Endothelial Cell Activation in Patients With Decompensated Heart Failure

Paolo C. Colombo, MD; Javier E. Banchs, MD; Sulejman Celaj, MD; Ashok Talreja, MD;
Justine Lachmann, MD; Shailesh Malla, MD; Nicholas B. DuBois, MD; Anthony W. Ashton, PhD;
Farhana Latif, MD; Ulrich P. Jorde, MD; J. Anthony Ware, MD; Thierry H. LeJemtel, MD

Background—Vascular endothelial functions, other than nitric oxide (NO)–mediated control of vasomotor tone, are poorly characterized in patients with chronic heart failure (CHF). Veins and arteries are exposed to the same circulating proinflammatory mediators in patients with CHF. The present study tested whether endothelial cell activation occurs in veins of patients with decompensated CHF and whether activation, if present, subsides with return to a clinically compensated state.

Methods and Results—Fifteen patients with decompensated CHF requiring transient inotropic support and 6 age-matched, healthy controls were studied. Endothelial cells and blood were collected from a forearm vein, and brachial artery flow–mediated dilation (FMD) was measured before and 24 hours after discontinuation of short-term inotropic therapy, when patients had returned to a steady compensated state. Nitrotyrosine immunoreactivity (an intracellular marker of oxidative stress), cyclooxygenase-2 (COX-2), and inducible NO synthase (iNOS) expression were significantly higher in venous endothelial cells of patients in clinical decompensation when compared with healthy subjects. Return to a compensated state resulted in a significant reduction in nitrotyrosine immunoreactivity, COX-2, and iNOS expression. Concomitantly, a significant increase in FMD and a decline in plasma total 8-isoprostane and bicycloprostaglandin E_2 levels were observed. Venous endothelial NOS expression was unaffected by clinical decompensation.

Conclusions—Clinical decompensation in CHF is associated with activation of the venous endothelium. Return to a compensated state after short-term inotropic therapy results in a significant reduction in endothelial nitrotyrosine formation, COX-2, and iNOS expression. (***Circulation*. 2005;111:58-62.**)

Key Words: heart failure ■ endothelium ■ inflammation

A sustained improvement in endothelial nitric oxide (NO)–dependent vasodilation accompanies the return to a compensated state in patients with chronic heart failure (CHF) hospitalized for clinical decompensation.[1] Oxidative stress modulates vascular endothelial function in CHF.[2] Systemic markers of oxidative stress and inflammation steadily increase as the functional status of patients with CHF deteriorates.[3,4] Increased oxidative stress and cytokines promote endothelial cell activation, with induction of several proinflammatory genes, including cyclooxygenase-2 (COX-2) and inducible NO synthase (iNOS).[5,6] COX-2 and iNOS may further impair endothelial function by increasing local production of reactive oxygen species and proinflammatory/vasoactive prostanoids.[7,8] Whether the vascular endothelium is activated and thereby might contribute to systemic inflammation in severely symptomatic patients with CHF is currently unknown.

The present study tested the hypothesis that venous endothelial cells are activated during an episode of decompensation in patients with CHF and that venous endothelial cell activation subsides with return to a compensated state. Accordingly, we measured nitrotyrosine formation (an intracellular marker of oxidative stress), COX-2, endothelial NOS (eNOS), and iNOS expression in venous endothelial cells from patients with CHF who required temporary inotropic therapy while hospitalized for fluid retention, hypotension, and peripheral hypoperfusion. Endothelial nitrotyrosine, COX-2, eNOS, and iNOS expression were measured before initiation and 24 hours after discontinuation of inotropic therapy when patients had returned to a steady compensated state. Brachial artery flow–mediated dilation (FMD), plasma total 8-isoprostane (a systemic marker of oxidative stress), and plasma prostaglandin (PG) E_2 were measured at the same time points.

Methods

Patient Population

Patients with CHF were eligible for the study when hospitalized for overt clinical decompensation with hemodynamic compromise (systolic blood pressure <90 mm Hg and peripheral hypoperfusion) and fluid retention (recent weight gain >5 lb). Patients with arrhythmias,

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From the Department of Medicine (J.E.B., S.C., A.T., J.L., S.M., N.B.D., A.W.A., F.L., J.A.W., T.H.L.), Division of Cardiology, Albert Einstein College of Medicine, Bronx, NY, and the Department of Medicine (P.C.C., U.P.J.), Division of Cardiology, New York–Presbyterian Hospital, New York, NY.

Correspondence to Thierry H. LeJemtel, MD, Albert Einstein College of Medicine, 1300 Morris Park Ave, Bronx, NY 10461. E-mail lejemtel@aecom.yu.edu

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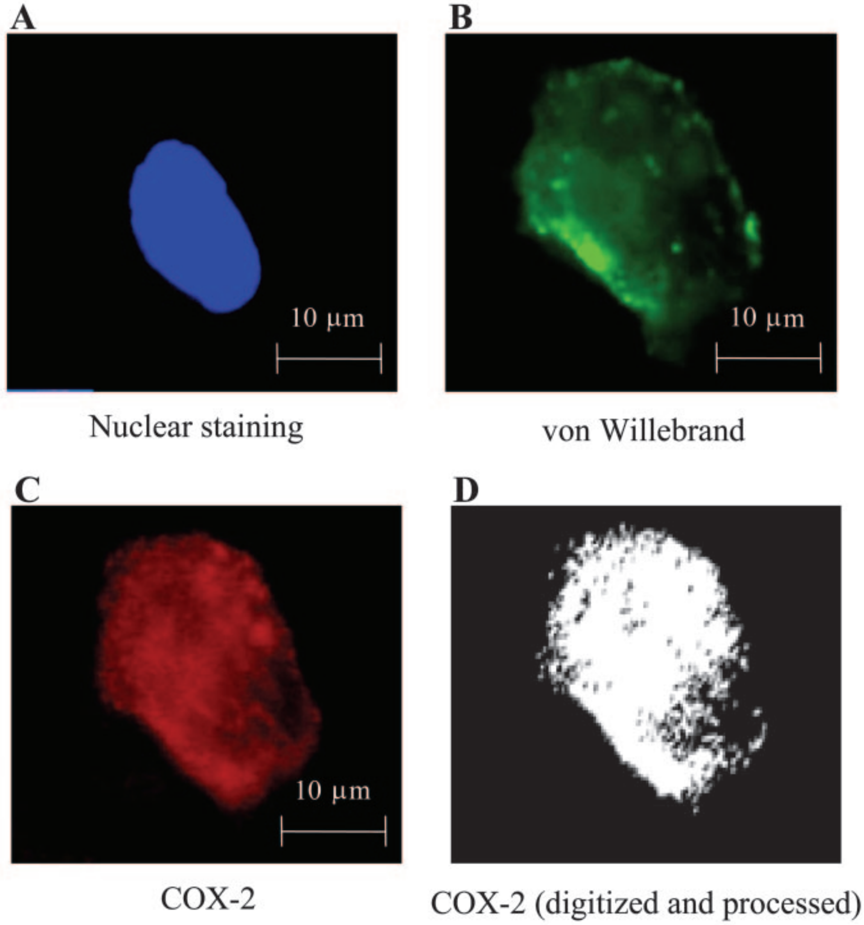

Figure 1. Quantitative immunofluorescence analysis of COX-2 protein expression in representative venous endothelial cell. Nuclear (blue) (A) and von Willebrand factor (green) (B) fluorescent staining identified cells and determined their endothelial origin. Cy-3 (red) fluorescent image of COX-2 (C) was digitized and processed (D). Aforementioned technique was also used to quantify nitrotyrosine immunoreactivity, eNOS, and iNOS expression. Abbreviations are as defined in text.

renal failure (serum creatinine value >2.0 mg/dL), infection, acute coronary syndrome, and inadequate compliance to diet and/or medications were excluded. Patients receiving β-adrenergic blockers were treated with milrinone at a mean infusion rate of 0.3 $\mu g \cdot kg^{-1} \cdot min^{-1}$ (range, 0.2 to 0.375 $\mu g \cdot kg^{-1} \cdot min^{-1}$) for 72 hours. The remaining patients were treated with dobutamine at a mean infusion rate of 3.5 $\mu g \cdot kg^{-1} \cdot min^{-1}$ (range, 2.5 to 5.0 $\mu g \cdot kg^{-1} \cdot min^{-1}$) for 72 hours. Cardiovascular treatment other than diuretic dosing remained unchanged throughout the index hospitalization. Clinical status was reassessed 24 hours after discontinuation of inotropic therapy. Blood and venous endothelial cells were collected, and brachial artery FMD measurements were obtained immediately before initiation and 24 hours after discontinuation of inotropic therapy in patients who had returned to a steady compensated state (systolic blood pressure >90 mm Hg, improved peripheral perfusion, and weight reduction >5 lb). Blood and venous endothelial cells were also collected from 6 healthy, age-matched subjects. Controls were matched by age to within 5 years with patients (1:2). Patients were contacted by telephone 4 weeks after hospital discharge. All subjects signed an informed consent document approved by the Albert Einstein College of Medicine Committee on Clinical Investigation.

Endothelial Cell and Blood Collection

With use of a 0.021-inch-diameter, J-shaped wire (Daig) inserted through an 18-gauge Angiocath (Becton Dickinson), endothelial cells and blood were collected from a superficial forearm vein.[9] The wire was transferred to dissociation buffer (0.5% bovine serum albumin, 2 mmol/L EDTA, and 100 μg/mL heparin in phosphate-buffered saline) at 4°C. Cells were rinsed, fixed in 3.7% formaldehyde, transferred to slides, air dried, and stored at -80°C. Plasma and serum were separated by centrifugation and stored at -80°C.

Protein Analysis

Quantitative immunofluorescence analysis of protein expression has been validated against immunoblotting in cultured endothelial cells.[9] When compared with immunoblotting, correlation coefficients (r_s) for nitrotyrosine, COX-2, eNOS, and iNOS were 0.93, 0.99, 0.93, and 0.91, respectively (all $P<0.005$). The overall coefficient of variation and measurement error in duplicate experiments for nitrotyrosine, COX-2, eNOS, and iNOS were 12% and 311 pixels, respectively.[9]

Cells on slides were rehydrated and rendered permeable with 0.1% Triton X-100. Monoclonal antibodies for anti-nitrotyrosine (Upstate Biotechnology), anti–COX-2 (Cayman), anti-eNOS (Transduction Laboratories), and anti-iNOS (Transduction Laboratories) were used, followed by Cy3-conjugated secondary antibodies. Negative control slides were generated with preimmune IgG. Polyclonal anti–von Willebrand factor antibodies (Dako) were then used, followed by secondary antibodies preconjugated with streptavidin–Oregon green. Nuclei were stained with diaminophenylindole (DAPI; Molecular Probes). Analysis was blinded by numerically coding each slide. Staining was visualized under UV light under a fluorescence microscope (Nikon Eclipse E600). Cy3 staining (red) of nitrotyrosine, COX-2, eNOS, and iNOS in endothelial cells was digitally captured by a COHU CCD camera. Image processing was performed with commercially available software.[9] The background was optimized by level and threshold functions (nonspecific extracellular signal was reduced to a uniform black background). These settings used to optimize image quality were then applied, as standards, for processing of all subsequent cell images. The intensity of Cy3 staining was quantified by determining the number of positive (bright) intracellular pixels (Figure 1). Slides were systematically read left to right, top to bottom. Only cells with both cellular and nuclear integrity were analyzed. Cellular and nuclear integrity was assessed morphologically. Intact cells were defined as those with a continuous, unbroken cell membrane, as observed with phase-contrast microscopy. Intact nuclei were defined as well circumscribed oval bodies as delineated by DAPI staining. Nitrotyrosine, COX-2, eNOS, and iNOS were sequentially measured. Expression of each protein was determined by analyzing at least 25 consecutive intact endothelial cells.[9] Between-experiment variability was standardized by using reference slides of human umbilical vein endothelial cells (HUVECs). Slides from patients and controls were stained concurrently with one slide of HUVECs. In preliminary experiments, no significant difference in nitrotyrosine, COX-2, eNOS, and iNOS immunoreactivity was observed in venous endothelial cells collected twice, at a 4-day interval, from 6 subjects with stable CHF.

Cell Culture

HUVECs were exposed for 72 hours to 20% sera from patients with decompensated CHF, with or without addition of milrinone (200 μg/mL) or dobutamine (100 μg/mL). Concentrations were analogous to plasma therapeutic concentrations in humans. Nitrotyrosine, COX-2, eNOS, and iNOS were quantified by immunoblotting.

Ultrasonography of the Brachial Artery

A broadband, 12-MHz, ultrasonic transducer connected to an ATL 5000 system (Advanced Technology Laboratories) was used to measure brachial artery diameters at baseline and 90 seconds after release of a cuff inflated around the arm to 50 mm Hg above systolic blood pressure for 5 minutes. Brachial artery diameters were analyzed as previously described and according to the guidelines of the International Brachial Artery Reactivity Task Force.[1,10] With the patient supine, the transducer was positioned ≈5 cm above the antecubital fossa. The brachial artery was identified and carefully scanned to determine its origin and course and the presence and extent of atheroma. Exclusion criteria included extensive arterial wall atheromatous changes and arterial narrowing. Once the optimal portion of the artery was visualized, the position of the transducer was marked on the skin. Depth and gain settings were optimized to identify the lumen-to–vessel wall interface and were kept constant during each study. All images were recorded on 31/2-inch super-VHS videotapes. Images were then digitally acquired for analysis (NovaMicrosonics). Arterial diameter was determined with electronic calipers as the internal dimension of the vessel wall from the anterior-to-posterior interface between the lumen and the intima. The mean diameter was calculated from 3 cardiac cycles incident with the R wave on a continuously recorded ECG.

Plasma Total 8-Isoprostane and Bicyclo-PGE_2

Plasma concentrations of total 8-isoprostane and bicyclo-PGE_2 were determined with the use of enzyme immunoassay kits (Cayman Chemicals).

Statistical Analysis

Data are presented as mean±SD. A pixel ratio was derived for analysis of endothelial nitrotyrosine, COX-2, eNOS, and iNOS. Pixel ratio was defined as the average pixel count per endothelial cell in a study subject divided by the average pixel count per HUVEC in a reference slide. Student's *t* test was used to compare endothelial, FMD, and plasma measurements.

Results

Sixteen patients responsive to treatment (clinically compensated 24 hours after discontinuation of inotropic therapy) and 6 age-matched, healthy subjects completed the study protocol. Controls and patients were matched by age to within 5 years (1:2) except for 3 patients who were >75 years old. Therefore, healthy subjects tended to be younger (56±13 versus 63±15 years, $P=0.3$). Endothelial cell sampling was inadequate in 1 patient. All 15 patients (left ventricular ejection fraction, 24±7%; systolic blood pressure, 82±5 mm Hg) were treated with furosemide, digoxin, and angiotensin-converting-enzyme inhibitors. Eight patients (53%) were also treated with β-blockers. All patients were discharged within 48 hours of discontinuation of inotropic therapy, and none was rehospitalized over the following 4 weeks.

Endothelial Cell Activation

The number of harvested endothelial cells was similar in all 3 groups: 399±267 (range, 154 to 912) in patients with decompensated CHF; 339±299 (range, 124 to 794) after return to a compensated state; and 456±320 (range, 112 to 884) in healthy subjects (P=NS). Nitrotyrosine, COX-2, and eNOS were quantified in all 15 patients and 6 healthy volunteers. iNOS expression was measured in only 9 patients and 4 healthy subjects. The number of harvested endothelial cells was insufficient for this last analysis in the remaining 6 patients and 2 healthy subjects.

Nitrotyrosine immunoreactivity, expressed as a pixel ratio (see Methods), averaged 3.1±1.5 (range, 1.1 to 5.9) in patients with decompensated CHF; 1.9±1.1 (range, 0.7 to 4.1) after return to a compensated state; and 1.2±0.6 (range, 0.3 to 2.0) in healthy subjects (Figure 2A). COX-2 averaged 4.5±2.8 (range, 0.6 to 10.9) in patients with decompensated CHF; 1.2±0.7 (range, 0.3 to 2.2) after return to a compensated state; and 1.0±0.7 (range, 0.1 to 1.8) in healthy subjects (Figure 2B). eNOS averaged 1.0±0.3 (range, 0.4 to 1.7) in patients with decompensated CHF; 1.0±0.4 (range, 0.4 to 1.9) after return to a compensated state; and 1.0±0.3 (range, 0.6 to 1.4) in healthy subjects (Figure 2C). iNOS averaged 1.2±0.5 (range, 0.5 to 2.1) in patients ($n=9$) with decompensated CHF; 0.9±0.4 (range, 0.3 to 1.6) after return to a compensated state; and 0.6±0.3 (range, 0.2 to 1.0) in healthy subjects ($n=4$; Figure 2D).

Therefore, when comparing patients with decompensated CHF and healthy subjects, endothelial oxidative stress, as measured by nitrotyrosine immunoreactivity, was 3-fold higher ($P<0.01$), COX-2 immunoreactivity 4-fold higher ($P<0.01$), and iNOS twice as high ($P<0.05$) in endothelial cells of decompensated patients, whereas eNOS was similar (P=NS).

Return to a compensated state was associated with a 1.6-fold reduction in nitrotyrosine immunoreactivity ($P<0.01$), a 4-fold decline in COX-2 expression ($P<0.01$), and a 1.5-fold reduction in iNOS expression ($P<0.05$). eNOS expression did not change (P=NS). After return to a compensated state, endothelial nitrotyrosine immunoreactivity tended to remain greater in patients with CHF than in healthy subjects ($P=0.08$), whereas endothelial COX-2 and iNOS immunoreactivity were similar.

To assess whether a direct effect of milrinone or dobutamine might be responsible for the changes in endothelial protein expression, milrinone (200 μg/mL) or dobutamine (100 μg/mL) was added to the culture media of HUVECs grown in 20% serum collected from patients with decompensated CHF. The addition of milrinone or dobutamine did not change endothelial nitrotyrosine immunoreactivity, COX-2, eNOS, and iNOS expression (data not shown). Therefore, return to a compensated state rather than stimulation of cAMP by dobutamine or milrinone appears to have mediated the endothelial changes observed in patients with CHF.

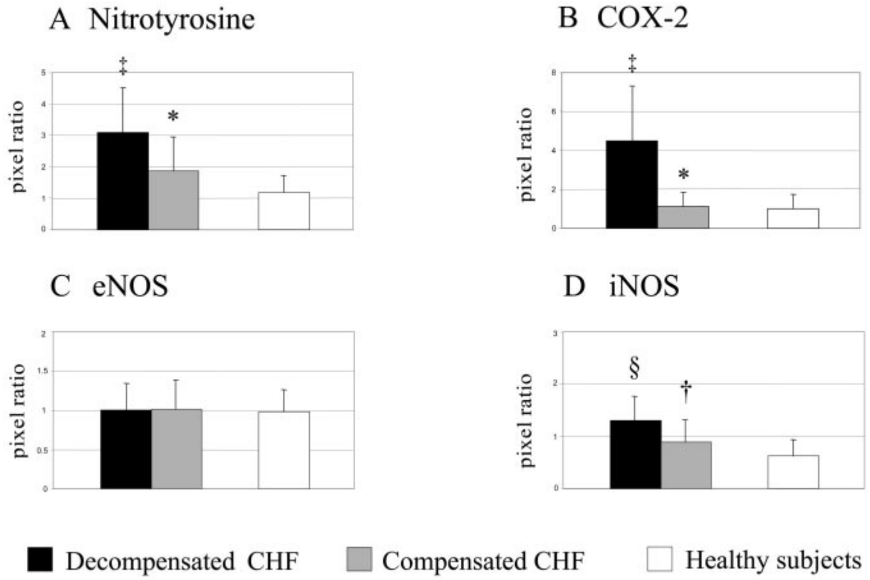

Figure 2. Increased endothelial oxidative stress, COX-2, and iNOS expression in patients with decompensated CHF. Quantitative analysis of nitrotyrosine immunoreactivity (A), COX-2 (B), eNOS (C), and iNOS (D) expression was performed in venous endothelial cells from patients with decompensated CHF before (black) and after (gray) return to steady compensated state and in endothelial cells from age-matched, healthy subjects (white). Results for nitrotyrosine, COX-2, eNOS, and iNOS are expressed as ratios to average pixel count in HUVECs (reference slides). Abbreviations are as defined in text. *$P<0.01$ vs decompensated CHF; †$P<0.05$ vs decompensated CHF; ‡$P<0.01$ vs healthy subjects; §$P<0.05$ vs healthy subjects.

FMD, Plasma Total 8-Isoprostane, and Bicyclo-PGE$_2$

Brachial artery FMD increased from 5.2±2.5% in clinical decompensation to 7.6±2.2% after return to a compensated state ($P<0.01$). Plasma total 8-isoprostane and bicyclo-PGE$_2$ values were higher in patients with decompensated CHF than in healthy subjects (413±173 versus 185±57 pg/mL, $P<0.01$; 54±11 versus 38±9 pg/mL, $P<0.05$; respectively). Return to a compensated state was associated with a substantial reduction in plasma total 8-isoprostane (413±173 versus 293±174 pg/mL, $P<0.01$) and in bicyclo-PGE$_2$ (54±11 versus 45±11 pg/mL, $P<0.05$). After return to a compensated state, plasma total 8-isoprostane tended to remain greater in patients with CHF than in healthy subjects ($P=0.06$), whereas plasma bicyclo-PGE$_2$ was similar.

Discussion

The present data provide the first direct evidence that venous endothelial cells are activated in patients hospitalized for overt clinical decompensation of CHF with hemodynamic compromise and fluid retention. Nitrotyrosine formation (an intracellular marker of oxidative stress), COX-2, and iNOS expression were higher in venous endothelial cells harvested from decompensated patients than in venous endothelial cells from healthy subjects. Increased nitrotyrosine formation, COX-2, and iNOS expression subsided toward levels of healthy subjects after return to a compensated state. These findings suggest that the venous endothelium may contribute to systemic inflammation in patients with severely symptomatic CHF.

Evaluation of vascular function has mostly focused on invasive and noninvasive assessment of endothelial NO-mediated control of vasomotor tone in patients with CHF. Venous endothelial biopsy coupled to quantification of protein expression by immunofluorescence allows evaluation of other functional aspects of the vascular endothelium. When compared with the arterial endothelium, the venous endothelium is exposed to lower pulsatile biomechanical forces but to the same circulating proinflammatory mediators (eg, angiotensin II, tumor necrosis factor-α, and interleukin-1β). In that regard, direct examination of the venous endothelium may increase our knowledge of the multifaceted aspects of vascular endothelial dysfunction in CHF.

The present study does not provide any physiological correlates of venous endothelial activation. Characterization of arterial endothelial cells would have been more relevant to determine the mechanisms responsible for the changes in brachial artery FMD. However, cannulation of the radial or brachial artery could not be justified in the absence of a clinical indication because of the risks of thrombosis and a chronic reduction in lumen diameter.[11] Although vascular responses may differ among vascular beds, it is interesting that recent studies have actually demonstrated that lowering COX-2 activity and antioxidant treatment with vitamin C improve endothelium-dependent vasodilation in patients with coronary artery disease, hypertension, and CHF.[7,12,13]

Basal NO production is enhanced in patients with most severe CHF.[14,15] Ishibashi and colleagues[16] recently showed that not only eNOS but also iNOS contributes to vascular NO production in patients with advanced CHF: selective inhibition of iNOS reduced forearm blood flow and venous plasma nitrites/nitrates in patients with CHF, but not in controls. Our study shows, for the first time, evidence of increased iNOS expression in the venous endothelium of patients with CHF and overt clinical decompensation, whereas eNOS expression was similar in patients with decompensated and compensated CHF and in healthy subjects.

Vascular NO availability is reduced in patients with CHF, despite an increase in NO synthesis.[17–19] Superoxide degrades NO with formation of peroxynitrate, a toxic metabolite that nitrosylates proteins on tyrosine residues. Oxidative stress may lead to decompensation through multiple pathways. It may exert a negative inotropic effect on the myocardium by increasing peroxynitrate formation, and/or it may compromise tissue perfusion by impairing endothelial function because of limited NO bioavailability.[20,2] The concomitant improvement in FMD and reduction in endothelial nitrotyrosine after return to a compensated state underline the potential role of this latter pathway.

The compensated state of CHF is characterized in the periphery by a preferential distribution of cardiac output to essential organs: heart, brain, and kidneys.[21] Decreased vascular NO availability that, in turn, relates to an increase in oxidative stress and excessive production of vasoactive PGs via the inducible pathway may precipitate decompensation by hindering the preferential distribution of limited cardiac output to essential organs.

The syndrome of decompensated CHF is complex and frequently associated with tissue hypoperfusion, leading to hypoxia that in turn may increase oxidative stress and inflammation. Monitoring the transition from compensated to decompensated heart failure may be preferable to gain insight into the vascular events that precede and eventually contribute to decompensation. However, such an approach is not practical for obvious logistic reasons. In contrast, the transition from decompensated to compensated CHF frequently occurs in a hospital setting, and thus is more readily amenable to investigation. Further prospective studies involving larger cohorts of patients with advanced CHF may evaluate the endothelial phenotype over time and determine whether activation of the vascular endothelium may help predict decompensation in patients with CHF, thereby offering new therapeutic targets.

The present study investigated the cellular mechanisms of endothelial dysfunction in patients hospitalized for overt clinical decompensation, characterized by hemodynamic compromise and severe fluid retention. Our results should not be extrapolated to patients with less severe CHF, who, while hemodynamically stable, present with fluid retention.

Activation of the venous endothelium is a further manifestation of the systemic inflammatory response that accompanies the syndrome of severe CHF. Preliminary evidence suggests that immunomodulating therapy may be beneficial.[22] Whether aggressive antiinflammatory and antioxidant therapy has an adjunctive role in managing patients with severe CHF remains to be investigated.

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