Lung Endothelial Dysfunction in Congestive Heart Failure
Role of Impaired Ca\(^{2+}\) Signaling and Cytoskeletal Reorganization

Alexander Kerem,* Jun Yin,* Stephanie M. Kaestle, Julia Hoffmann, Axel M. Schoene, Baljit Singh, Hermann Kuppe, Mathias M. Borst, Wolfgang M. Kuebler

Rationale: Congestive heart failure (CHF) frequently results in remodeling and increased tone of pulmonary resistance vessels. This adaptive response, which aggravates pulmonary hypertension and thus, promotes right ventricular failure, has been attributed to lung endothelial dysfunction.

Objective: We applied real-time fluorescence imaging to identify endothelial dysfunction and underlying molecular mechanisms in an experimental model of CHF induced by supracoronary aortic banding in rats.

Methods and Results: Endothelial dysfunction was evident in lungs of CHF rats as impaired endothelium-dependent vasodilation and lack of endothelial NO synthesis in response to mechanical stress, acetylcholine, or histamine. This effect was not attributable to downregulation of endothelial NO synthase. Imaging of the cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)) revealed a singular impairment of endothelial [Ca\(^{2+}\)]\(_{i}\) homeostasis and signaling characterized by a lack of [Ca\(^{2+}\)]\(_{i}\) oscillations and deficient or attenuated [Ca\(^{2+}\)]\(_{i}\) responses to mechanical stress, histamine, acetylcholine, or thapsigargin. Reconstitution of a [Ca\(^{2+}\)]\(_{i}\) signal by ionophore treatment restored endothelial NO production, but lack of endothelial responsiveness was not primarily attributable to downregulation of Ca\(^{2+}\) influx channels in CHF. Rather, we identified a massive remodeling of the endothelial cytoskeleton in the form of an increased expression of beta-actin and F-actin formation which contributed critically to endothelial dysfunction in CHF because cytoskeletal disruption by cytochalasin D largely reconstituted endothelial [Ca\(^{2+}\)]\(_{i}\) signaling and NO production.

Conclusions: Our findings characterize a unique scenario of endothelial dysfunction in CHF that is caused by a singular impairment of [Ca\(^{2+}\)]\(_{i}\) signaling, and identify cytoskeletal reorganization as a major regulator of endothelial signaling and function. (Circ Res. 2010;106:1103-1116.)

Key Words: congestive heart failure ■ pulmonary hypertension ■ endothelial dysfunction ■ Ca\(^{2+}\) regulation ■ cytoskeletal dynamics

Congestive heart failure (CHF) is a major and growing cause of morbidity and mortality in most affluent countries, with a prevalence approaching 10 per 1000 among those older than 65 years of age.\(^1\) Approximately 60% to 80% of patients with CHF of systolic or diastolic origin develop pulmonary hypertension owing to left heart disease (previously known as pulmonary venous hypertension).\(^2,3\) Importantly, this form of pulmonary hypertension is not solely caused by a “passive” increase in pulmonary vascular pressures, but is frequently aggravated by a concomitant “reactive” increase in pulmonary vascular resistance (PVR). Incidence and magnitude of this “reactive” component vary from 20% to 100% and from supranormal to extreme PVR values, and seem to worsen with progressive duration and severity of the underlying heart disease.\(^4,7\) The “reactive” PVR increase in CHF results both from an elevated tone and extensive remodeling of lung resistance vessels.\(^8,9\) The resulting increase in right ventricular afterload limits right ventricular output, and may ultimately cause fatal right ventricular failure.\(^2\) The clinical relevance of this scenario is highlighted by epidemiological studies which identified reduced right ventricular ejection fraction as potent and independent predictor of mortality in CHF.\(^10\)

The results of several preclinical studies suggest that CHF may cause lung endothelial dysfunction, as indicated by an impaired endothelium-dependent relaxation of pulmonary artery segments in response to acetylcholine (ACh) in CHF rats.\(^11,12\) Consequently, lung endothelial dysfunction has been

Original received October 26, 2008; resubmission received October 5, 2009; revised resubmission received January 4, 2010; accepted February 9, 2010.

From the Institute of Physiology (A.K., J.Y., S.M.K., J.H., W.M.K.), Charité—Universitätsmedizin Berlin, Germany; German Heart Institute Berlin (J.Y., W.M.K., H.K.), Germany; Keenan Research Centre (J.Y., W.M.K.), Li Ka Shing Knowledge Institute, St Michael’s Hospital, Toronto, Ontario, Canada; Department of Cardiology, Angiology and Pneumology (A.M.S., M.M.B.), University of Heidelberg, Germany; Department of Veterinary Biomedical Sciences (B.S.), Western College of Veterinary Medicine, University of Saskatchewan, Canada; and Department of Surgery (W.M.K.), University of Toronto, Ontario, Canada.

*Both authors contributed equally to this work.

Correspondence to Prof Dr Wolfgang M. Kuebler, The Keenan Research Centre, Li Ka Shing Knowledge Institute, St Michael’s Hospital, 30 Bond St, Bond Wing 2-021, M5B 1W8 Toronto, Ontario, Canada. E-mail kueblerw@smh.toronto.on.ca

© 2010 American Heart Association, Inc.

Circulation Research is available at http://circres.ahajournals.org

DOI: 10.1161/CIRCRESAHA.109.210542

1103
proposed to contribute critically to increased PVR and right ventricular afterload in CHF patients.13 The cellular mechanisms underlying endothelial dysfunction in CHF are yet unclear but may present potential targets for prevention or treatment of pulmonary hypertension in CHF.

Here, we applied real-time fluorescence imaging of lung endothelial function and underlying signaling pathways in an experimental model of CHF in rats. Our findings identify a fundamental impairment of endothelial [Ca\(^{2+}\)]\(_i\) signaling that is in part attributable to an extensive reorganization of the endothelial actin cytoskeleton as a new pathogenic factor in pulmonary hypertension and a novel mechanism of endothelial dysfunction in heart failure.

**Methods**

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

In brief, CHF was induced in Sprague–Dawley rats by supracoronary aortic banding 9 weeks before the investigations.8,14 Sham-operated rats served as controls. Isolated lungs of CHF and control rats were prepared for real-time fluorescence microscopy as reported.15,16 NO synthesis in small pulmonary arterioles and lung venules capillaries was quantified by fluorescence imaging of the NO-sensitive dye DAF-FM as change in fluorescence intensity (F) over time relative to its individual baseline (F\(_b\)). Ca\(^{2+}\)\(_i\) concentration in cytosol ([Ca\(^{2+}\)]\(_c\)) and endosomal stores of lung endothelial cells was quantified by ratiometric imaging of fura-2 and fura-2FF, respectively.17 The endothelial F-actin cytoskeleton was visualized by Alexa 568 phalloidin staining as described.18 Immunofluorescence staining for α-smooth muscle actin in lung microvessels was performed as reported.19 Endothelium-dependent and -independent vasodilation was determined as dose-dependent reduction in pulmonary arterial pressure (ΔP\(_{PA}\)) in response to ACh and sodium nitroprusside (SNP), respectively, in lungs preconstricted with U466195,20 Pulmonary and systemic hemodynamics were measured in vivo in CHF and control rats as described,8,14 and the vasorelaxant effect of inhaled nitric oxide was determined as dose-dependent reduction in P\(_{PA}\). Western blot analyses from whole lung homogenate and fresh lung endothelial cells (FLECs) and immunohistochemical analyses were performed as outlined in the Online Data Supplement.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Control</th>
<th>CHF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>355±7</td>
<td>352±6</td>
</tr>
<tr>
<td>Heart weight/body weight, %</td>
<td>0.31±0.01</td>
<td>0.47±0.02*</td>
</tr>
<tr>
<td>Fulton’s index (RV/[LV+S]), %</td>
<td>26.6±1.0</td>
<td>29.2±0.6*</td>
</tr>
<tr>
<td>Mean arterial pressure, mm Hg</td>
<td>99±4</td>
<td>98±5</td>
</tr>
<tr>
<td>P(_{VED}), mm Hg</td>
<td>5±1</td>
<td>11±1*</td>
</tr>
<tr>
<td>P(_{PA}), mm Hg</td>
<td>11.0±0.6</td>
<td>18.4±1.2*</td>
</tr>
<tr>
<td>PVR, dyn·sec/cm(^5)</td>
<td>15.2±1.8</td>
<td>24.0±4.5*</td>
</tr>
<tr>
<td>Static lung compliance, mL/mm Hg</td>
<td>0.97±0.02</td>
<td>0.62±0.02*</td>
</tr>
<tr>
<td>Wet-to-dry lung weight ratio</td>
<td>4.75±0.01</td>
<td>5.22±0.02*</td>
</tr>
<tr>
<td>Epithelial lining fluid, µL</td>
<td>195±37</td>
<td>198±46</td>
</tr>
</tbody>
</table>

Group data are from 10 control and CHF rats each except for epithelial lining fluid data, which are from 5 rats each. *P<0.05 vs control. Fulton’s index was calculated as ratio of right ventricular weight (RV) over left ventricle (LV) plus septal (S) weight. P\(_{VED}\) indicates left ventricular end-diastolic pressure; P\(_{PA}\), pulmonary arterial pressure.

The experimental protocols included the following: (1) pharmacological interventions; (2) pressure elevation; (3) tuning of endothelial [Ca\(^{2+}\)]\(_i\); and (4) actin depolymerization.

**Pharmacological Interventions**

Histamine (10\(^{-5}\) to 10\(^{-3}\) mol/L), ACh (10\(^{-10}\) to 10\(^{-4}\) mol/L), S-nitroso-N-acetylpenicillamine (SNAP) (10\(^{-4}\) to 10\(^{-3}\) mol/L), SNAP (10\(^{-6}\) to 10\(^{-6}\) mol/L), or the transient receptor potential vanilloid (TRPV)\(_4\) activator 4α-phorbol-12,13-didecanoate (4αPDD) (10 µmol/L) was added to the perfusate. 1-Arginine (250 µmol/L), atropine (10 µmol/L), or (+)-tubocurarine (100 µmol/L) was added 10 minutes before stimulation with ACh. Thapsigargin (2 µmol/L), which releases Ca\(^{2+}\) from endosomal stores, was infused in nominally Ca\(^{2+}\)-free HEPES solution.

**Pressure Elevation**

Baseline recordings were obtained at left atrial pressure (P\(_{LA}\)) of 5 cmH\(_2\)O, and P\(_{LA}\) was raised to 15 cmH\(_2\)O for 30 minutes by adjusting the height of the venous outflow. In a subset of experiments, P\(_{LA}\) in CHF lungs was 15 cmH\(_2\)O at baseline and raised to 25 cmH\(_2\)O.

**Tuning of Endothelial [Ca\(^{2+}\)]\(_i\)**

Lungs were infused with nominally Ca\(^{2+}\)-free HEPES solution containing 2% dextran and EGTA (0.5 mmol/L) or 200 mmol/L Ca\(^{2+}\) in HEPES solution in the presence or absence of the Ca\(^{2+}\) ionophore 4-bromo A-23187 (5 µmol/L).

**Actin Depolymerization**

Cytochalasin D (100 µmol/L) was added to the perfusate 10 minutes before ACh, histamine, or P\(_{LA}\) elevation. All data are presented as means±SEM.

**Results**

Cardiopulmonary Characterization

Nine weeks after aortic banding, CHF rats had a higher heart-to-body weight ratio as compared to controls reflecting an increase in cardiac weight as the body weight of CHF and control animals was similar (Table). Cardiac hypertrophy was more pronounced in right than left ventricles, as demonstrated by an increased Fulton index. Elevated left ventricular end-diastolic pressure, increased P\(_{PA}\) and PVR, and reduced static lung compliance in CHF rats reflect the characteristics of pulmonary hypertension owing to left heart disease, whereas...
systemic arterial pressure was unaltered. A slight increase in wet-to-dry lung weight ratio indicates mild edema formation in CHF lungs, whereas overt alveolar flooding was absent, as indicated by similar volumes of epithelial lining fluid (Table).

**Lung Endothelial Dysfunction in CHF**

Real-time imaging of DAF-FM in venular capillaries of the isolated lung revealed a deficient endothelial NO response to ACh stimulation in CHF rats (Figure 1A). Whereas ACh caused a dose-dependent increase in endothelial fluorescence in controls, CHF lungs showed no response (Figure 1B). A similar deficiency in endothelial NO production after stimulation with ACh was evident in small pulmonary arterioles of 25 to 50 μm diameter (Figure 1C). In line with previous studies implicating the muscarinic M₃ receptor in the endothelium-dependent vasodilatory response to ACh,²¹ NO production in control lungs was blocked by atropine but not tubocurarine (Figure 1D). Yet, the lack of an NO response in CHF lungs was not attributable to a downregulation of the M₃ ACh receptor, as demonstrated by equal expression in FLECs from control and CHF animals (Figure 1E).

Endothelial dysfunction in CHF lungs was confirmed by hemodynamic measurements in isolated perfused rat lungs demonstrating a complete lack of the endothelium-dependent vasorelaxant effect of ACh (Figure 2A), whereas the endothelium-independent response to SNP was preserved (Figure 2B). To test the in vivo relevance of impaired endothelial NO production in CHF, we measured the lung hemodynamic response to inhaled NO. Consistent with the notion that the lung vascular bed is fully dilated under physiological conditions, NO inhalation did not affect PᵥA in control animals. Yet in CHF rats, NO inhalation caused a dose-dependent reduction in PᵥA, substantiating that lung vascular tone is increased in CHF and suggesting that restoration of endothelial NO production may mitigate pulmonary hypertension in CHF (Figure 2C).

The lack of endothelial NO production in CHF lungs was not exclusive for stimulation with ACh but was equally evident after stimulation with histamine despite unaltered expression of the histamine H₁ receptor (Figure 2D). The endothelial NO response to nonpharmacological stimulation by mechanical stress was similarly abrogated in CHF. Whereas acute elevation of lung microvascular pressure stimulates endothelial NO production in control lungs,¹⁶ this response was absent in lungs of CHF rats (Figure 2E). The lack of response was not attributable to diminished endothelial stretch, because amplitude and time response of vessel distension were similar in control and CHF lungs (Figure 2F). Infusion of the exogenous NO donor SNAP resulted in identical dose-dependent increases in DAF-FM fluorescence in control and CHF lungs (Figure 2G), effectively ruling out that insufficient dye loading or an impaired dye sensitivity may have contributed to the lack of fluorescence increase in CHF lungs.

Endothelial dysfunction was not attributable to downregulation of endothelial nitric oxide synthase (eNOS), as demonstrated by immunohistochemistry and Western blot analyses of both lung homogenates and FLECs which rather indicated an increase of eNOS expression in CHF lungs (Figure 3A and 3B). The inducible NOS isoform (iNOS) was not detectable in lungs of control or CHF rats, with iNOS induction by intravenous lipopolysaccharide serving as positive control (Figure 3C). A critical lack of L-arginine as essential substrate for NO synthesis was excluded by experiments in which L-arginine addition to the perfusate failed to reconstitute NO production in response to ACh in CHF lungs (Figure 3D).

**Lung Endothelial [Ca²⁺]ᵢ Signaling in CHF**

Next, we addressed the option of impaired posttranslational eNOS activation by Ca²⁺/calmodulin signaling in CHF. Fura-2 imaging revealed that already the baseline endothelial [Ca²⁺]ᵢ profile differed considerably in CHF lungs as compared to controls. The endothelial [Ca²⁺]ᵢ profile in control lungs is characterized by spontaneous oscillations¹⁵ whereas in CHF lungs, spontaneous [Ca²⁺]ᵢ oscillations were almost entirely absent, and mean endothelial [Ca²⁺]ᵢ was ~15% lower as compared to controls (Figure 4A). Mechanical stimulation of endothelial cells by elevation of PᵥLA from 5 to 15 cmH₂O increased mean endothelial [Ca²⁺]ᵢ, and the amplitude of endothelial [Ca²⁺]ᵢ oscillations (data not shown) in control animals as reported previously,¹⁵ but had no effect on endothelial [Ca²⁺]ᵢ in CHF lungs (Figure 4B). The apparent lack of a [Ca²⁺]ᵢ response to hydrostatic stress was not attributable to adaptation to an increased offset pressure in CHF rats, because PᵥLA elevation from 15 to 25 cmH₂O equally failed to evoke a detectable [Ca²⁺]ᵢ signal (Figure 4C). Recently, we and others have shown that the lung endothelial [Ca²⁺]ᵢ response to hydrostatic stress is mediated by activation of mechanosensitive TRPV4 channels.¹⁷,²² Consistent with the absence of a pressure-induced [Ca²⁺]ᵢ response in CHF, pharmacological activation of TRPV4 by 4aPDD increased endothelial [Ca²⁺]ᵢ in control but not in CHF lungs (Figure 4D).

In agreement with the lack of an NO response to ACh, the atropine-sensitive endothelial [Ca²⁺]ᵢ response to ACh was likewise absent in CHF lungs (Figure 5A). Similarly, the distinctive biphasic endothelial [Ca²⁺]ᵢ response to stimulation with histamine was largely abrogated in CHF lungs and could not be recovered by up to 100-fold higher pharmacological doses (Figure 5B). Analogous to endothelial dysfunction, impaired endothelial [Ca²⁺]ᵢ signaling was not restricted to lung venular capillaries, but was equally evident in small pulmonary arterioles as exemplified by the lack of a histamine response (Figure 5C). To test whether downregulation of endothelial Ca²⁺ entry channels may account for the impaired Ca²⁺ response to mechanical and pharmacological stimulation, we analyzed the expression of cation channels of the TRPV and transient receptor potential canonical (TRPC) family in lung endothelial cells. TRPV2 and TRPV4 (Figure 5D) were markedly downregulated in CHF as compared to control lungs, suggesting that a lower expression of these mechanosensitive cation channels may contribute to the lack of endothelial [Ca²⁺]ᵢ responses to hydrostatic stress or 4aPDD. However, TRP channels of the canonical superfami-

family including, TRPC1, TRPC4, and TRPC6, which have been implicated in the [Ca²⁺]ᵢ response to ligands such as histamine, thrombin, or ACh,²³–²⁴ were not differentially expressed
in CHF and control lungs (Figure 5E). Next, we addressed whether depletion of endosomal Ca$^{2+}$ stores may contribute to the impaired endothelial [Ca$^{2+}$]$_i$ response in CHF lungs. This possibility was effectively ruled out by fura-2FF imaging of endosomal Ca$^{2+}$, which revealed an equivalent Ca$^{2+}$ release from endosomal stores after thapsigargin treatment in Ca$^{2+}$-free perfused CHF and control lungs (Figure 5F). However, the concomitant increase in endothelial [Ca$^{2+}$]$_i$
was markedly attenuated in CHF lungs suggesting rapid binding or reuptake of Ca\(^{2+}\) subsequent to its release into the cytosol.

To test the relevance of impaired [Ca\(^{2+}\)]\(_i\) signaling for endothelial dysfunction in CHF, we tuned endothelial [Ca\(^{2+}\)]\(_i\) by lung perfusion with HEPES buffer containing 0 or 200 nmol/L Ca\(^{2+}\) in the presence or absence of Ca\(^{2+}\) ionophore 4-bromo A-23187. Perfusion of ionophore-treated lungs at 200 nmol/L Ca\(^{2+}\) tuned endothelial [Ca\(^{2+}\)]\(_i\) to 180 to 200 nmol/L (n=3; data not shown) and induced a marked increase in endothelial NO production (Figure 5G). Conversely when lungs were perfused with nominally Ca\(^{2+}\)-free buffer or with 200 nmol/L Ca\(^{2+}\) in the absence of Ca\(^{2+}\)-ionophore, no increase in DAF-FM fluorescence was detectable. Tuning of endothelial [Ca\(^{2+}\)]\(_i\) by 4-bromo A-23187 induced essentially similar NO responses in CHF and control lungs, demonstrating that NO synthesis in CHF is restored if an adequate endothelial [Ca\(^{2+}\)]\(_i\) signal can be generated.

**Cytoskeletal Reorganization**

Based on the notion that actin filaments play a fundamental role in intracellular Ca\(^{2+}\) homeostasis\(^{5,26}\) and may regulate Ca\(^{2+}\) influx via store-operated and TRP channels,\(^{27}\) we addressed the effects of CHF on the actin cytoskeleton and its role in endothelial dysfunction. Imaging of fluorescently labeled phalloidin in control lungs showed circular F-actin filaments which were particularly pronounced at microvascular bifurcations and more prominent in small pulmonary arterioles as

---

**Figure 2. Lung endothelial dysfunction in CHF.** A and B, Group data show endothelium-dependent vasorelaxation response to ACh (A) and endothelium-independent response to SNP (B) in isolated perfused rat lungs preconstricted by U46619 (50 pmol/min). Vasorelaxation was determined as dose-dependent pressor response and is expressed as reduction in pulmonary arterial pressure (ΔPPA) relative to the respective vehicle control. Dose–responses are constructed from 4-parameter logistic curve fits, and EC\(_{50}\) was calculated as 2.7×10\(^{-7}\) for the ACh response in control lungs. Data from n=5 each; *P<0.05 vs control. C, Group data show dose-dependent changes in pulmonary arterial pressure (ΔPPA) in response to inhalation of NO in ventilated control and CHF rats. Data from n=5 each; *P<0.05 vs control. D, Group data show changes in DAF-FM fluorescence in lung venular capillaries in response to 10 μmol/L histamine. Data from n=5 each; *P<0.05 vs baseline. Representative immunoblot shows equal expression of the histamine H1 receptor in FLECs of control and CHF rats. Ponceau staining is shown as loading control. Replicated 3 times, ie, in a total of n=6 independent samples. E, Group data show changes in capillary DAF-FM fluorescence in response to P\(_{LA}\) elevation from 5 to 15 cmH\(_2\)O for 30 minutes in control and CHF lungs. Data from n=5 each; *P<0.05 vs baseline at t=0 minutes. F, Representative time profiles of lung microvascular diameter changes in response to P\(_{LA}\) elevation from 5 to 15 cmH\(_2\)O in control and CHF lungs demonstrating equal amplitude and time constant of pressure-induced microvascular distension. Replicated in n=5 each. G, Dose-dependent changes in capillary DAF-FM fluorescence in response to the exogenous NO donor SNAP show equal and reproducible increases in fluorescence in control and CHF lungs. Data from n=32 measurements in n=4 rats each.
compared to venular capillaries (Figure 6A and 6B). In CHF lungs, phalloidin staining was enhanced several-fold in both small pulmonary arterioles and venular capillaries as compared to controls, and spirally arranged actin microfilaments were distributed over the entire length of microvascular segments (Figure 6A and 6B). Phalloidin staining was specific for actin microfilaments, because fluorescence intensity was reduced by several orders of magnitude when microvessels were pretreated with cytochalasin D, which cleaves actin microfilaments, whereas degradation of elastic fibers with elastase had no effect (Figure 6C). Although a potential contribution of smooth muscle cells to the phalloidin signal in small pulmonary arterioles cannot be excluded because of methodological constraints, the increased phalloidin fluorescence in venular capillaries of CHF as compared to control lungs was clearly attributable to cytoskeletal reorganization in the microvascular endothelium, because the respective microvessels were devoid of α-smooth muscle actin (Figure 6D). The notion of a significant reorganization of the endothelial actin cytoskeleton in CHF was substantiated by Western blot analyses of FLECs demonstrating a ≈13-fold increase in β-actin expression in FLECs of CHF as compared to controls (Figure 6E).

To test whether cytoskeletal reorganization contributes to endothelial dysfunction, we pretreated CHF and control lungs with cytochalasin D. In CHF lungs, cytochalasin D restored the endothelial [Ca²⁺], increase in response to hydrostatic stress as demonstrated by representative images (Figure 7A) and single cell tracings (Figure 7B), yet did not re-establish endothelial [Ca²⁺] oscillations. Likewise, cytochalasin D restored the endothelial [Ca²⁺] increase in response to histamine in both lung venular capillaries (Figure 7C) and

$\frac{48}{H1108}$ Circulation Research April 2, 2010

by guest on November 1, 2017 http://circres.ahajournals.org/ Downloaded from
Figure 4. Endothelial [Ca\(^{2+}\)] oscillations and response to mechanical stress. A, Representative single cell tracings of endothelial [Ca\(^{2+}\)] in lung venular capillaries show spontaneous [Ca\(^{2+}\)] oscillations in control but not in CHF lungs (left). Group data demonstrate reduced mean endothelial [Ca\(^{2+}\)] (top right) and virtual absence of [Ca\(^{2+}\)] oscillations (bottom right) in CHF lungs as compared to control. Data from n=7 each; *P<0.05 vs control. B, Representative images show fura-2–loaded endothelial cells color-coded for [Ca\(^{2+}\)] in lung venular capillaries. Images were obtained at P\(_{LA}\) of 5 cmH\(_2\)O (left) and 30 minutes after P\(_{LA}\) elevation to 15 cmH\(_2\)O (right) in control (top) and CHF (bottom) lungs. Vessel margins are depicted by dotted lines. C, Group data show mean endothelial [Ca\(^{2+}\)], at baseline (P\(_{LA}\)=5 cmH\(_2\)O) and over 30 minutes of P\(_{LA}\) elevation to 15 cmH\(_2\)O in venular capillaries of control and CHF lungs or in CHF lungs perfused at a baseline P\(_{LA}\) of 15 cmH\(_2\)O, with a subsequent increase to 25 cmH\(_2\)O (CHF, offset). Data from n=5 to 6 each; *P<0.05 vs baseline at t=0 minutes. D, Group data show mean endothelial [Ca\(^{2+}\)], at baseline and after TRPV4 activation by 4αPDD (10 μmol/L) in venular capillaries of control and CHF lungs. Data from n=4 each; *P<0.05 vs baseline at t=0 minutes.
Figure 5. Endothelial [Ca\textsuperscript{2+}]i signaling in CHF. A, Group data show mean endothelial [Ca\textsuperscript{2+}]i in venular capillaries of control and CHF lungs at baseline and 10 minutes following stimulation with 5×10\textsuperscript{-6} mol/L ACh in the presence or absence of the muscarinic and nicotinergic ACh receptor inhibitors atropine (10 \mu mol/L) or (-)-tubocurarine (100 \mu mol/L), respectively. Data from n=5 each; *P<0.05 vs basal endothelial [Ca\textsuperscript{2+}]i in control lungs, #P<0.05 vs ACh stimulation in the absence of ACh receptor inhibitors in control lungs. B, Representative single cell tracings of endothelial [Ca\textsuperscript{2+}]i in venular capillaries show characteristic biphasic [Ca\textsuperscript{2+}]i response to histamine (10 \mu mol/L) in control lungs, but almost complete attenuation in CHF lungs even when stimulated with excessive histamine doses (up to 1000 \mu mol/L). Replicated in n=4 each. C, Group data show endothelial [Ca\textsuperscript{2+}]i in small pulmonary arterioles at baseline and peak [Ca\textsuperscript{2+}]i response to histamine (10 \mu mol/L) in control and CHF lungs. Data from n=4 each; *P<0.05 vs baseline, #P<0.05 vs control. D, Representative immunoblots (top) and bar charts (bottom) of TRPV2 and TRPV4 expression in FLECs from control and CHF lungs. Data from n=4 each; *P<0.05 vs control. E, Representative immunoblots of TRPC1, TRPC4, and TRPC6 in FLECs from control and CHF lungs. Replicated 3 times each. F, Group data show changes in endosomal Ca\textsuperscript{2+} and endothelial [Ca\textsuperscript{2+}]i, as determined by fura-2FF and fura-2 ratio imaging, respectively, in lung venular capillaries. Changes from the individual baseline were measured after 10 minutes in Ca\textsuperscript{2+}-free perfused control or CHF lungs in the absence (-Tg) or presence (+Tg) of thapsigargin (2 \mu mol/L). Data from n=4 each; *P<0.05 vs untreated (-Tg), #P<0.05 vs control. G, Group data show changes in capillary DAF-FM fluorescence in response to tuning of endothelial [Ca\textsuperscript{2+}]i by lung perfusion with defined Ca\textsuperscript{2+} concentrations ([Ca\textsuperscript{2+}]\textsubscript{free}) of nominally 0 or 200 nmol/L in the presence or absence of the Ca\textsuperscript{2+} ionophore 4-bromo A-23187 (5 \mu mol/L). Data from n=5 each; *P<0.05 vs lung perfusion with 200 nmol/L Ca\textsuperscript{2+} in the absence of 4-bromo A-23187.
Figure 6. Cytoskeletal reorganization. A, Representative images show endothelial actin cytoskeleton stained by Alexa 568 phalloidin in venular capillaries of control (left) and CHF (right) lungs. Vessel margins are depicted by dotted lines. B, Group data show Alexa 568 phalloidin fluorescence, quantified as number of fluorescent pixels per area in lung venular capillaries and pulmonary arterioles of control and CHF rats. Data from n=4 to 6 each; *P<0.05 vs control, #P<0.05 vs capillaries. C, Representative images show Alexa 586 phalloidin–labeled microvessels in CHF lungs after pretreatment with pancreatic elastase (200 U/mL) (left) or cytochalasin D (100 μmol/L) (right). Replicated in n=3 each. D, Representative images (top) and group data (bottom) show in situ immunofluorescence staining for α-smooth muscle actin in fixed and permeabilized venular capillaries of control and CHF lungs and in pulmonary arterioles as positive control. FITC fluorescence is quantified as fluorescent pixels per area, and nonspecific staining is assessed by an isotype-matched nonspecific control antibody. E, Representative immunoblot (top) and bar chart (bottom) of β-actin expression in FLEC from control and CHF lungs. Ponceau staining is shown as loading control. Replicated 3 times; *P<0.05 vs control.
Figure 7. Cytoskeletal disruption reconstitutes endothelial [Ca\textsuperscript{2+}], signaling and NO production. A, Representative images show fura-2–loaded endothelial cells color-coded for [Ca\textsuperscript{2+}], in a lung venular capillary. Images from a CHF lung pretreated with cytochalasin D (100 μmol/L) were obtained at baseline P\textsubscript{LA} of 5 cmH\textsubscript{2}O (left) and 30 minutes after P\textsubscript{LA} elevation to 15 cmH\textsubscript{2}O (right). Note pressure-induced [Ca\textsuperscript{2+}] increase as compared to untreated CHF lung in Figure 4B. B, Representative single cell tracings of the endothelial [Ca\textsuperscript{2+}], response to P\textsubscript{LA} elevation to 15 cmH\textsubscript{2}O in a control lung, a CHF lung, or a cytochalasin D–pretreated CHF lung. Replicated in n=5 each. C and D, Group data show endothelial [Ca\textsuperscript{2+}] in lung venular capillaries (C) and small pulmonary arterioles (D) at baseline and peak [Ca\textsuperscript{2+}], response to histamine (10 μmol/L) in control and CHF lungs with or without cytochalasin D pretreatment. Data from n=4 each; *P<0.05 vs baseline. E, Representative images of DAF-FM–loaded lung venular capillaries show endothelial cells color-coded for NO production. Images from a CHF lung pretreated with cytochalasin D (100 μmol/L) were obtained at baseline P\textsubscript{LA} of 5 cmH\textsubscript{2}O (left) and 30 minutes after P\textsubscript{LA} elevation to 15 cmH\textsubscript{2}O (right). F, Representative single cell tracing of endothelial DAF-FM fluorescence in response to P\textsubscript{LA} elevation to 15 cmH\textsubscript{2}O in a control lung, a CHF lung, or a cytochalasin D–pretreated CHF lung. Replicated in n=5 each. G and H, Group data show changes in endothelial DAF-FM fluorescence in lung venular capillaries (G) and small pulmonary arterioles (H) of control and CHF lungs at baseline and in response to ACh (5×10\textsuperscript{6} mol/L) with or without cytochalasin D pretreatment. Data from n=4 each; *P<0.05 vs baseline.
small arterioles (Figure 7D) of CHF lungs, but had no detectable effect on baseline [Ca\textsuperscript{2+}], or the histamine response in control lungs.

Consistent with the notion that restoration of endothelial [Ca\textsuperscript{2+}], signaling can reverse endothelial dysfunction in CHF, cytochalasin D partially reconstituted endothelial NO production in response to hydrostatic stress as shown in representative images (Figure 7E) and single cell tracings (Figure 7F), respectively. Similarly, microfilament disruption partially restored the endothelial NO response to ACh in venular capillaries (Figure 7G) and small arterioles (Figure 7H) of CHF lungs without detectable effects on NO production in control lungs.

**Discussion**

Here, we show that CHF causes endothelial dysfunction in pulmonary microvessels as demonstrated by a lack of NO synthesis in response to mechanical or receptor-mediated stimulation in lung venular capillaries and small arterioles, and by the lack of an endothelium-dependent vasorelaxant response to ACh. Reduced expression or eNOS substrate deficiency, poor dye loading, insufficient stimulation attributable to reduced receptor expression or lesser mechanical stretch could be ruled out as potential underlying mechanisms. Instead, the almost complete lack of an endothelial [Ca\textsuperscript{2+}], response to mechanical stress, ACh, or histamine and the reconstitution of endothelial NO synthesis by Ca\textsuperscript{2+}-ionophore treatment revealed that endothelial dysfunction was caused by impaired endothelial [Ca\textsuperscript{2+}], signaling. Cytoskeletal reorganization in lung endothelial cells, which was evident as many-fold increases in F-actin content and β-actin expression, was identified to play a critical role in this scenario, because cytochalasin D largely reconstituted endothelial [Ca\textsuperscript{2+}], signaling and NO synthesis in response to appropriate stimulation. Although underlying molecular mechanisms remain to be elucidated, our results identify regulation of [Ca\textsuperscript{2+}], signaling and NO synthesis by cytoskeletal reorganization as a novel and clinically relevant mechanism of endothelial cell adaptation in cardiovascular disease.

**Lung Endothelial Dysfunction in CHF**

In systemic blood vessels of CHF patients or animal models, endothelial dysfunction contributes critically to impaired coronary and systemic perfusion with fundamental impact on morbidity and mortality.28 In the lung, endothelial dysfunction in CHF has been proposed to induce a reactive increase in PVR, causing pulmonary hypertension and ultimately right ventricular failure.13 Here, we used our established real-time imaging techniques to directly visualize deficient endothelial NO production in lung venular capillaries of CHF rats,16,29 The additional demonstration of an essentially similar dysfunction in small pulmonary arterioles constitutes to our knowledge the first reported real-time imaging of pulmonary arterial endothelial cells in the intact lung.

In normal lungs, capillary NO production in response to ACh was sensitive to atropeine but not tubocurarine, suggesting a role for capacitative Ca\textsuperscript{2+} influx in the NO response.30 In capillaries and arterioles of CHF lungs, endothelial dysfunction was manifest as lack of NO production in response to ACh, histamine or mechanical stress. Likewise, endothelial dysfunction was functionally evident as lack of endothelium-dependent vasodilation in response to ACh in the presence of a preserved endothelium-independent vasorelaxation. Of note, the half-maximal effective ACh concentration (EC50) for capillary NO production exceeds the EC50 for the ACh-dependent reduction in PPA by almost 10-fold. Although this discrepancy may be attributable to a lesser sensitivity of lung capillaries to ACh as compared to pulmonary resistance vessels, it may equally suggest that endothelial NO production is yet submaximal when ACh-dependent vasorelaxation is complete. Our experiments rule out downregulation of eNOS or lack of eNOS substrate which have been shown to underlie endothelial dysfunction in pulmonary arterial hypertension,31,32 and consequently suggest that lung endothelial dysfunction in CHF may be attributable to impaired posttranslational regulation of eNOS.

**Impaired Lung Endothelial [Ca\textsuperscript{2+}], Signaling in CHF**

By endothelial fura-2 imaging in CHF lungs, we identified a fundamental impairment of endothelial [Ca\textsuperscript{2+}], homeostasis and signaling that was evident as lower basal [Ca\textsuperscript{2+}], a lack of spontaneous and agonist-induced [Ca\textsuperscript{2+}], oscillations, and the virtual absence of a detectable [Ca\textsuperscript{2+}], response to mechanical or pharmacological stimulation by hydrostatic stress, ACh or histamine. We considered the possibility that continuous exposure to elevated vascular pressures may have caused an offset shift in endothelial mechanosensitivity. Yet even at an elevated baseline P\textsubscript{LA} of 15 cmH\textsubscript{2}O corresponding to the higher left ventricular end-diastolic pressure in CHF rats, an additional pressure increment failed to elicit a detectable [Ca\textsuperscript{2+}], response. Likewise, direct activation of TRPV4 which mediates the endothelial [Ca\textsuperscript{2+}], response to hydrostatic stress in control lungs17,22 failed to elicit a [Ca\textsuperscript{2+}], increase in CHF lungs. TRPV4 and TRPV2 were both downregulated in CHF, seemingly supporting the notion that downregulation of these cation channels may underlie the loss of endothelial mechanosensitivity. Yet, our subsequent finding that cytochalasin D largely reconstituted endothelial [Ca\textsuperscript{2+}], responses demonstrates that this downregulation of TRPV channels does not critically limit the endothelial response to hydrostatic stress.

The notion that downregulation of Ca\textsuperscript{2+} influx channels does not constitute the predominant mechanism underlying the impaired endothelial [Ca\textsuperscript{2+}], responsiveness in CHF is substantiated by several lines of evidence. First, both components of the biphasic response to histamine, ie, the initial Ca\textsuperscript{2+} release from intracellular stores and the subsequent Ca\textsuperscript{2+} influx via store-operated Ca\textsuperscript{2+} channels,33 were virtually absent in CHF lungs. Hence, the lack of an endothelial [Ca\textsuperscript{2+}], response is not simply attributable to impaired capacitative Ca\textsuperscript{2+} entry, because the defect is already evident at the level of endosomal Ca\textsuperscript{2+} release. Second, several members of the TRPC channel superfamily previously implicated in the [Ca\textsuperscript{2+}], response to histamine23,24 were equally expressed in CHF and control lungs. This finding is in notable contrast to data from Alvarez et al demonstrating downregulation of endothelial TRPC1, TRPC3, and TRPC4 in an aortocaval...
fistula model of heart failure in rats, indicating considerable differences between lung vascular adaptation in high-flow (fistula) and low-flow (aortic banding) CHF models. Third, endothelial cells of CHF lungs lacked both spontaneous and agonist-induced [Ca\(^{2+}\)] oscillations that are largely independent from extracellular Ca\(^{2+}\) influx. Last, whereas thapsigargin triggered an equal Ca\(^{2+}\) release from endosomal stores in CHF and control lungs, the concomitant increase in endothelial [Ca\(^{2+}\)] was markedly attenuated in CHF rats. Taken together, these findings suggest a singular impairment of endothelial Ca\(^{2+}\) homeostasis which cannot be attributed primarily to downregulation of Ca\(^{2+}\) influx channels.

The notion of an impaired endothelial [Ca\(^{2+}\)], signaling is in line with previous studies reporting an attenuated increase in lung endothelial permeability following endosomal Ca\(^{2+}\) store depletion in animal models of heart failure. In these studies, administration of a Ca\(^{2+}\) ionophore increased permeability equally in control and heart failure lungs effectively ruling out endothelial Ca\(^{2+}\) desensitization. These data are in line with the lack of endothelial [Ca\(^{2+}\)], responsiveness demonstrated by real-time imaging in the present study. Our finding that the Ca\(^{2+}\) ionophore A-23187 restored NO production in CHF lungs stresses the functional relevance of this adaptation as the predominant cause of lung endothelial dysfunction in CHF and provides a mechanistic explanation for previously reported, yet so far inexplicable data that demonstrated an attenuated vasodilation of pulmonary arterial rings from CHF rats in response to Ach, although the relaxation response to A-23187 was preserved. Notably, a similar impairment of endothelial Ca\(^{2+}\) homeostasis, ie, reduced basal [Ca\(^{2+}\)], and store-operated Ca\(^{2+}\) entry, was recently reported in pulmonary endothelial cells from chronically hypoxic rats, suggesting that this mechanism of endothelial dysfunction may also be relevant to noncardiogenic forms of pulmonary hypertension.

Cytoskeletal Reorganization

We focused on adaptive changes of the endothelial actin cytoskeleton in CHF and their potential role in impaired endothelial [Ca\(^{2+}\)], signaling based on: (1) the consideration that F-actin constitutes the only Ca\(^{2+}\) binding system able to store considerable amounts of Ca\(^{2+}\) ions at physiological [Ca\(^{2+}\)], and store-operated and TRPC3 channels; and (3) the fact that cytoskeletal organization and actin expression in endothelial cells adapt to chronically altered mechanical stress, eg, in simulated microgravity.

Endothelial actin microfilaments were most prominent at vascular bifurcations in line with their ability to provide structural support at sites of mechanical stress. Surprisingly, endothelial F-actin filaments enfolded lung capillaries in circular and spiral arrangements which is in contrast to data from cultured pulmonary artery endothelial cells where microfilaments align parallel to flow. Although this arrangement is reminiscent of smooth muscle cells, a contamination of the phalloidin signal could be effectively excluded by the negative immunostaining for α-smooth muscle actin in lung capillaries. Importantly, a spiral arrangement may be advantageous in providing both structural stability and elasticity in an organ in which microvessels continuously undergo cyclic longitudinal stretch resulting from respiratory movements.

Phalloidin imaging and Western blot analyses from FLECs revealed an abundant increase in F-actin content and β-actin expression in endothelial cells of CHF lungs, and microfilament disruption by cytochalasin D largely reconstituted endothelial [Ca\(^{2+}\)], and NO responses. These findings demonstrate that lung endothelial cells adapt to CHF by cytoskeletal reorganization with profound implications on cell signaling and function. The molecular mechanism by which actin expression and arrangement regulate endothelial [Ca\(^{2+}\)], signaling and NO synthesis remains to be elucidated, but may involve one or more of the following scenarios. First, rapid Ca\(^{2+}\) binding by G-actin monomers and Ca\(^{2+}\) storage by F-actin polymers may act as physiological sink to buffer Ca\(^{2+}\) influx and release in endothelial cells that express more than 10-fold higher actin levels as normal. This hypothesis is in agreement with our finding of an attenuated [Ca\(^{2+}\)], response to thapsigargin in CHF despite a comparable Ca\(^{2+}\) release from endosomal stores, and with previous data demonstrating that cytochalasin D prolongs [Ca\(^{2+}\)], elevations in response to thapsigargin or Ca\(^{2+}\) ionophores in T-cells. Second, the identification of the Stim/Orai coupling machinery as molecular mechanism of store-operated Ca\(^{2+}\) entry raises the possibility that translocation of the endosomal Ca\(^{2+}\) sensor Stim1 to or interaction with pore-forming Orai proteins in the plasma membrane may be impaired by a dense cortical actin layer. Such a scenario could explain some aspects of impaired [Ca\(^{2+}\)], signaling in CHF, eg, the lack of a store-operated Ca\(^{2+}\) entry following histamine stimulation, but does not provide a mechanistic explanation for the absence of the initial histamine response or the attenuated [Ca\(^{2+}\)], response to thapsigargin. For similar reasons, internalization of TRPC Ca\(^{2+}\) channels by cytoskeletal reorganization, as recently described in neutrophils, may contribute to, but cannot solely explain the impaired [Ca\(^{2+}\)], signaling in CHF.

Although reconstituting the endothelial [Ca\(^{2+}\)], responses to hydrostatic stress or histamine, cytochalasin D failed to reset basal [Ca\(^{2+}\)], levels or to restore endothelial [Ca\(^{2+}\)], oscillations in CHF lungs. The fungal metabolite cytochalasin D inhibits actin polymerization and disrupts actin microfilaments, and was therefore used in our study to provide proof-of-principle for a functional role of cytoskeletal remodeling in impaired [Ca\(^{2+}\)], signaling and endothelial dysfunction in CHF. Yet, at the same time, this intervention can be considered too crude and generalized to restore all facets of the complex regulation of endothelial [Ca\(^{2+}\)], signaling and may simultaneously trigger secondary responses, which in turn affect endothelial [Ca\(^{2+}\)], homeostasis. Furthermore, additional, yet unidentified adaptive mechanisms can be expected to contribute to the unique lung endothelial phenotype in CHF.

In summary, we identify a fundamental impairment in endothelial [Ca\(^{2+}\)], homeostasis and signaling as novel mechanism underlying lung endothelial dysfunction in CHF. The functional relevance of this defect is illustrated by our finding that inhalation of exogenous NO decreased P\(_{PA}\) and thus mitigated pulmonary hypertension in CHF rats. This singular adaptation of endothelial cell responsiveness in CHF is primarily caused by a massive reorganization of the endothelial
lial cytoskeleton. Hence, actin microfilament expression and arrangement constitute a major regulator of endothelial cell signaling and function.

Acknowledgments
We thank Renate Noske-Reimers and Argid and Raimund Rutenberg for excellent technical assistance.

Sources of Funding
This study was supported by Deutsche Forschungsgemeinschaft (Graduate Program 865: Mechanisms of Vascular Regulation), the European Commission under the Sixth Framework Programme (contract no. LSHM-CT-2005-018725, PULMOTENSION), and the Kaiser-Friedrich Foundation Berlin.

Disclosures
None.

References
What Is Known?

- The majority of patients with congestive heart failure develop secondary pulmonary hypertension.
- Pulmonary hypertension owing to left heart disease is frequently aggravated by a reactive increase in pulmonary vascular resistance.
- Congestive heart failure causes lung endothelial dysfunction which may trigger this increase in lung vascular resistance, yet underlying mechanisms are unclear.

What New Information Does This Article Contribute?

- Endothelial-dependent vasodilation and NO synthesis are impaired in lungs of rats with congestive heart failure.
- Lung endothelial dysfunction in heart failure constitutes the functional consequence of a fundamental impairment in endothelial Ca\(^{2+}\) signaling.
- The impaired Ca\(^{2+}\) signaling is attributable to a massive remodeling of the endothelial cytoskeleton.

In heart failure, lung endothelial dysfunction has been proposed to contribute to secondary pulmonary hypertension, but its characteristics and mechanisms remain unclear. In rats with heart failure, we identified lung endothelial dysfunction and lack of endothelial NO synthesis. These effects resulted from impaired regulation of endothelial NO synthase by cytosolic Ca\(^{2+}\), because lung endothelial cells of heart failure rats showed a deficient Ca\(^{2+}\) response to appropriate stimulation. Impaired Ca\(^{2+}\) signaling was attributable to a massive upregulation and remodeling of the endothelial actin cytoskeleton. From our results, three key novel findings emerge. First, we substantiate the concept that congestive heart failure causes lung endothelial dysfunction, which aggravates pulmonary hypertension. Second, we identify impaired endothelial Ca\(^{2+}\) signaling as a novel mechanism of endothelial dysfunction in vivo. Third, we report that cytoskeletal remodeling constitutes a major regulator of cytosolic Ca\(^{2+}\) homeostasis and signaling. Taken together, these findings suggest that changes in the endothelial cytoskeleton contribute profoundly to cardiovascular disease. Further exploration and mechanistic analysis of this effect may drive the identification of new therapeutic targets in endothelial dysfunction. Meanwhile, preservation of endothelial function presents a promising strategy for the treatment of pulmonary hypertension secondary to left heart disease.
Lung Endothelial Dysfunction in Congestive Heart Failure: Role of Impaired Ca2+ Signaling and Cytoskeletal Reorganization
Alexander Kerem, Jun Yin, Stephanie M. Kaestle, Julia Hoffmann, Axel M. Schoene, Baljit Singh, Hermann Kuppe, Mathias M. Borst and Wolfgang M. Kuebler

Circ Res. 2010;106:1103-1116; originally published online February 18, 2010;
doi: 10.1161/CIRCRESAHA.109.210542

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2010 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/106/6/1103

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2010/02/18/CIRCRESAHA.109.210542.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/
Lung endothelial dysfunction in congestive heart failure: Role of impaired Ca\textsuperscript{2+} signaling and cytoskeletal reorganization

Alexander Kerem, Jun Yin, Stephanie M. Kaestle, Julia Hoffmann, A.M. Schoene, Baljit Singh, Hermann Kuppe, Mathias M. Borst, Wolfgang. M. Kuebler
Detailed Methods Section

**Animals.** Male Sprague-Dawley (SD) rats of 80-90 g body weight (bw) were obtained from local breeding facilities of the academical institution. All experiments were performed in accordance with the "Guide for the Care and Use of Laboratory Animals" (Institute of Laboratory Animal Resources, National Academy press, Washington D.C., 1996) and were approved by the local government authorities.

**Materials.** The NO-sensitive fluorophore 4-amino-5-methylamino-2'-7'-difluorofluorescein diacetate (DAF-FM DA), the acetoxymethyl esters of the Ca^{2+}-sensitive dye fura-2 and the endosomal Ca^{2+} dye fura-2FF, the F-actin dye Alexa 568 phalloidin, the Ca^{2+} ionophore 4-bromo A-23187, the endoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin, and the exogenous NO donor S-nitroso-N-acetylpenicillamine (SNAP) were obtained from Molecular Probes (Invitrogen GmbH, Karlsruhe, Germany). Human serum albumin (20%) was from Octa Pharma GmbH (Langenfeld, Germany), NO from Linde AG (Pullach, Germany), and pancreatic elastase type I from ICN Biomedicals (Cleveland, OH).

Anti-eNOS/NOS type III polyclonal antibody was purchased from Becton Dickinson GmbH (Heidelberg, Germany), monoclonal anti-β-actin antibody from Sigma Aldrich (Taufkirchen, Germany), and polyclonal antibodies against the transient receptor potential vanilloid (TRPV) channels TRPV2 and TRPV4 from Calbiochem (San Diego, CA) and Alomone Labs (Jerusalem, Israel), respectively. Antibodies directed against the transient receptor potential canonical (TRPC) channels TRPC1, TRPC4, and TRPC6 were from Abcam Inc. (Cambridge, MA), and those against iNOS/NOS type II, histamine H1 receptor, acetylcholine M3 receptor, and horseradish-peroxidase conjugated 2nd antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). FITC-conjugated mouse monoclonal antibody to α-smooth muscle actin, isotype-matched nonspecific control from murine myeloma, and all other probes and chemicals were purchased from Sigma-Aldrich.

**Supracoronary aortic banding.** CHF was induced by supracoronary aortic banding nine weeks prior to investigations as previously described. In brief, juvenile male SD rats (91±9 g bw) were anesthetized by intraperitoneal injection of ketamine (87 mg/kg; Pharmacia GmbH, Erlangen, Germany) and xylazine (13 mg/kg; Rompun, Bayer AG, Leverkusen, Germany). Following a left hemithoracotomy, the aorta was banded by supracoronary implantation of a titanium clip with an internal diameter of 0.8 mm (Weck Closure Systems; Research Triangle Park, NC). Wounds were surgically closed and post-operative analgesia was performed with 4 mg/kg Carprofen (Rimadyl®, Pfizer GmbH, Karlsruhe, Germany) given subcutaneously for 3 days. Sham-operated rats (without implantation of a clip) of similar body weight served as controls. Nine weeks after supracoronary aortic banding or sham operation at a mean body weight of 354±5 g, rats were utilized for experimental analyses.

**Isolated perfused lung preparation.** Experimental procedures have previously been described. In brief, lungs from anesthetized SD rats were continuously pump-perfused with autologous heparinized blood at 14 mL/min and 37°C. Lungs were constantly inflated with a gas mixture of 21% O_2, 74% N_2 and 5% CO_2 at a positive airway pressure of 5 cmH_2O. At baseline, left atrial pressure (P_LA) and P_PA were continuously recorded (DASYlab 32; Datalog GmbH, Moenchengladbach, Germany). Agents were infused into lung venular capillaries or small pulmonary arterioles, respectively, in 2% dextran (70 kDa) HEPES solution containing 150 mMol/L Na⁺, 5 mMol/L K⁺, 1.5 mMol/L Ca^{2+}, and 20 mMol/L HEPES (Serva, Heidelberg, Germany) at pH 7.4 and osmolarity of 295 mOsmol/L. For Ca^{2+}-free conditions, a nominally Ca^{2+}-free HEPES solution containing 2% dextran and EGTA (0.5 mMol/L) was infused.

**In situ fluorescence imaging.** Isolated perfused lungs were positioned under an upright fluorescence microscope (Axiotech Vari 100HD; Zeiss, Jena, Germany) on a custom-built stage and superfused with normal saline at 37°C. For dye loading to lung venular capillaries, a microcatheter (Ref. 800/110/100; SIMS Portex Ltd., Kent, UK) was advanced via the left atrium and wedged in a pulmonary vein draining a capillary area on the lung surface. For dye delivery to small pulmonary arterioles, the microcatheter was
advanced from the right ventricle over the bifurcation of the pulmonary trunk and wedged in an intrapulmonary artery. Via the microcatheter, the NO and Ca\(^{2+}\) sensitive fluorescence dyes DAF-FM DA, fura-2 AM, or fura-2FF AM (each 5 µMol/L) were infused for 20 min as previously described\(^3\). Fluorescence probes were excited by monochromatic illumination (Polychrome IV; T.I.L.L. Photonics, Martinsried, Germany) at 340, 360 and 380 nm for fura-2 and fura-2FF, 480 nm for DAF-FM and FITC, and 570 nm for Alexa 568 phalloidin. Fluorescence was collected through an approachmat objective (UAPO 40x W2/340; Olympus, Hamburg, Germany), dichroic (FT 425, 510, and 580; all Zeiss, Jena, Germany) and emission filters (BP 505-530, LP 515, and LP 590; all Zeiss) by a CCD camera (Senicam; PCO, Kehlheim, Germany), and subjected to digital image analysis (TILLvisION 4.01; T.I.L.L. Photonics). Single venular capillaries of 14-30 µm in diameter and small pulmonary arterioles of 25-50 µm in diameter were viewed at a focal plane corresponding to their maximum diameter.

NO concentration was determined by real-time imaging of endothelial DAF-FM fluorescence\(^4\). Weakly fluorescent DAF-FM is converted in an NO-dependent, irreversible reaction to an intensely fluorescent benzotriazole derivative with the fluorescence increase linearly reflecting NO concentration\(^3\). DAF-FM fluorescence intensity (F) was expressed relative to its individual baseline (F\(_0\)). Ca\(^{2+}\) concentration in cytosol ([Ca\(^{2+}\)]) and endosomal stores of lung endothelial cells was quantified by ratiometric imaging of fura-2 and fura-2FF, respectively\(^2\). The endothelial F-actin cytoskeleton was visualized by Alexa 568 phallolidin as previously described\(^3\). In brief, endothelial cells of lung venular capillaries or pulmonary arterioles, respectively, were fixed in situ by local infusion of 3.7% formaldehyde, permeabilized by 0.1% saponin, and stained for F-actin by 5 U/mL Alexa 568 phallolidin followed by a wash with buffer infusion. Control staining for smooth muscle actin in fixed and permeabilized lung microvessels was performed analogously by infusion of an FITC conjugated mouse monoclonal antibody to α-smooth muscle actin (10 µMol/L) or a respective isotype-matched nonspecific control antibody from murine myeloma (10 µMol/L) and subsequent wash.

**Endothelial dysfunction.** Endothelium-dependent and –independent vasodilation was determined in isolated perfused rat lungs as previously reported\(^6\). In brief, the pulmonary vasculature was preconstricted by infusion of the endoperoxide analog U46619 (9,11-Dideoxy-11α,9α-epoxymethanoprostaglandin F2α; 50 pMol/min). Endothelium-dependent and –independent vasodilation was determined as the pressor response to acetylcholine (10\(^{-8}\)-10\(^{-5}\) Mol/L) and sodium nitroprusside (10\(^{-9}\)-10\(^{-6}\) Mol/L), respectively, and expressed as reduction in PPA (ΔPPA) as compared to the respective vehicle control.

**Cardiopulmonary characterization.** Hearts from CHF and control animals were excised, and Fulton's index was calculated as ratio of right ventricular weight over left ventricle plus septal weight (RV/[LV+S]). For hemodynamic measurements, rats were anesthetized by urethane (1.5 g/kg bw ip; Fluka, Buchs, Switzerland) and ketamine (0.5 mg/kg bw im). Arterial pressure, pulmonary arterial pressure, left atrial pressure and aortic flow were determined via implanted catheters (Sims Portex, Portex Ltd, Hythe, UK) and an ultrasonic flowprobe (Transonic Systems, Ithaca, NY) placed around the ascending aorta distal to the branching of the coronary arteries as previously described\(^1,7\). Pulmonary vascular resistance (PVR) was calculated as arteriovenous pressure difference over flow. Left ventricular end-diastolic pressure was recorded by a 2.2 French catheter-tip transducer (Millar SPR249; Millar Instruments, Houston, TX) that was advanced via the right carotid artery. To measure pulmonary vasorelaxation in response to inhaled NO in CHF and control rats, NO gas was introduced into the inspiratory limb of the ventilation circuit as previously described to yield concentrations of 10–50 ppm\(^7\). Inspired NO concentrations were continuously monitored (Dräger Pac III; Dräger Safety AG & Co. KGaA, Lübeck, Germany), and pulmonary vasorelaxation was determined as dose-dependent reduction in PPA (ΔPPA). Static lung compliance was calculated by linear regression analysis of the airway pressure response to 1 ml incremental lung volume steps in the isolated lung preparation. Wet-to-dry lung weight ratio was determined by use of the microwave drying technique\(^1\), and the degree of alveolar edema was estimated from measurements of the lung epithelial lining fluid volume by a double indicator dilution technique as previously described\(^8\).
**Western blot analysis.** For protein analyses from whole lung tissue, aliquots of frozen rat lungs were powdered, lysed and homogenized in phosphate buffered saline (PBS) containing protease inhibitor mixture (Complete Mini, Roche Diagnostics GmbH, Mannheim, Germany), 1 mMol/L phenylmethansulfonylfluoride (PMSF), and 1% Triton-100. Samples were shock frozen in liquid nitrogen and thawed on ice for three times. Lysates were collected by pelleting the cellular debris for 15 min at 19,800 g. For protein analyses from fresh lung endothelial cells (FLEC), endothelial cells were separated by a magnetic bead immunosorting technique yielding an endothelial cell fraction of > 97% as determined by FACS analysis of CD31 positive cells and a cell viability of > 96% as assessed by trypan blue exclusion².

Total protein concentration was determined by the Bradford Protein Assay (Bio-Rad Protein Assay; Bio-Rad, Munich, Germany). Sample proteins (50 µg/slot homogenate and 30 µg/slot FLEC) and a pre-stained protein-weight marker (Bio-Rad) were size-fractionated by SDS polyacrylamide gel electrophoresis (10%) and transferred to a nitrocellulose membrane (Protran; Schleicher & Schuell, Dassel, Germany) with a Protean II wet blotter (Bio-Rad). Because classical housekeeping genes such as GAPDH (data not shown) or β-actin (fig. 6E) are markedly regulated in CHF lungs, equal protein loading of lanes was confirmed by Ponceau S staining (Sigma)⁹. Membranes were blocked at room temperature with 5% dry milk powder (Töpfer, Dietmannsried, Germany) in PBS containing 1% TWEEN (PBST), incubated with matching primary antibody (1:1000 in 5% dry milk powder PBST over night at 4°C) and washed three times with PBST (5 min each). Subsequently, NC membranes were incubated with horseradish peroxidase conjugated 2nd antibodies, washed 5 times, and protein bands were visualized by enhanced chemiluminescence (ECL; Perkin Elmer GmbH, Freiburg, Germany). Quantitative analysis of protein bands was performed with Image J (NIH, Bethesda, MD).

**Immunohistochemistry.** Immunohistochemical analyses were performed as previously described¹⁰. Briefly, rat lungs were fixed by tracheal instillation of 4% paraformaldehyde (PFA) at an airway pressure of 5 cmH₂O for 30 min. Tissue sections of 1 cm³ thickness were excised from surface and central lung areas and fixed for another 16 h in 4% PFA followed by three washes in phosphate-buffered saline (PBS). Dehydrated tissues were embedded in paraffin, and 5 µm thick sections were cut and mounted onto slides coated with 10% poly-L-lysin (Sigma Aldrich). Lung sections were deparaffinized and rehydrated prior to quenching of endogenous hydrogen peroxide. After treatment with pepsin and blocking with bovine serum albumin, sections were incubated with primary anti-eNOS antibody (1:400; Santa Cruz Biotechnology, Inc.) for one hour. Tissues were exposed to appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (1:150; Dako, Glostrup, Denmark) followed by color development. Appropriate controls including omission of primary antibody, staining for α-smooth muscle actin or von Willebrand factor were performed (data not shown).

**Statistical analysis.** Data are given as mean ± SEM. Values of several groups were compared by Wilcoxon and Friedman tests for dependent groups and by Kruskal-Wallis and Mann-Whitney U-tests for independent groups (SigmaStat 3.10; Systat Software Inc., Erkrath, Germany). Non-linear regression analysis, four parameter logistic curve fitting and calculation of half maximal effective concentration (EC₅₀) was performed using SigmaPlot software (Version 9.0, Systat Software Inc.). Statistical significance was assumed at P< 0.05.
**Supplemental references**


