Capillary Density of Skeletal Muscle
A Contributing Mechanism for Exercise Intolerance in Class II–III
Chronic Heart Failure Independent of Other Peripheral Alterations

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OBJECTIVES
The study was conducted to determine if the capillary density of skeletal muscle is a potential contributor to exercise intolerance in class II–III chronic heart failure (CHF).

BACKGROUND
Previous studies suggest that abnormalities in skeletal muscle histology, contractile protein content and enzymology contribute to exercise intolerance in CHF.

METHODS
The present study examined skeletal muscle biopsies from 22 male patients with CHF compared with 10 age-matched normal male control patients. Aerobic capacities, myosin heavy chain (MHC) isoforms, enzymes, and capillary density were measured.

RESULTS
The patients with CHF demonstrated a reduced peak oxygen consumption when compared to controls (15.0 ± 2.5 vs. 19.8 ± 5.0 ml/kg–1·min–1, p <0.05). Using cell-specific antibodies to directly assess vascular density, there was a reduction in capillary density in CHF measured as the number of endothelial cells/fiber (1.42 ± 0.28 vs. 1.74 ± 0.35, p = 0.02). In CHF, capillary density was inversely related to maximal oxygen consumption (r = 0.479, p = 0.02). The MHC IIx isoform was found to be higher in patients with CHF versus normal subjects (28.5 ± 13.6 vs. 19.5 ± 9.4, p <0.05).

CONCLUSIONS
There was a significant reduction in microvascular density in patients with CHF compared with the control group, without major differences in other usual histologic and biochemical aerobic markers. The inverse relationship with peak oxygen consumption seen in the CHF group suggests that a reduction in microvascular density of skeletal muscle may precede other skeletal muscle alterations and play a critical role in the exercise intolerance characteristic of patients with CHF. (J Am Coll Cardiol 1999;33:1956–63) © 1999 by the American College of Cardiology

Exertional fatigue or exercise intolerance is a common presenting symptom in over one-third of patients with chronic heart failure (CHF). Although medical therapy has reduced mortality, exercise intolerance remains a major source of morbidity in this population. It is well accepted that the severity of left ventricular systolic dysfunction, as measured by left ventricular ejection fraction (LVEF) or pulmonary capillary wedge pressure, cannot alone explain the compromised functional capacity observed in chronic heart failure (1–4). Left ventricular ejection fraction does not correlate with exercise tolerance, and increases in pulmonary capillary wedge pressure during exercise are not responsible for exercise limitation in patients limited by dyspnea. Therefore, although previously studied by many groups, the complete mechanisms for exercise intolerance in patients with CHF remain unknown.

Previous studies comparing class II–IV CHF patients to active normals have demonstrated differences in exercise tolerance accompanied by alterations in skeletal muscle histology and biochemistry (5–9). These alterations include a reduction of type I fibers, decreased aerobic enzymes and contractile protein changes. Studies are conflicting regarding changes in capillary density in the skeletal muscle of CHF patients. Our laboratory (5) previously reported that the number of capillaries per muscle fiber was decreased for type I and type IIA fibers in CHF, but the ratio of capillaries to cross-sectional fiber area was not different in CHF (due to a concomitant reduction in fiber diameter) when compared with normal subjects. On the basis of these findings,
Skeletal Muscle Capillary Density in Heart Failure

Abbreviations and Acronyms

A-VO$_2$ = arteriovenous-oxygen difference
CHF = chronic heart failure
LVEF = left ventricular ejection fraction
MET = metabolic equivalent
MHC = myosin heavy chain
NMR = nuclear magnetic resonance

we concluded that overall oxygen diffusion distances were unchanged in patients with CHF. Mancini et al. (8) observed no difference in the number of capillaries per muscle fiber, but did observe an increase in capillaries/mm$^2$ cross-sectional area in CHF. Lipkin et al. (9) observed capillaries per muscle fiber to be normal in CHF, whereas Drexler et al. (7) found capillary length density (number of capillaries per unit volume of muscle tissue) to be reduced in CHF. Vascular density in these studies was assessed by methods that detect components of the basement membrane (i.e., periodic acid–Schiff staining), not by a direct examination for endothelial cells. Therefore, capillary density warrants further investigation to explain the exercise intolerance seen in patients with CHF.

The vascular bed in skeletal muscle functions mainly to supply oxygen to muscle fibers. Capillary density strongly correlates with human skeletal muscle oxidative capacity (10,11). Therefore, it is also possible that it may be important in determining exercise tolerance in CHF independent of other biochemical or histological changes in the skeletal muscle. The purpose of this investigation was to determine if the capillary density of skeletal muscle is a potential contributor to exercise intolerance in patients with ambulatory class II–III CHF. We hypothesized that capillary density in skeletal muscle of patients with CHF would be reduced and positively correlated with measures of exercise tolerance.

METHODS

Patient population. Twenty-two male patients (12 from Duke Medical Center in Durham, North Carolina and 10 from Henry Ford Hospital in Detroit, Michigan) with class II–III CHF due to left ventricular systolic dysfunction (LVEF < 35%) and 10 (from Duke Medical Center) age–matched male sedentary normals participated in the study. All subjects with CHF were on a stable medical regimen for a minimal of three months before the study. Twenty were on digoxin, 21 were taking diuretics, 21 angiotensin-converting enzyme inhibitors, and 12 were taking long-acting nitrates. All subjects with CHF were symptomatic limited by dyspnea or leg fatigue. Thirteen patients had ischemic cardiomyopathy and nine had idiopathic cardiomyopathy. All subjects were free of claudication, rales and peripheral bruits. Exclusion criteria included insulin-independent diabetes mellitus, clinically significant chronic obstructive pulmonary disease and peripheral vascular disease. All patients with CHF were sedentary and not involved in any form of regular physical activity. There was no indication of cardiopulmonary dysfunction in normal subjects by either history or physical examination, and none exhibited symptoms of ischemic heart disease. Normal subjects were on no medications and none were engaged in any type of regular aerobic exercise.

Study protocol. All studies were performed under research protocols approved by the Institutional Review boards of the Duke University, the Durham Veterans Administration Medical Center and Henry Ford Hospital. Each subject was informed of testing protocols and the potential risks and benefits of participation. All subjects provided written consent before participation.

Exercise testing. All patients with CHF underwent graded upright bicycle exercise to a symptom–limited maximum on a cycle ergometer (Fitron, Lumex, Ronkonkoma, New York, or Monarck, Varberg, Sweden) with a 12-lead electrocardiogram as previously described in our laboratory (5). The workload began at 150 kpm/min and advanced in 3-min stages of 150 kpm/min. Equilibrium radionucleotide angiograms were obtained for Duke University subjects at rest using a low-energy mobile gamma camera. Expired gases were analyzed continuously using a Sensormedics 4400 unit (Yorba Linda, California). Henry Ford patients were included if LVEF was <35% via equilibrium radionucleotide angiograms or catheterization within six months, or an echocardiogram showing LVEF <30%.

Muscle biopsies. Biopsy samples were obtained from the vastus lateralis using a modified Bergstrom needle technique (12). Biopsy sites were anesthetized with a 2% lidocaine solution, and 0.5-cm incisions were made through the skin and fascia lata. The needle was consistently inserted to a depth of 40 to 60 mm. Samples were then mounted in cross section, in optimal cutting temperature (OCT) compound (Miles Pharmaceutical, West Haven, Connecticut) beds, and snap-frozen at −80°C.

Immunohistochemical analysis of vascular density. Vascular density, expressed as endothelial cells/muscle fiber, was determined by examining the total number of endothelial cells relative to the total number of muscle fibers via light microscopy. Endothelial cells were identified in histologic sections using immunohistological techniques with an endothelial-cell–specific monoclonal antibody in methods previously described (13). Sections were cut throughout the tissue block for analysis, ensuring a homogenous sample of the block. Briefly, slides were allowed to come to room temperature, placed in ice-cold acetone for 2 min and phosphate buffered saline (PBS) for 5 min. Blocking solution (10% horse serum in PBS) was applied for 1 h at room temperature. The primary antibody CD-31 (DACO) was applied for 30 min at room temperature, followed by sequential incubation with a biotinylated anti-mouse IgG.
and ABC reagent, according to the manufacturer's specifications (Vectastain ABC kit, Vector Laboratories, Burlingame, California). Levamisole was added to block endogenous alkaline phosphatase activity and immune complexes were localized using the chromogenic alkaline phosphatase substrate Vector Red (Vector Laboratories). When counterstained with hematoxalin, dehydrated and mounted with Permount (Fisher Scientific) the antigen appears to be red and the nuclei appears to be blue. A murine IgG monoclonal antibody served as a negative control (13). A minimum of six 200× fields were counted. The mean number of fibers counted for each sample was 40. Only fields that were in cross section were counted. Fields not oriented in cross section were excluded from analysis. A random number of samples were chosen for a blinded repeat analysis. Intraobserver variability on samples chosen for blind repeat analysis was 5%. To exclude differences in cross-sectional area of muscle fibers between CHF and normals, randomly selected slides in cross section were projected and digitized. The stained slide of human muscle was placed on the Olympus 1X70 microscope (Sterling, Virginia) and transferred onto a computer screen at a magnification of 100× through an Optronics Engineering DEI-750 camera (Goleta, California) to the Adobe Premiere 4.2.1 program (San Jose, California). Within this program the image of the muscle fiber was captured and saved to the Adobe Photoshop LE program, then opened into the NIH Image 1.6/ppc program. In the NIH Image program the individual muscle fiber was outlined and the total area was calculated.

Myosin heavy-chain analysis. Muscle biopsy samples (~30 mg each) were minced with razor blades on a chilled glass plate and transferred to microcentrifuge tubes. The samples were washed and resuspended twice in a rigor solution containing protease inhibitors (100 mmol/liter NaCl; 15 mmol/liter Tris pH 8.0; 2 mmol/liter MgCl2; 4 mmol/liter EGTA; 7 mmol/liter B-mercaptoethanol, 0.05 mmol/liter EDTA and diluted (1:100) in 20 mmol/liter imidazole buffer with 0.02% BSA (17).

Phosphofructokinase and succinic dehydrogenase activities were performed on the fresh homogenates while the enzymes malate dehydrogenase, 3-hydroxyl-CoA dehydrogenase, phosphorylase, hexokinase, and lactate dehydrogenase were performed on the frozen homogenates stored (~80°C) until the time of analysis. Enzyme assays were performed fluorometrically using an end point assay at a temperature of 23°C as outlined previously (17,18).

The maximal activity of one enzyme, cytochrome C oxidase, was measured spectrophotometrically at 38°C using a reaction medium that consisted of 970 μl of 10 mmol/liter potassium phosphate buffer (pH 7.0) and 20 μl of 1 mmol/liter solution of reduced cytochrome C (Sigma C-2506 (St. Louis, Missouri)). The assay was initiated by adding 10 μl of the sample (diluted 10:1 in phosphate buffer) to the reaction medium (1 mmol/liter) and the decrease in absorbance was measured at a wavelength of 550 nm for 3 min. Maximum activity was calculated using the measured slope and the extinction coefficient of reduced cytochrome C at 550 nm.

Statistical analysis. Intergroup comparisons were performed using the unpaired Student t test. Intragroup CHF comparisons were made using the nonparametric Mann-Whitney U test. A p value <0.05 was considered significant.

To determine the relation of variables, Pearson correlations were performed on linear regressions between the measured biochemical and histologic markers and aerobic capacity. For purposes of the analysis of relationships between aerobic capacity and vascular density we divided the CHF patients into three groups: those with a range in exercise capacity of 4 METs, 4 to 5 METs, and >5 METs. Thus, the groups analyzed were those with exercise capacities <4 METs, 4 to 5 METs, and >5 METs.

RESULTS

Table 1 contains demographic data for the CHF patients and normal controls. There were no significant differences in age, weight, height or body surface area (BSA) between the two groups. Subjects with CHF had a peak VO2 of 15.0 ± 2.5 ml/kg/min versus 19.8 ± 5.0 for normals (p < 0.05) and a LVEF of 22.5 ± 8.4% vs. 56.0 ± 7.9 (p < 0.001).

Table 2 provides a summary of the relative content of MHC type I, type IIa, and type IIx in the skeletal muscles of individuals with CHF and normal subjects. Relative percentages for MHC I and MHC IIa were all similar between groups. The relative percentage of MHC IIx

Enzymology. For the measurement of the maximal activities of several enzymes representative of different energy pathways, frozen tissue samples were homogenized in a phosphatase buffer (pH 7.4) containing 0.02% bovine serum albumin (BSA), 5 mmol/liter B-mercaptoethanol, and 0.05 mmol/liter EDTA and diluted (1:100) in 20 mmol/liter imidazole buffer with 0.02% BSA (17).

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isoform was significantly higher in the CHF group (patients with CHF 28.3 ± 13.6% vs. normals 21.5 ± 6.5%, p < 0.05).

Table 3 contains aerobic and glycolytic enzyme activities in the two groups. One of four glycolytic enzymes measured, phosphorylase, was found to be elevated in CHF (p < 0.05). Two out of five aerobic enzymes, 3-hydroxyl-CoA dehydrogenase (p = 0.001) and succinic dehydrogenase (p < 0.05), were lower in patients with CHF.

Vascular density measurements for each of the individual patients with CHF and normal subjects are shown in Figure 1. Overall, the number of endothelial cells/muscle fiber was 1.42 ± 0.28 for patients with CHF versus 1.76 ± 0.36 (p = 0.02) in normals. There was no difference in fiber area between the groups. Figure 2 illustrates capillaries/mm² for all subjects categorized by MET level. No differences were detected in fiber diameter between groups.

Pearson correlation analysis was performed separately on patients with CHF and on normal subjects to demonstrate relationships between all markers of aerobic capacity and capillary density. In normal subjects, these results were unexpected and counterintuitive. On the basis of exercise training studies, we expected a strong positive relationship between capillary density and oxidative capacity. These results suggest that a maladaptive process is active in the peripheral skeletal muscle in CHF.

**Capillary density and aerobic capacity.** The capillary bed in skeletal muscle functions mainly to supply oxygen to muscle fibers. Therefore, skeletal muscle capillary density has a strong positive relationship with peak VO₂ in normal subjects. In humans, exercise training results in an increase in the number of capillaries/muscle fiber and an increase in the number of capillaries/mm² of muscle (11). Interestingly, in other animals, increases in capillary density precede changes in the activity of oxidative enzymes and changes in contractile protein composition in models of fiber type transformation (19). Therefore, angiogenesis probably plays a critical role in adaptive responses to functional demand, where it correlates with improvements in endurance and oxidative capacity (10,11,20).

The results of this study suggest that patients with CHF and with severely compromised aerobic capacities may have a compensatory, maladaptive angiogenic response to exercise intolerance. In addition, we postulate that as CHF worsens, capillary density changes may occur before major enzyme or contractile protein changes. These two postulates are supported by the weak but consistent trend that four of five aerobic enzymes are decreased in patients with CHF and two aerobic markers, maximal oxygen consumption and total exercise time in patients with CHF (Fig. 3). Taking into account previous studies in normal subjects, these results were unexpected and counterintuitive.
compared with normals, and MHC IIx is greater in CHF without a concomitant decrease in MHC I. Worsening CHF may completely shift the enzyme panel toward a significant decrease in all aerobic enzymes and a reduction in MHC I compared to normal subjects.

**Role of peripheral hemodynamics and metabolism.** Although it is known that patients with CHF have a reduced blood flow to exercising skeletal muscle when compared to normal subjects, several studies have demonstrated that anaerobic metabolism in skeletal muscle during exercise in patients with CHF is not totally due to hypoperfusion (21–24). These observations have been confirmed by 31P-NMR (nuclear magnetic resonance) studies showing early anaerobic metabolism in patients with CHF when compared with normals, even with total occlusion of limb blood flow (25,26).

Furthermore, prior work from our laboratory demonstrated that patients with CHF have the ability to optimally extract oxygen from arteriole blood, as demonstrated by maximal arteriovenous-oxygen differences (\(\Delta V_{O2}\)) (2). This compensatory response allows patients with CHF to obtain similar oxygen uptakes at rest and submaximal workloads when compared with normals. The increases seen in the capillary bed cause an adjunctive increase in blood volume, thereby enhancing oxygen extraction. This study demonstrates that the number of fibers being subserved by capillaries is greater in CHF muscle compared to normal muscle (the capillary-to-fiber ratio is lower), but lower as the CHF group’s aerobic capacity decreases. Therefore, there is a greater potential for maximizing oxygen removal in CHF. We postulate that a decreased capillary density to the working muscles may contribute to the earlier onset of skeletal muscle anaerobic metabolism in ambulatory patients with class II–III CHF.

**Interplay between cardiac output and skeletal muscle.** This study has identified reduced capillary density as a possible contributor to exercise intolerance in patients with CHF. This study also demonstrates that capillary density can be altered independent of other major skeletal muscle alterations. These findings do not exclude an interplay between cardiac reserve and the periphery skeletal muscle. One limitation of this study was that cardiac output was not measured. Wilson et al. (27) have demonstrated that patients with CHF with normal cardiac output response to exercise had skeletal muscle abnormalities. Interestingly, this subgroup responded well to exercise training. Because no study has thoroughly examined central hemodynamic and peripheral alterations simultaneously or the feasibility of reversing skeletal muscle alterations with a long-term exercise program, it is difficult to ascertain the extent each is contributing to the observed exercise intolerance. Decreased cardiac reserve is a likely mechanism for early fatigue in patients with CHF; however, identifying the peripheral changes that occur, and their potential reversibility due to deconditioning, would not only help solve the puzzle of skeletal muscle pathophysiology in CHF compared to deconditioned normal subjects, but also aid in therapeutic interventions for all patients with CHF.

*Figure 1.* Comparison of endothelial cell/muscle fiber ratio (mean ± SD) between patients with ambulatory class II–III CHF and normal subjects (*p = 0.02).*
Possible explanations for the inverse relationship between capillary density and aerobic capacity. Increased capillary density for patients with low MET levels may reflect the body’s attempt to maintain metabolic demands of the periphery that are compromised as a result of an abnormal cardiac output response to exercise. This may explain the tiered levels of endothelial cell/muscle fiber seen in CHF in Figure 2 and the inverse relationships illustrated in Figure 3. These inverse relationships were not found in the normal subjects (data not shown).

In addition, endothelial growth may also be stimulated by an increased need to clear waste products, primarily lactate, shown to accumulate in CHF. In subgroups of patients with CHF, activities of daily living are performed at high percentages of an individual’s maximal capacity, at or near anaerobic threshold. It is possible that this represents an exercise training stimulus for the skeletal muscle. For example, home activities such as grocery shopping, food preparation while standing, dish washing, simple lawn care or light walking require MET levels of 2.5 to 3.5, equivalent to 60% to 70% of maximal oxygen consumption in a patient with a MET level of 4. This is well within a training range to initiate a training response in skeletal muscle. Most of patients with CHF in our study reported an independent life that included a number of activities requiring 2.5 to 3.5 MET levels. In response to performing continuous bouts of activity per day that are 60% to 70% of maximal oxidative capacity, endothelial cell proliferation may occur in patients with CHF that parallel exercise training at or above anaerobic threshold in normals.

Myosin heavy-chain isoforms and enzymes. Despite finding significant differences in vascular density, we did not observe markedly significant differences in two other traditional indicators of aerobic capacity, myosin heavy-chain (MHC) isoforms and enzymatic profile. Although two of five aerobic enzymes, 3-hydroxyl-CoA dehydrogenase and succinic dehydrogenase, were found to be higher in normals versus CHF patients, we do not believe this would account for the difference in aerobic capacity. The MHC isoforms show no relationship to oxygen consumption, but MHC IIx in CHF patients is higher compared to normals.

Our previous study showed 3-hydroxyl-CoA dehydrogenase and succinic dehydrogenase to be different, and fiber typing (comparable to MHC in the present study) to be shifted more toward a glycolytic fiber type. Careful examination of other studies measuring capillary density reveals similar findings. Drexler et al. (7) reported a reduced capillary length density in CHF subjects with MET levels of 4.6, and Lipkin et al. (9) reported normal values for CHF patients with MET levels of 3.3. These three studies, along with the present study, demonstrate a potential inverse relationship between capillary density and aerobic capacities <4 METs.

Sensitivity of CD-31. The recognition of the importance of vascularity to tumor growth (metastasis) has led to the development of cell-specific monoclonal antibodies that can

![Figure 2](image-url)
be used to directly identify endothelial cells in histologic sections. Even among cell-specific antibodies, antigen heterogeneity is an important feature, and CD-31 is regarded as the most sensitive and specific marker for endothelial cells. Using immunohistochemical techniques, Wlinder et al. (28) reported that microvascular density in tumors was correlated with a decrease in survival. Horak et al. (29) likewise used immunohistochemical techniques and cell-specific monoclonal antibodies to demonstrate that neovascularization in a tumor was associated with a decreased survival in patients. In their study, CD-31 was more sensitive than von Willebrand factor in the quantification of vascular density. Tenaglia and colleagues (30) recently reported that the presence of neovascularization, as demonstrated by CD-31 (not von Willebrand factor) immuno-staining, in coronary atherectomy samples was associated with unstable coronary syndromes. Our study is the first to report vascular density in human skeletal muscle utilizing endothelial cell-specific monoclonal antibodies.

Conclusions. This is the first study that has attempted to compare extremely sedentary normal subjects (MET <6) to mild to moderate class II–III CHF patients. The results of the present study provide a possible explanation for leg fatigue, one of the hallmark symptoms of exercise intolerance in patients with ambulatory class II–III CHF. The results also suggest substrate delivery to skeletal muscle can be impaired at the microvascular level (i.e., capillary density) without other major concomitant intrinsic abnormalities in skeletal muscle. We postulate that within groups of CHF patients there may be a discordant change in capillary density that is inversely related to aerobic capacity. The stimulus for this proposed angiogenesis remains unknown. This study does not exclude the possibility that an attenuated cardiac reserve may be primarily responsible for exercise intolerance in CHF and supports the need for longitudinal studies that simultaneously examine how cardiac output and skeletal muscle changes affect aerobic capacity in compensatory CHF patients.

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