1 and 7 μm (small) vs. the percentage capillaries with a diameter ranging from 8 to 50 μm (large). A significant difference was only observed between young and type 2 diabetic patients ($P = 0.003$).

**Endothelial glycocalyx.** Endothelial glycocalyx permeability, expressed as the thickness of the erythrocyte perfused boundary region (PBR), was measured with SDF imaging. Average values of the groups were calculated with the use of capillaries identified with a diameter between 1 and 50 μm (Fig. 4). On average, PBR was greater in the type 2 diabetic patients compared with the young controls (2.00 ± 0.05 μm vs. 1.79 ± 0.04 μm, $P < 0.05$). No significant differences were observed between the young and older. Significant correlations were observed between the PBR thickness and HbA$_{1c}$ levels

<table>
<thead>
<tr>
<th>Table 1. Subjects’ characteristics</th>
<th>Young ($n=15$)</th>
<th>Older ($n=15$)</th>
<th>Type 2 Diabetes ($n=15$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>24 ± 1</td>
<td>70 ± 2*</td>
<td>70 ± 1*</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>77.2 ± 2.3</td>
<td>82.8 ± 2.3</td>
<td>86.2 ± 2.9*</td>
</tr>
<tr>
<td>Body mass index, kg/m$^2$</td>
<td>23.2 ± 0.6</td>
<td>26.6 ± 0.5*</td>
<td>27.4 ± 0.6*</td>
</tr>
<tr>
<td>Leg volume, liters</td>
<td>8.0 ± 0.2</td>
<td>8.2 ± 0.4</td>
<td>7.7 ± 0.3</td>
</tr>
<tr>
<td>HbA$_{1c}$, %</td>
<td>5.2 ± 0.1</td>
<td>5.6 ± 0.1*</td>
<td>6.6 ± 0.2†</td>
</tr>
<tr>
<td>Fasting glucose, mmol/l</td>
<td>5.0 ± 0.1</td>
<td>5.4 ± 0.1</td>
<td>7.8 ± 0.4†</td>
</tr>
<tr>
<td>2 h Glucose (OGTT), mmol/l</td>
<td>4.9 ± 0.3</td>
<td>6.6 ± 0.5*</td>
<td>15.3 ± 1.1†</td>
</tr>
<tr>
<td>OGTT AUC glucose, mmol·l$^{-1}$·2 h$^{-1}$</td>
<td>718 ± 29</td>
<td>933 ± 53*</td>
<td>1636 ± 86†</td>
</tr>
<tr>
<td>Fasting insulin, mU/l</td>
<td>14.6 ± 0.2</td>
<td>21.3 ± 3.1</td>
<td>24.5 ± 3.3</td>
</tr>
<tr>
<td>2 h Insulin (OGTT), mU/l</td>
<td>31 ± 4</td>
<td>61 ± 8*</td>
<td>70 ± 8*</td>
</tr>
<tr>
<td>OGTT AUC insulin, U·l$^{-1}$·2 h$^{-1}$</td>
<td>5,263 ± 340</td>
<td>10,570 ± 1,567*</td>
<td>6,806 ± 612</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>3.27 ± 0.34</td>
<td>5.83 ± 0.82</td>
<td>7.30 ± 0.88†</td>
</tr>
<tr>
<td>OGIS</td>
<td>443 ± 13</td>
<td>364 ± 14*</td>
<td>291 ± 10†</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>125 ± 2</td>
<td>133 ± 3*</td>
<td>134 ± 4*</td>
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<tr>
<td>Diastolic blood pressure, mmHg</td>
<td>66 ± 2</td>
<td>74 ± 2*</td>
<td>74 ± 3*</td>
</tr>
<tr>
<td>PAL index</td>
<td>1.44 ± 0.03</td>
<td>1.52 ± 0.04</td>
<td>1.39 ± 0.07</td>
</tr>
</tbody>
</table>

Values represent means ± SE. HbA$_{1c}$, glycated hemoglobin; OGTT, oral glucose-tolerance test; OGTT$_{120}$, plasma glucose concentrations 120 min after ingesting a 75 g glucose load; HOMA-IR, homeostasis model of assessment-insulin resistance; OGIS, oral glucose insulin sensitivity; PAL index, physical activity level. Analysis of variance (ANOVA) was used for statistical analysis of the data, using Bonferroni post hoc testing. *Significantly different from young, $P < 0.05$. †Significantly different from the older healthy subjects, $P < 0.05$.

<table>
<thead>
<tr>
<th>Table 2. Muscle and fiber characteristics</th>
<th>Fiber</th>
<th>Young ($n=15$)</th>
<th>Older ($n=15$)</th>
<th>Type 2 Diabetes ($n=15$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quadriceps muscle CSA, cm$^2$</td>
<td></td>
<td>84.0 ± 3.6</td>
<td>710 ± 2.8*</td>
<td>67.6 ± 6.5*</td>
</tr>
<tr>
<td>Fiber area, μm$^2$</td>
<td>Mixed</td>
<td>7172 ± 432</td>
<td>6303 ± 311</td>
<td>5616 ± 259*</td>
</tr>
<tr>
<td></td>
<td>Type I</td>
<td>6660 ± 522</td>
<td>6533 ± 386</td>
<td>5558 ± 275*</td>
</tr>
<tr>
<td></td>
<td>Type II</td>
<td>7570 ± 478</td>
<td>6107 ± 354</td>
<td>5649 ± 315*</td>
</tr>
</tbody>
</table>

Values represent means ± SE. CSA, cross-sectional area. Differences between groups (quadriceps muscle CSA and mixed muscle fiber area) were determined by one-way ANOVA, and pairwise comparisons by Bonferroni post hoc testing were used to assess where significant differences were located. Fiber type-specific results were determined by a two-way repeated-measures ANOVA, with fiber type (type I vs. type II) as within-subject factor and group (young vs. older vs. type 2 diabetic patients) as between-subjects factor. *Significantly different from the young (Bonferroni post hoc test, $P < 0.05$).

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and OGIS values [0.36 ($P < 0.05$) and $-0.36$ ($P < 0.05$), respectively], suggesting an association between the level of insulin sensitivity and the endothelial glycocalyx thickness. Likewise, a significant correlation was observed between the PBR thickness and muscle cross-sectional area ($r = 0.57; P < 0.01$) and the proportion of small capillaries ($r = -0.39; P < 0.01$).

**DISCUSSION**

The present study confirms reduced muscle mass and lower insulin sensitivity in healthy older men and age-matched type 2 diabetic patients compared with young controls. The greater insulin resistance at a more advanced age was accompanied by a lower capillary density in skeletal muscle tissue and an increased permeability of the endothelial glycocalyx layer.

In agreement with earlier research (38, 45), we show that insulin sensitivity (OGIS and HOMA-IR) is reduced in both healthy as well as type 2 diabetic older adults compared with healthy young controls. Similar observations were made for skeletal muscle mass, with significantly less skeletal muscle tissue in the healthy and type 2 diabetic older men compared with the younger controls (Table 2). These results are in line with earlier findings from our lab (40) as well as others (4, 18, 19, 50), showing muscle loss with aging, and an even more rapid muscle loss in a clinically compromised type 2 diabetic elderly subpopulation (27). Besides assessing muscle mass on a macroscopic scale, we performed additional microscopic analyses to assess various muscle fiber type characteristics (Tables 2 and 3). In agreement with previous work from our group (37, 48) as well as others (26), we observed that muscle fiber size was reduced in the older and older type 2 diabetic men when compared with the young controls. In addition, muscle fiber size tended to be smaller in the type 2 diabetic patients compared with the age-matched healthy controls. Differences in muscle fiber size between groups seemed to be particularly evident for the type II muscle fibers. In line, we observed a strong tendency for a fiber type × group interaction ($P = 0.071$), suggesting that type II fibers are more prone to muscle atrophy in the older and/or older type 2 diabetes patients.

The reduced muscle mass in the older populations have been, at least partly, attributed to a reduced sensitivity of the muscle protein synthetic machinery to the anabolic response to dietary protein consumption (10, 13). Underlying mechanisms responsible for this so-called anabolic resistance include impairments in (micro)vascular function and subsequent attenuated increase in postprandial perfusion (5, 17, 34). Impairments in microvascular function may be attributed to a lower capillary density (29, 32) and/or impairments in endothelial wall function (41). An intact glycocalyx is required to allow trans-

![Fig. 3. Relative number of vessels (expressed as percentage) per vessel diameter. Values are expressed as means ± SE. T2D, type 2 diabetes. *Significantly different from young (Bonferroni’s post hoc test, $P < 0.05$).](chart.png)
capillary distribution of insulin into the interstitial fluid, thereby allowing associated myocellular signaling, subsequent amino acid uptake, and myofibrillar protein synthesis. In agreement with previous work in various groups, a thicker perfused boundary region is associated with endothelial dysfunction as observed in various organs in multiple patient groups (6, 14a, 15, 47, 52). In line with these findings we report a thicker perfused boundary region in the type 2 diabetic vs. the young. The greater PBR is accompanied by a reduced endothelial glycocalyx thickness, compromising nutrient exchange over the endothelium. We propose that a compromised nutrient exchange in the postprandial state in the older and/or type 2 diabetic patient may contribute substantially to the anabolic resistance to feeding and, as such, may be one of the underlying mechanisms responsible for the loss of muscle mass with aging (and “accelerated” aging in the older type 2 diabetes patient). In the present study we compare muscle mass and muscle fiber type characteristics with several parameters of microvascular function including muscle capillary density, as well as the thickness of the endothelial glycocalyx layer in healthy young, older, and older type 2 diabetic patients. This observational study is the first to combine various quantitative and qualitative assessment of microvascular characteristics of muscle tissue.

In the muscle biopsy samples, we quantified microvascular perfusion capacity according to the method of Hepple and colleagues (21). Fewer capillary-to-fiber contacts and a smaller ratio between capillaries and the number of muscle fibers they perfuse were observed in the older and older type 2 diabetes patients. Consequently, aging and insulin resistance seem to be accompanied by a structural reduction in perfusion capacity, thereby reducing the potential for amino acid delivery to the muscle. In addition to the muscle biopsy analysis, sidestream darkfield (SDF) imaging was applied to visualize real-time perfusion of the sublingual microvasculature. Using SDF imaging, capillaries with a diameter range between 1 and 50 μm were visualized. Currently this method cannot be applied in human leg muscle tissue for in vivo measurements of muscle perfusion, but previous work has shown that these measurements are representative for many other tissues such as liver, brain, and kidney (33, 44, 47). Smaller capillaries tended to be less prevalent in the older and older type 2 diabetes patients compared with the healthy young controls (Fig. 3). The observation of a lower capillary density in leg muscle tissue in the older and older type 2 diabetes patients seems to agree with the apparent loss of well-perfused small nutritive capillaries visualized with SDF imaging. The capillary loss seems to occur without concomitant arteriolar/venular loss in the old and diabetics (Fig. 3, inset). An alternative explanation for the observed shift in the distribution of vessel size with a relative loss of small vessels could be dilation of the small capillaries.

The main reason to apply SDF imaging in the present study was to assess some of the qualitative aspects of the microcirculation in the young, older, and older type 2 diabetic patients. The endothelial glycocalyx layer is increasingly being recognized for its important function in the transport capacity (i.e., glucose and/or insulin) over the endothelial wall (16, 43). Eskens and colleagues (16) have demonstrated that the loss of endothelial glycocalyx results in elevated permeability of the endothelial layer, thereby compromising protective function and transport capacity over the endothelium. In the present study we show that the endothelial glycocalyx was more compromised in older type 2 diabetes patients compared with the healthy young controls (Figs. 1 and 4). In the healthy young subjects we observed an average perfused boundary region thickness of 1.79 ± 0.04 μm, which is similar to previously reported values in healthy young subjects (18–40 yr) (35). An increased perfused boundary region, reflecting deeper penetration of red blood cells into the luminal part of the endothelial glycocalyx, was observed in the older (1.89 ± 0.05 μm) and the older type 2 diabetic patients (2.00 ± 0.05 μm) compared with healthy young subjects. To the best of our knowledge, this is the first report of a direct comparison of the endothelial glycocalyx layer between young, older, and older age-matched type 2 diabetic patients. Furthermore, we show that the thickness of the perfused boundary region correlates well with both the OGIS and HbA1c values taken as a measure for insulin resistance (r² = 0.36; P < 0.05). These correlations strongly support our hypothesis that the integrity of the endothelial glycocalyx layer interacts with the degree of insulin sensitivity and glucose uptake during an OGTT (Table 1).

Previously, Broekhuizen et al. (8) and Nieuwdorp et al. (36) showed that hyperglycemia is accompanied with changes in glycocalyx characteristics. With the present data we extend on these observations by showing that diabetes and hyperglycemia are associated with loss of muscle mass, lower microvascular density, and increased endothelial glycocalyx permeability. It has recently been suggested (49) that impaired tissue perfusion due to abnormality of the microvascular system is present along with other more conventional cardiovascular risk factors, including hypertension and diabetes (12, 14, 55). However, the pathological processes involved in the vascular dysfunction in diabetes are complex and far from being understood. Loss of glycocalyx function may be a common factor in the established relationship between insulin resistance and endothelial dysfunction in patient populations. Furthermore, the results from the present study imply that such microvascular complications may also play a key role in the etiology of anabolic resistance.
and the associated loss of skeletal muscle mass. Therefore, improving microvascular perfusion and glycocalyx integrity could represent new potential targets to prevent or decrease muscle loss and support healthy aging.

We conclude that aging and type 2 diabetes are accompanied with a reduced muscle mass, lower insulin sensitivity, reduced skeletal muscle capillary density, and compromised endothelial wall function. These observations indicate that both quantitative and qualitative changes in the microvasculature may contribute to the loss of skeletal muscle mass and the development of insulin resistance at a more advanced age.

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DISCLOSURES

H. Vink is Chief Scientific Officer at GlycoCheck BV, The Netherlands.

AUTHOR CONTRIBUTIONS


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