Vascular rarefaction in peripheral skeletal muscle after experimental heart failure

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Vascular rarefaction in peripheral skeletal muscle after experimental heart failure. Am J Physiol Heart Circ Physiol 285: H1554–H1562, 2003; 10.1152/ajpheart.01045.2002.—A decrease in vascular density in peripheral skeletal muscle has been associated with exercise intolerance in humans with congestive heart failure (CHF). The purpose of this study was to determine whether CHF results in a reduction in vascular density in peripheral skeletal muscle. In this established model, CHF was induced by coronary artery ligation in New Zealand White rabbits and sham rabbits that underwent an identical surgical procedure without ligation of the coronary artery. At study termination, rabbits underwent hemodynamic testing and skeletal muscle analysis. The first series of rabbits was divided into sham (n = 6) and CHF (n = 6) 21 days postoperatively. Ten CHF rabbits were then examined 3 (n = 3), 7 (n = 3), and 14 days (n = 4) postoperatively. Vascular density in sham tibialis anterior muscle was 347 ± 41 capillaries/mm² or 1.20 ± 0.11 capillaries/muscle fiber. In 21-day CHF rabbits, the capillary density was significantly lower, 236 ± 14 capillaries/mm² or 0.84 ± 0.04 capillaries/muscle fiber (both P < 0.0001 vs. sham); PECAM protein was 2-fold lower (P < 0.0001) in muscle protein lysates; the fraction of apoptotic cells was greater, 3.8 ± 2.2 vs. 0.69 ± 0.56 (P < 0.02 vs. sham) with many TdT-mediated dUTP-biotin nick-end labeling-positive endothelial cells; and Bax protein was 2.8-fold greater (P < 0.0001). By regression analysis, vascular density tended to decrease over time (r² = 0.572, P < 0.0001). Vascular rarefaction and endothelial apoptosis develop after experimental CHF and may contribute to the skeletal muscle abnormalities in this disease. Modulating vascular density may provide new approaches to treat exercise intolerance in CHF.

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Congestive heart failure (CHF) is the leading hospital discharge diagnosis for patients over 65 yr of age in the United States (30). In patients with CHF, pharmacological therapies such as angiotensin-converting enzyme inhibitors have resulted in marked reductions in mortality rates, but intolerance remains a major cause of disability and decreased quality of life (9, 35). Exercise intolerance or exertional fatigue is often the presenting symptom in patients with CHF (10). In addition to morbidity and lifestyle limitations, exertional fatigue often prevents regular physical activity, which is critical to limit many of the comorbid diseases that patients with CHF suffer, including obesity, hypertension, and glucose intolerance (1, 35).

The mechanisms for exercise intolerance in CHF are not completely understood. Increases in intrapulmonary pressure produce shortness of breath (dyspnea) in acute CHF; however, several lines of evidence indicate that increased intrapulmonary pressures is not the primary factor limiting exercise in the majority of patients with chronic heart failure due to left ventricular dysfunction. First, traditional central hemodynamic parameters, i.e., left ventricular ejection fraction and pulmonary capillary wedge pressures, have been shown to be poor predictors of exercise capacity (26, 36). Second, a favorable “central” hemodynamic response to pharmacological therapy does not predict or correlate with an improvement in exercise tolerance (27, 36). Third, there is no direct relationship between pulmonary artery pressures and peak oxygen consumption, a marker of exercise capacity (39). Finally, pulmonary artery capillary wedge pressure is not higher in CHF patients limited by dyspnea compared with those limited by fatigue (39, 13). These findings indicate that peripheral skeletal muscle abnormalities play a role in limiting exercise capacity in patients with CHF, and several studies (25, 38, 42) have reported abnormalities in peripheral skeletal muscle in patients with CHF.

The vascular bed in peripheral skeletal muscle functions to supply oxygen and remove waste products from skeletal muscle fibers, and therefore the relative capillary density of a muscle group strongly correlates with the oxidative or endurance capacity of the muscle (16, 18). The association of increases in vascular density with increases in functional demand in skeletal
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muscle is well established. In humans, exercise conditioning results in an increase in the number of capillaries per muscle fiber and an increase in the number capillaries per millimeter squared of muscle (18). The presence of an angiogenic response directly correlates with improvements in endurance capacity (17, 18). Conversely, reductions in vascular density occur in the setting of decreases in functional demand in skeletal muscle. For example, immobilization results in a decrease in vascular density and aerobic capacity in the muscle of the target limb (19).

Vascular rarefaction is defined as a decrease in vessels per unit area, and vascular rarefaction in muscle has been associated with pathophysiological sequelae (31–33). In humans with CHF and mild-to-moderate exercise intolerance, we (12) reported a reduction in vascular density in the peripheral skeletal muscle of these men compared with matched controls. In addition, we (11) recently demonstrated that a reduction in vascular density in peripheral skeletal muscle was present in men with CHF compared with those without CHF, even when we controlled for the patients’ exercise capacity, which was measured by their maximum oxygen consumption. The vascular rarefaction in peripheral skeletal muscle in these patients with CHF could not be explained by deconditioning alone (11). Therefore, the central hypothesis to be tested in the present study was that CHF may cause a reduction in vascular density in peripheral skeletal muscle, and this reduction in vascular density occurs with an increase in endothelial cell apoptosis. To directly test this central hypothesis, we chose to investigate the changes in capillary density in peripheral skeletal muscle in an established rabbit model of CHF.

METHODS

Rabbit CHF model. This CHF model was originally described by Mahaffey et al. (24) and has been used, and is described in detail, in prior publications from our group (24, 28, 37). Briefly, New Zealand White rabbits (3–5 kg body wt, males) underwent ligation of the left circumflex (LCx) coronary artery to produce a myocardial infarction (CHF) or an identical procedure except the coronary artery had a suture placed around the vessel but the vessel was not ligated (sham). Animals were housed under standard conditions and were fed ad libitum. Rabbits were pretreated with an intramuscular injection of 500,000 units of penicillin, anesthetized with a mixture of ketamine (30 mg/kg) and xylazine (2 mg/kg), intubated, and then mechanically ventilated. A left thoracotomy was performed through the third and fourth intercostals space, and the large marginal branch of the LCX coronary artery was identified and ligated with a 5-0 Prolene suture. In sham animals, the LCx artery was enclosed with a suture, but the suture was not tightened and therefore no ligation occurred. The chest was evacuated of residual air after the central he-

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the capillary-to-muscle fiber ratio (8). Slides were coded to prevent group recognition. All vascular density analyses were performed by a single reader (D. J. Nusz) who was provided coded slides without animal numbers. A random sample of slides was analyzed in duplicate on the same day and repeated on separate days with a variability of <10%. Muscles from both the right and left limbs were available in all but two rabbits, including one sham and one 21-day CHF rabbit, and therefore the average of the left and the right limb was used in all but two samples. In the two cases where only one limb was available, the value from that that limb was used. Comparisons between CHF and sham, and CHF at different time points postoperatively, were made.

**Platelet endothelial cell adhesion molecule (or CD-31) protein expression.** To confirm the results of the histological vascular density measures, protein extracts (50 μg) were separated with an 8% SDS-PAGE run at 150–200 V for 1 h. Proteins were transferred to nitrocellulose paper by electrotransfer. Blots were blocked overnight in 5% nonfat dried milk overnight. A mouse monoclonal CD-31 antibody (clone 9G11, R&D Systems) was used at a concentration of 1 μg/ml for 1 h at room temperature. The blot was washed and incubated with a goat anti-mouse IgG horseradish peroxidase-conjugated antibody (Promega; Madison, WI) and enhanced chemiluminescence detection system (Amersham). An immunoreactive band was detected just above the 122-kDa molecular mass marker. To reconfirm that equivalent amounts of protein were loaded, the blots were washed at 95°C for 3 min and reexposed to X-ray film for 20 min, and, if no signal was detected, the blot was probed with a murine monoclonal antibody against skeletal muscle actin (cat. no. sc-8432, Santa Cruz Biotechnology) at a concentration of 1 μg/ml for 1 h at room temperature. In addition, a subset of blots was stripped and probed with goat anti-rabbit IgG horseradish peroxidase-conjugated antibody (Santa Cruz Biotechnology). Quantification was performed using densitometry and methods previously described (8).

**Detection of apoptosis in histological sections.** To establish the rate of apoptosis, TdT-mediated dUTP-biotin nick-end labeling (TUNEL) with ApopTag (Intergen; Purchase, NY) was performed and analyzed as previously described (10). Briefly, tissue sections were incubated with 20 μg/ml proteinase K (Sigma), followed by 2% H2O2 to block endogenous peroxidase activity. Slides were then incubated for 60 min at 37°C with 0.3 EU/μl TdT and 0.04 nmol/μl peroxidase activity. Slides were then incubated for 60 min at room temperature, placed in ice-cold acetone for 10 min, air dried for 10 min, and placed in PBS for three 3-min washes. Blocking solution (10% horse serum in PBS) was applied for 20 min at room temperature. Murine anti-human proliferating cell nuclear antigen (PCNA; Dako; Carpinteria, CA) was diluted 1:1,000 in 1× PBS and applied to tissue sections for 18 h at 37°C. Murine anti-human myosin heavy chain (slow) antibody (Novocastra Laboratories; Newcastle, UK) was diluted (1:40) in 1× PBS, or anti-myosin heavy chain (fast) antibody (Novocastra Laboratories) was diluted (1:20) in 1× PBS and applied to tissue sections for 1 h at room temperature. The incubation with the primary antibody was followed by sequential incubations with a biotinylated anti-mouse IgG and ABC reagent according to the manufacturer’s specifications (Vectorstain Avidin-Biotinylated Enzyme Complex kit, Vector Laboratories; Burlingame, CA). Levamisole was added to block endogenous alkaline phosphatase activity, and immune complexes were visualized with the use of the chromogenic alkaline phosphatase substrate Vector red (Vector Laboratories). Sections were counterstained with hematoxylin, dehydrated, and mounted with Permount (Fisher Scientific). In this method, the antigen appears red, and the cell nuclei are blue. For the determination of rates of proliferation in histological sections, as was done in the apoptosis assay, three random areas were analyzed on each sample slide under a ×40 objective. The total number of positive nuclei was expressed as the percentage of the counted nuclei, similar to methods previously described (10).

After immunohistochemical staining with the slow myosin antibody, the percentages of oxidative fibers (red staining), glycolytic fibers (no red staining), and “transition” fibers (staining in between red and white) were determined using MetaMorph (Universal Imaging; Downingtown, PA) software. The sectioned were viewed on an inverted microscope and photographed at ×200 magnification. A total of 10 different ×200 fields was used, and a minimum of 200 fibers/sample was counted. The staining, photography, and image analysis were performed by a single operator (A. M. Pippen) with no knowledge of the coding system. A “normal staining” fiber was assigned an intensity score of 2,000, and a glycolytic fiber was assigned a score of 0. The program then assigned an intensity level to each fiber. This enabled us to count just the fibers with an intensity of <2,000, fibers with an intensity of ≥2,000, and uncolored fibers. Endothelial cell counts per normal red fibers and per transitional fibers were determined by counting the endothelial cells surrounding the fibers that MetaMorph differentiated with the use of intensity scores.

**bFGF and VEGF detection by ELISA.** Total VEGF and bFGF protein concentrations in skeletal muscle protein lysates was determined by a solid-state ELISA system using Quantikine kits (R&D Systems), as previously described (8). The sensitivities of the assays were 5 and 10 pg/ml for recombinant VEGF and bFGF protein, respectively. All assays were performed in duplicate, and the average was used. In preliminary studies, we validated this assay for 25–100 μg total protein/well.
Statistical analysis. All data are presented as means ± SD. Statistical differences between the sham and CHF rabbits were assessed by Student’s t-test for unpaired variables. We used the SAS statistical software package to calculate the simple linear regression of vascular density versus the number of days after the induction of CHF.

RESULTS

Vascular rarefaction in 21-day CHF versus sham rabbit skeletal muscle. Representative examples of the vascular density in the TA muscle from a sham and a 21-day CHF rabbit are shown in Fig. 1. In the sham rabbits (n = 6, 21 days after sham surgery), the vascular density was 347.3 ± 40.5 capillaries/mm² muscle or 1.20 ± 0.11 capillaries/muscle fiber. In the CHF rabbits (n = 6) at 21 days postoperatively, the capillary density was significantly lower, 236.0 ± 24.5 capillaries/mm² muscle (P < 0.00001 vs. sham) and 0.84 ± 0.04 capillaries/muscle fiber (P < 0.0001 vs. sham). Similar results were obtained by examining PECAM (CD-31) levels in whole muscle protein lysates by Western blotting (Fig. 2). Quantitative analysis of the Western blots demonstrated that PECAM protein levels were 2.0-fold greater in sham muscle (17,284 ± 2,257 U/µg soluble protein) versus CHF muscle (8,499 ± 1,967 U/µg soluble protein, P < 0.0001). To confirm that protein loads were equal, the same blots were stripped and reprobed with an antibody against skeletal muscle actin. These blots were scanned as well, and the data were also analyzed as the intensity of PECAM staining relative to the intensity of skeletal muscle actin staining from the same blot. The relative differences between sham and 21-day CHF samples were the same (data not shown).

Increase in apoptosis in skeletal muscle from CHF rabbits. In association with the decrease in vascular density in the CHF versus sham rabbits at 21 days postoperatively, the fraction of apoptotic (TUNEL-positive) cells was greater in 21-day CHF compared with sham rabbits (3.8 ± 2.2 vs. 0.69 ± 0.56, P < 0.02; Fig. 1, D vs. C). The TUNEL-positive nuclei values obtained from skeletal muscle of the sham rabbits was similar to those obtained in a recent report (10) from our group showing that apoptosis (i.e., TUNEL-positive nuclei) was increased in rabbit TA skeletal muscle subjected to unilateral hindlimb ischemia, and in that report the frequency of TUNEL-positive nuclei in the nonischemic limb was 0.96 ± 0.40. Similar to this previous report, the average number of nuclei examined per
sample in the present study was 239 (range 150–350). By double staining, many (range 53–69%) of the apoptotic nuclei were Tie-2-positive (endothelial) cells (Fig. 1). In association with the histological evidence of an increase in apoptosis, Bax protein expression was also significantly increased (2.8-fold, \(P < 0.0001\)) in the same skeletal muscle of the 21-day CHF versus sham rabbits. An unexpected finding was that within the 21-day CHF rabbits, a fraction of slow myosin staining (oxidative) fibers demonstrated a lower than expected intensity of staining (Fig. 4B, arrow). With the use of computer-assisted image analysis, this particular pattern of staining was present in 1.6 ± 0.72% of the fibers from sham rabbits compared with 13.45 ± 4.45% of the fibers from the CHF rabbits. Figure 4C demonstrates that the 8.1-fold difference between the sham and 21-day CHF groups

**PCNA-positive cells** was 1.0 ± 1.0 in the sham skeletal muscle and 0.3 ± 0.5 (\(P = 0.13\), not significant (NS)) in the 21-day CHF rabbit skeletal muscle. Skeletal muscle levels of VEGF were similar (102.3 ± 83.0 and 61.2 ± 15.6, \(P = 0.1\), NS) in the 21-day CHF and sham rabbits, respectively. Likewise, muscle levels of bFGF were similar (35.2 ± 23.9 and 27.7 ± 11.5, \(P = 0.43\), NS) in the 21-day CHF and sham rabbits, respectively. Therefore, the reduction in vascular density in TA skeletal muscle 21 days after the onset of CHF was not accompanied by a change in proliferation or in the levels of growth factors that have been associated with angiogenesis in skeletal muscle.

**Contractile protein changes.** As shown in Fig. 4, and as predicted, the majority of skeletal muscle fibers in the TA muscle were glycolytic, and there was no difference in this fraction of fast-twitch (glycolytic) fibers between the sham and 21-day CHF rabbits. An unexpected finding was that within the 21-day CHF rabbits, a fraction of slow myosin staining (oxidative) fibers demonstrated a lower than expected intensity of staining (Fig. 4B, arrow). With the use of computer-assisted image analysis, this particular pattern of staining was present in 1.6 ± 0.72% of the fibers from sham rabbits compared with 13.45 ± 4.45% of the fibers from the CHF rabbits. Figure 4C demonstrates that the 8.1-fold difference between the sham and 21-day CHF groups

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**Fig. 2.** A: representative Western blot from TA skeletal muscle protein lysates from one sham (lane 1) and one 21-day heart failure (HF) rabbit (lane 2). In A, 50 \(\mu\)g of protein were subjected to electrophoresis through 8% PAGE under nondenaturing conditions. The protein was transferred to a nitrocellulose membrane, the filtrate was probed with an anti-human CD-31 antibody (top), and the position of the 122-kDa molecular mass marker is shown by the arrow. To confirm that equal amounts of protein were loaded, the blot was stripped and probed with a murine monoclonal antibody against skeletal muscle actin (bottom). The positions of the 42- and 85-kDa molecular mass markers are shown. B: results of the quantitative assessment (integrated density/\(\mu\)g protein) of the PECAM images. *\(P < 0.0001\), sham (bar 1) compared with CHF rabbits (bar 2).

**Fig. 3.** A: representative Western blot demonstrating that Bax protein is increased in 21-day CHF rabbit skeletal muscle (lanes 1 and 3; HF) compared with sham-operated (lanes 2 and 4) rabbit skeletal muscle. Protein (25 \(\mu\)g) was subject to electrophoresis through 12% SDS-PAGE. The protein was transferred to a nitrocellulose membrane and probed with a murine monoclonal antibody against Bax protein. The positions of the 32- and 18-kDa molecular mass markers are shown. B: the blot was stripped and reprobed with an antibody against skeletal muscle actin, and the positions of the 42- and 85-kDa molecular mass markers are shown.
was statistically significant \((P < 0.00001)\). With the use of a combination of alkaline phosphatase staining and immunohistochemistry, the number of capillaries associated with normal- and low-intensity staining slow fibers was determined. In the 21-day CHF rabbits, there were \(6.17 \pm 0.68\) capillaries around the normal-staining red fibers compared with \(4.41 \pm 0.56\) \((P < 0.0001)\) capillaries around the low-intensity-staining fibers.

**Time course of vascular density changes in peripheral skeletal muscle.** Finally, we sought to determine whether vascular rarefaction occurred earlier than 21 days after the onset of CHF. A series of rabbits underwent coronary artery ligation and were randomly assigned to hemodynamic assessment and death at 3, 7, or 14 days postoperatively. There was no evidence of inflammation or other structural changes in peripheral skeletal muscles in the CHF rabbits at these time points (data not shown) or the 21-day time point. As shown in Fig. 5, changes (the reduction) in vascular density tended to increase over time. Regression analyses indicated a significant relationship between the number of days of heart failure and the number of capillaries per muscle fiber \((P < 0.0001, r^2 = 0.572)\), with the least squares line intercepting vascular density at 1.167, and this value being reduced by 0.0179 for each day of CHF. As shown in Fig. 6, top, Bax protein was increased in skeletal muscle protein lysates at the early time points. Bax protein was expressed at 1.69, 1.62, and 2.20 higher levels than in sham rabbits. An additional marker of apoptosis (TUNEL staining) was not significantly greater in the CHF muscle than the levels found in the muscle of the sham rabbits until the 14-day time point (Fig. 6B). Compared with the sham rabbits, there was no evidence for an increase in PCNA staining in the muscle of CHF rabbits at any time point.

**DISCUSSION**

In an attempt to explain the mechanisms that account for the exercise intolerance seen in patients with heart failure, studies in human and animal models have reported histological and biochemical abnormalities in peripheral skeletal muscle in the setting of heart failure that include fiber atrophy, a relative loss
of oxidative fibers, and a loss in oxidative enzyme activity (25–27, 35, 38, 42). Vascular rarefaction is defined as a decrease in vessels per unit area. Vascular rarefaction plays a role in development and some physiologic conditions (23); however, vascular rarefaction has been shown to lead to perfusion abnormalities in several different organs and is an established pathologic process in several systemic disease states (22, 31, 33). In this study, we hypothesized, and were able to demonstrate, that vascular rarefaction can occur in peripheral skeletal muscle after the induction of heart failure using an established preclinical model, and to support this we showed 1) a decrease in vascular density in the TA muscle measured both as the number of capillaries per area of muscle and the number of capillaries per muscle fiber; 2) a reduction in vascular density that increased over time; and 3) an increase in apoptosis in the same skeletal muscle that included but was not limited to endothelial cells.

The capillary bed in peripheral skeletal muscle functions to supply oxygen and remove toxins from muscle fibers (16). Therefore, a reduction in capillary density in muscle is a plausible explanation for fatigue and exercise intolerance in CHF. A prior human study (12) from our group showed that a decrease in capillary density was the predominant skeletal muscle abnormality present in adult men with CHF compared with age- and gender-matched controls. In animal models of hypertension and diabetes, studies (6, 15, 32) have demonstrated decreases in capillary density or vascular flow to organs with sequelae including myocardial dysfunction, stroke, and renal failure. Similarly, humans with hypertension and diabetes have been shown to have vascular rarefaction in several organs, and the vascular rarefaction may result in functional deficits in cardiac perfusion and contribute to the excess cardiac mortality observed in these patients in the absence of large vessel atherosclerosis (6, 20). In the present study, we used an established preclinical model of CHF and demonstrated evidence for vascular rarefaction in peripheral skeletal muscle. This finding was present when we measured the number capillaries per millimeter squared of muscle and was confirmed when we evaluated two complimentary measures: the number of capillaries per muscle fiber or the endothelial mass per unit of soluble muscle protein. Vescovo et al. (41) examined the TA muscle of rats after the onset of CHF induced by an intraperitoneal injection of monocrotaline. Although the group did not measure vascular density by alkaline phosphatase or immunohistochemistry, they did report an increase in TUNEL-positive staining in nuclei from interstitial cells with lamine-positive basal lamina anatomically identifiable as capillary vessels. The results of the present study demonstrate that changes in vascular density occur after the onset of heart failure in rabbits, and this finding shares similarities to the skeletal muscle abnormalities found in humans with heart failure (11). The findings in this study now provide a model to examine approaches to modulate vascular density to examine the effects on peripheral skeletal muscle in heart failure.

In humans with CHF, deconditioning has been proposed as a potential explanation for, or a confounding factor in, the assessment of changes in peripheral skeletal muscle of patients with heart failure (7). A report (11) from our group demonstrated that changes in vascular density in the peripheral skeletal muscle of patients with CHF persisted even when we controlled for the patients’ maximal exercise capacity. Although activity was not specifically monitored in the present preclinical study, changes in activity in these housed rabbits was not likely to have contributed to the changes in vascular density seen in the peripheral skeletal muscle. Therefore, the results of our study provide support for the hypothesis that a reduction in vascular density in peripheral skeletal muscle occurs as a result of the induction of CHF.

When skeletal muscle is subjected to increases in functional demand, for example, through continuous motor nerve stimulation, increases in capillary density are seen in the target muscle as early as 3 days, and these changes precede the alterations in oxidative enzymes or contractile protein composition (16, 34).
humans, the presence of an angiogenic response directly correlates with improvements in the endurance capacity of the muscle (17, 18). Conversely, decreases in functional demand in skeletal muscle, i.e., immobilization, result in a decrease in vascular density and aerobic capacity (19). Although the significance of this finding remains to be determined, in the CHF model used in the present report vascular rarefaction was spatially associated with changes in slow (oxidative) myosin fibers. Although the data are not shown, these alterations in slow myosin staining interestingly were not observed in the same rabbit skeletal muscle group that was subjected to motor nerve stimulation (4) or hindlimb ischemia (8). Future studies will be needed to determine the effects of blocking or reversing vascular rarefaction in peripheral skeletal muscle in heart failure.

In skeletal muscle, when the capillary density is stable, the rate of endothelial cell proliferation is equal to the rate of endothelial cell death. A net decrease in vascular density can occur because of an increase in cell death or a reduction in proliferation. We found no evidence for changes in endothelial proliferation or a significant increase in the endothelial cell mitogen VEGF in the setting of the reduction in vascular density in the peripheral skeletal muscle from heart failure rabbits. However, we did find evidence for an increase in apoptosis in the skeletal muscle from the heart failure versus sham animals. There was an increase both in TUNEL staining and in Bax protein expression. Therefore, increases in apoptosis would appear to be present in endothelial and nonendothelial cells. Nevertheless, our data suggest that increases in endothelial cell death, as opposed to a decrease in endothelial proliferation, is a mechanism that contributes to vascular rarefaction in peripheral skeletal muscle after the induction of CHF. Because VEGF can also serve as a survival factor for endothelial cells (14), our finding suggests that VEGF is unable to fully protect endothelial cells located in peripheral skeletal muscle from the injury that occurs in the setting of heart failure. Additional studies will be needed to delineate the precise molecular mechanisms that regulate apoptosis in skeletal muscle and to evaluate approaches to limit apoptosis as a means to modulate skeletal muscle abnormalities in heart failure.

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DISCLOSURES

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