Simvastatin Treatment Attenuates Increased Respiratory Variability and Apnea/Hypopnea Index in Rats With Chronic Heart Failure

Karla K.V. Haack,* Noah J. Marcus,* Rodrigo Del Rio,* Irving H. Zucker, Harold D. Schultz

See Editorial Commentary, pp 909–910

Abstract—Cheyne–Stokes respiration and cardiac arrhythmias are associated with increased morbidity and mortality in patients with chronic heart failure (CHF). Enhanced carotid body chemoreflex (CBC) sensitivity is associated with these abnormalities in CHF. Reduced carotid body (CB) nitric oxide and nitric oxide synthase (NOS) levels play an important role in the enhanced CBC. In other disease models, Simvastatin (statin) treatment increases endothelial NOS, in part, by increasing Krüppel-like Factor 2 expression. We hypothesized that statin treatment would ameliorate enhanced CBC sensitivity as well as increased respiratory variability, apnea/hypopnea index, and arrhythmia index, in a rodent model of CHF. Resting breathing pattern, cardiac rhythm, and the ventilatory and CB chemoreceptor afferent responses to hypoxia were assessed in rats with CHF induced by coronary ligation. CHF was associated with enhanced ventilatory and CB afferent responses to hypoxia as well as increased respiratory variability, apnea/hypopnea index, and arrhythmia index. Statin treatment prevented the increases in CBC sensitivity and the concomitant increases in respiratory variability, apnea/hypopnea index, and arrhythmia index. Krüppel-like Factor 2 and endothelial NOS protein were decreased in the CB and nucleus tractus solitarii of CHF animals, and statin treatment increased the expression of these proteins. Our findings demonstrate that the increased CBC sensitivity, respiratory instability, and cardiac arrhythmias observed in CHF are ameliorated by statin treatment and suggest that statins may be an effective treatment for Cheyne–Stokes respiration and arrhythmias in patient populations with high chemoreflex sensitivity. (Hypertension. 2014;63:1041-1049.) • Online Data Supplement

Key Word: carotid body ■ Cheyne–Stokes Respiration ■ simvastatin ■ systolic heart failure

Patients with chronic heart failure (CHF) often exhibit Cheyne–Stokes respiration (CSR), a form of episodic breathing during sleep or wakefulness characterized by recurrent central apneas and a crescendo-decrescendo pattern of tidal volume. These individuals have a higher mortality rate, which may result from apnea-related hypoxemia and arrhythmias precipitated by CSR or apneic episodes. Increased arrhythmia incidence may be driven by higher tonic levels of sympathetic nervous system activity (SNA) as well as surges in SNA associated with CSR.

Overactivation of the sympathetic nervous system is a major contributor to the progression of CHF in both humans and animal models. Increased afferent input from the carotid body (CB) chemoreceptors enhances peripheral chemoreflex function, which reflexively contributes further to sympathetic activation. Oscillatory breathing patterns observed in CHF may contribute directly to tonic and episodic elevations in SNA by their effect on the CB chemoreflex (CBC). Furthermore, alterations in afferent input from the CBC to cardiovascular control centers of the brain, such as the nucleus tractus solitarii (NTS), alter central neural function as well, which may also contribute to cardiovascular and cardiorespiratory perturbations in CHF.

We have identified several mechanisms underlying this exacerbation in CBC sensitivity that occurs in CHF. Decreased neuronal nitric oxide synthase-nitric oxide (nNOS-NO), decreased antioxidant enzyme expression, and increased CB angiotensin II and an upregulation of the angiotensin II type 1 receptor (AT1R) in the CB all contribute to the enhanced CBC sensitivity in CHF. In a disease model that mimics the hypoxia associated with recurrent central apneas that often occur in CHF, inflammation has been identified as a potential contributor to enhanced CBC sensitivity as well.

HMG Co-A (3-hydroxy-3-methylglutaryl-coenzyme A) reductase inhibitors (statins) are a class of cholesterol-lowering...
drugs that are beneficial in the prevention of coronary heart disease. Statins have several pleiotropic effects and have been shown to decrease sympathetic tone in both CHF animals and human CHF patients. Statin treatment increases endothelial NOS (eNOS) expression via the mechanosensitive transcription factor Krüppel-like Factor 2 (KLF2) in the endothelium. KLF2 is also a potent inhibitor of many proinflammatory signals; KLF2 activation can inhibit nuclear factor-κB and activator protein 1, 2 transcription factors involved in pro-inflammatory responses and upregulation of AT1R.

The first goal of this study was to determine whether statin treatment can reduce exaggerated CBC sensitivity and disorders breathing patterns in a rat model of CHF. In addition, considering the relationship between arrhythmia incidence and CSR in clinical populations, we sought to determine a potential salutary effect of statin treatment on the incidence of arrhythmias in CHF. Because a hallmark of CHF is a decrease in central and peripheral NOS (ostensibly via KLF2) and a concomitant increase in central and peripheral AT1R, a second goal of this study was to examine the expression of KLF2, eNOS, and AT1R both in the CB and in its primary central relay center, the NTS. We hypothesized that statin treatment would normalize protein expression of KLF2, eNOS, and AT1R in the CB and NTS.

Methods

More details of the methods are provided in the online-only Data Supplement.

Animals

Twenty-two male Sprague-Dawley rats weighing 220 to 280 g (Sasco Breeding Laboratories, Omaha, NE) were fed and housed according to institutional guidelines. Protocols were approved by the University of Nebraska Institutional Animal Care and Use Committee and were in accordance with the American Physiological Society’s Guiding Principles in the Care and Use of Laboratory Animals. Rats were given rat chow (Teklad, Houston, TX) and water ad libitum and were housed in a room with a 12-hour light–dark cycle. Rats were allowed to acclimatize for ≥1 week before cardiac surgery. There were a total of 4 to 6 animals per sham and CHF group. Details of each surgical procedure can be found in the online-only Data Supplement.

Induction of Heart Failure

Rats were assigned randomly to either a sham-operated control group or a CHF group. CHF was induced by ligation of the left coronary artery as has been described previously.

Radiotelemetry Implantation

After confirmation of CHF, a subset of animals was implanted with a Data Sciences International radiotelemetry pressure transducer (model # TA11PA-C40) in the femoral artery with the tip of the catheter advanced into the abdominal aorta. Recordings were taken while animals were resting quietly in their home cages or in the Plexiglas chamber during resting breathing measurements.

Simvastatin Administration

Sham and CHF rats were assigned randomly to either statin or vehicle treatment 6 weeks after coronary artery ligation surgery and confirmation of ejection fraction by echocardiography. Simvastatin (5 mg/kg as used in other studies, graciously donated from Merck Laboratories) was mixed into one eighth of a teaspoon of peanut butter, and rats were allowed to consume the statin plus peanut butter (vehicle). All rats consumed all the peanut butter/statin within 1 to 2 minutes of presentation. Rats were fed either statin or vehicle for 14 days before final echocardiography and experimentation.

Resting Breathing and Chemoreflex Evaluation With Minute Ventilation (V̇E)

To assess resting breathing pattern and its relationship to CBC sensitivity, resting breathing and changes in ventilation in response to hypoxia and hypercapnia were measured in sham-vehicle, sham-statin, CHF-vehicle, and CHF-statin animals in the conscious resting state and compared cross-sectionally.

Carotid Sinus Nerve Recording

Before termination, some animals underwent carotid sinus nerve recording as previously described.

Determination of Arrhythmia Incidence

The arterial pressure trace was used to evaluate the incidence of arrhythmias during the resting baseline period before chemoreflex testing. Using peak recognition software (Labchart, ADI instruments, Australia), the mean beat-to-beat interval was calculated during the 2-hour baseline period. Premature beats or delayed beats were tallied if the beat-to-beat interval exceeded 3 SDs above or below the mean. All events meeting the stated criteria were tallied and combined to derive a single index.

Micropunch of the NTS and Isolation of Carotid Bifurcation Protein for Western Blot Measurements

Micropunches and isolation of CB tissue were performed as described previously.

Western Blot Measurement of AT1R, eNOS, and KLF2 Proteins

Western blots were performed as described previously. Additional details can be found in the online-only Data Supplement.

CB Immunofluorescence and Chemoreceptor Cell Immunocytochemistry

To confirm and localize the protein expression seen in the carotid bifurcation experiments, we isolated the carotid bodies and performed immunostaining as previously described.

Statistical Analysis

Data are presented as mean±SE. The data were subjected to 1-way ANOVA followed by comparison for individual group differences using the Newman–Keuls test or Bonferroni correction. Statistical significance is indicated by a value of P<0.05.

Results

Cardiac Function in CHF-Vehicle, CHF-Statin, Sham-Vehicle, and Sham-Statin Rats

Table S1 in the online-only Data Supplement summarizes the echocardiographic measurements, heart rate, and body weights of all 4 groups of animals. Briefly, both ejection fraction and fractional shortening were similar between the CHF-vehicle and CHF-statin groups before and after statin treatment and significantly lower compared with sham groups. Statin treatment did not improve hemodynamic parameters in the CHF animals.

Effects of Statin Treatment on Respiratory Variability and Apnea/Hypopnea Index

Representative plethysmographic records (Figure 1A) illustrate the abnormal breathing patterns observed in CHF animals and...
the effect of statin to prevent these changes in CHF animals. Increases in respiratory variability associated with CHF are illustrated in the Poincare plots shown in Figure 1B. Short-term (SD1) and long-term (SD2) breath interval variability (Figure 1C) show that statin treatment prevented the increases in respiratory variability associated with CHF. CHF animals had a significantly higher apnea/hypopnea index compared with sham groups, and this effect was blunted by statin treatment (Figure 1A and 1D). Apneas immediately after sighs were excluded from the apnea/hypopnea index. There was no difference in the incidence of post-sigh apneas in any of the groups (13±1 sham vehicle; 10±2 CHF vehicle; 10±2 CHF statin; and 11±2 sham vehicle). Taken together, these data indicate that statin treatment normalized the abnormal respiratory patterns seen in the CHF state.

Effect of Statin Treatment on Arrhythmia Incidence
Because there is a correlation between heightened sympathetic activation, disordered breathing patterns, and increased incidence of ventricular arrhythmias, we also assessed the effect of statin treatment on arrhythmia incidence. There were no sustained tachy/brady-arrhythmias identified in our tracings, and qualitatively the increases in arrhythmias we observed in CHF were ectopic beats. Tachograms are shown in Figure 2A, which illustrate a greater number of arrhythmias in CHF-vehicle animals relative to sham-vehicle animals (Figure 2C). Statin treatment significantly reduced the number of arrhythmias per hour in CHF rats. The use of the arterial pressure waveform to identify arrhythmias limited our ability to detect specific types of alterations in heart rhythm; however, our methods did allow us to reliably detect a relative increase or decrease in arrhythmia incidence.

Effect of Statin Treatment on Chemoreflex Control of Ventilation
Representative tracings in Figure 3A and summary data in Figure 3B show that the reflex ventilatory response to isocapnic hypoxia was greater in CHF-vehicle animals compared to sham vehicle. Statin treatment completely normalized the exaggerated response to hypoxia in CHF animals. Chemoreflex ventilatory responses to CO₂ under either
hyperoxic or normoxic conditions were unaffected by CHF or statin treatment (data not shown).

Effect of Statin Treatment on CB Chemoreflex Sensitivity

Given that statin treatment restored resting breathing variability and normalized the exaggerated CBC ventilatory responses to hypoxia seen in CHF, we next determined whether these effects correlated with a change in CB afferent nerve discharge in response to hypoxia. As expected, at maximal oxygen levels (100%), CB afferent discharge was quiescent across all groups. However, at the lower ranges of FiO$_2$ (5%–15%), CB chemosensory activity was significantly increased in CHF-vehicle animals compared with sham vehicle (Figure 4). The CB afferent response to acute hypoxia in the CHF-statin group was significantly reduced compared with the CHF-vehicle group ($P<0.05$). Indeed, statin treatment normalized CB afferent discharge in CHF rats to levels observed in sham animals.

Effect of Statin on KLF2, eNOS, and AT1 Receptor Expression

In both the NTS and the CB, KLF2 and eNOS protein were significantly decreased in the CHF vehicle animals compared with sham-vehicle animals (Figure 5A and 5B). Statin treatment increased both KLF2 and eNOS to sham levels. Because the AT$_1$R can also contribute to the enhanced CB sensitivity
AT1R was upregulated in the CB and NTS of CHF-vehicle and NTS during CHF and after statin treatment. As expected, the molecular mechanisms responsible for enhanced CBC sensitivity in CHF are not completely understood, but previous work from our laboratory has clearly shown that decreasing carotid blood flow to the extent observed in CHF increases hypoxia-evoked CB discharge, increases angiotensin II and AT1R expression, and reduces eNOS expression in the CB. These findings indicate that a blood flow–dependent mechanism contributes to enhanced CBC sensitivity in CHF and provide a potential link between KLF2 and CBC sensitivity in CHF. Because KLF2 is required for statin-dependent increases in eNOS in the endothelium, it is likely that the salutary effect of statin treatment on CBC sensitivity results, in part, from KLF2/eNOS-dependent mechanisms.

The statin-mediated increase in KLF2 can also normalize the expression of several proinflammatory targets, such as E-selectin and vascular cell adhesion molecule 1, as well as indirect inhibition of proinflammatory cytokines like nuclear factor-κB and activator protein 1, which have been implicated in the hypoxic activation of the AT1R pathway in CHF. In addition to the above, KLF2 has been shown to inhibit the proinflammatory transcription factor HIF1α (hypoxia inducible factor 1-alpha) in the setting of hypoxia; thus, the statin-mediated upregulation of KLF2 could improve chemoreflex function via a variety of mechanisms.

Our study also has revealed that KLF2 expression is not limited to endothelial cells but is also expressed in CB glomus cells, and is suppressed in CHF. Statin treatment was effective in normalizing expression of KLF2 in glomus cells. To our knowledge, this is the first demonstration that KLF2 is localized to the glomus cells of the CB. The mechanisms responsible for regulating KLF2 expression and its function in CB glomus cells, or neurons in general, are not known. It will be important to further assess the role of KLF2 within glomus cells on CB excitability in normal, CHF, and other states.

Mechanisms of Statin-Mediated Improvement in CBC Sensitivity in CHF

We have shown that increased angiotensin II and reactive oxygen species and downregulation of NOS and superoxide dismutase enzymes contribute to enhanced CBC in CHF. Interventions that upregulate eNOS or superoxide dismutase or that block the renin-angiotensin system in the CB reduce chemoreflex sensitivity in CHF. Statin therapy has been shown to reduce sympathetic outflow and inflammatory mediators, and oxidative stress. Our results indicate that statins reduce enhanced CBC sensitivity in CHF likely by multiple KLF2-related mechanisms (increased eNOS, decreased renin-angiotensin system, decreased oxidative stress, and decreased inflammation). This improvement in CBC sensitivity is beneficial in normalizing disordered breathing patterns, sympato-excitation, and increased arrhythmia incidence, which are common in the CHF state.

There are still several unknown mechanisms by which statins can exert their pleiotropic effects. The Western blot data strongly suggest that KLF2 and eNOS protein are
increased after statin therapy. In addition, the precise molecular mechanism responsible for the upregulation in KLF2 and eNOS are not completely clear. For KLF2 to increase eNOS, it is suggested that inhibition of small GTP-binding proteins, such as rat sarcoma homolog gene family, member A (RhoA), is required.20 Future studies will examine the link between the RhoA/RhoA-associated kinase II (ROCKII) pathway and KLF2 more directly.

Role of Statins in Reducing Arrhythmia Incidence in CHF

Previous reports indicate that statin therapy reduces arrhythmia incidence and mortality in patients with heart failure46; however, these studies did not determine the impact that statin treatment may have had on CSR. Our studies show for the first time that statin treatment concomitantly improves disordered breathing patterns and decreases arrhythmia incidence, The mechanisms by which statins reduce arrhythmia incidence are unclear; however, reductions in disordered breathing patterns may contribute to this effect. In support of this idea, evidence indicates that arrhythmia incidence is higher during episodes of CSR,4 and reduction of apnea incidence with either continuous positive airway pressure or adaptive servo ventilation results in reductions in premature ventricular contraction frequency as well as measures of sympathetic activation.47–49 Our study did not specifically address the effect of statin therapy on SNA; however, previous studies have shown that statin therapy reduces SNA as well.18

Clinical Implications

Patients with severe CHF commonly experience disordered breathing patterns, which may be characterized by both obstructive and central apneas.1–3,50 Disordered breathing patterns are correlated with increases in arrhythmia incidence, and morbidity and mortality in CHF, and contribute to the progression of the disease.51 Continuous positive airway pressure and adaptive servo-ventilation are accepted therapeutic treatments for sleep disordered breathing; however, because of their cumbersome

Figure 5. Simvastatin normalized Krüppel-like Factor 2 (KLF2), endothelial nitric oxide synthase (eNOS), and angiotensin II type 1 receptor (AT1R) protein expression in the carotid body (CB) and nucleus tractus solitarii (NTS) in chronic heart failure (CHF). Representative Western blots (upper) and mean data (lower) showing (A) KLF2, (B) eNOS, and (C) AT1R expression in sham, CHF, and statin-treated groups. *P<0.05 vs sham vehicle, #P<0.05 vs CHF statin, n=4 to 6 rats in each group.
nature, compliance is not optimal. Our results suggest that statins have the potential to be used in tandem with continuous positive airway pressure and adaptive servo-ventilation to improve the efficacy of treatment for sleep disordered breathing. Although statin therapy has not been shown to increase survival of patients with CHF (Controlled Rosuvastatin Multinational Trial in Heart Failure [CORONA] Trial), they have been shown to decrease cardiovascular events and improve quality of life, especially in the elderly population.

In the current study, statin treatment had no effect on hemodynamic parameters, despite one previous study from our group showing that statin therapy can significantly increase ejection fraction. This may be because of differences in the model of CHF used (the previous study was performed in a rapid ventricular pacing model, whereas the current study in a coronary artery ligation rat model). In addition, the duration of treatment in the present study (2 weeks) may not have been sufficient to affect cardiac function. However, other studies in both animal models and humans have shown that statin treatment had no effect on hemodynamic parameters. Despite a lack of improvement in hemodynamic parameters, we posit that the beneficial pleiotropic physiological effects of statins to abate arrhythmias and disordered breathing in CHF rats may be from the increased expression of KLF2 and eNOS centrally and peripherally.

In the current study, the dose of statin used was higher than that typically given to humans; however, the metabolism and dosage of any drug between species is not a linear relationship. Previous studies have used a wide range of statin doses in rodent models (1–50 mg/kg) to assess various effects, and we therefore chose a median dose of 5 mg/kg in our study. We have demonstrated previously that even at low doses (0.5 mg/kg in a rapid ventricular pacing model of CHF in the rabbit), simvastatin still improved autonomic imbalance. Future studies will examine whether statin doses <5 mg/kg in a rodent model can also improve arrhythmia incidence and disordered breathing patterns.

The antiarrhythmic effects of statins in human subjects with coronary artery disease are a bit more controversial, with the protective effects of statins being largely dependent on both the type of arrhythmia seen and the severity of the coronary artery disease. However, the overall data suggest that in most cases, statins are thought to be antiarrhythmogenic. In concordance with these findings, we have shown that statin treatment is an effective means for reducing the incidence of arrhythmias in CHF. Furthermore, our recent study in patients with nonischemic CHF suggested that simvastatin reduces sympathetic nerve activity and oxidative stress, which may explain in part the reduction in arrhythmia incidence. Although most patient studies have shown a concomitant reduction in plasma cholesterol, animal studies suggest that the pleiotropic effects of statins are not mediated by their effect on plasma lipids.

Perspectives
In a rat model of CHF, oral simvastatin treatment improved incidence of arrhythmias, CB chemoreflex sensitivity, and apnea/hypopnea index. This corresponded to an increase in KLF2 and eNOS expression and a decrease in AT1R expression in both the CB and the NTS. These data suggest that oral simvastatin treatment may be an effective therapeutic strategy in patients with high chemoreflex sensitivity.

Acknowledgments
We thank Johnnie F. Hackley and Pamela L. Curry for their technical assistance.

Sources of Funding
This research was supported by National Heart, Lung, and Blood Institute grants P01-HL62222 (I.H. Zucker and H.D. Schultz), HL038690 (I.H. Zucker), F32 HL116172 (K.K.V. Haack), and F32 HL108592 (N.J. Marcus).

Disclosures
None.

References

Figure 6. Immunolocalization of Krüppel-like Factor 2 (KLF2) in the carotid body (CB) of chronic heart failure (CHF) rats. Representative image from a CB from a sham-vehicle rat, a CHF-vehicle, and a CHF-statin rat. KLF2 staining was localized to tyrosine hydroxylase positive glomus cells (arrow) and blood vessels (asterisk). Apparent decreases in KLF2 immunostaining were evident in the CBs from CHF rats. Note that CHF rats treated with statin displayed an increased KLF2 staining in the CB. Scale bar, 100 μm. Negative control was performed by omission of the primary antibody.


Haack et al Statins Normalize Respiratory Variability in CHF


**Novelty and Significance**

**What Is New?**

- Oral statin treatment normalizes arrhythmias, enhanced carotid body chemoreflex sensitivity, and respiratory instability observed in chronic heart failure.

**What Is Relevant?**

- Statin therapy may be an effective treatment for Cheyne–Stokes respiration in patient populations with high chemoreflex sensitivity, including patients with hypertension and chronic heart failure.

**Summary**

In a rat model of chronic heart failure, oral simvastatin treatment improved carotid body chemoreflex sensitivity, apnea/hypopnea index, and incidence of arrhythmias. This corresponded to an increase in Krüppel-like Factor 2 and endothelial nitric oxide synthase expression and a decrease in angiotensin II type 1 receptor expression in both the carotid body and the nucleus tractus solitarii.
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Hypertension. 2014;63:1041-1049; originally published online February 10, 2014;
doi: 10.1161/HYPERTENSIONAHA.113.02535

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/63/5/1041

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SIMVASTATIN TREATMENT ATTENUATES INCREASED RESPIRATORY VARIABILITY AND APNEA/HYPOPNEA INDEX IN RATS WITH CHRONIC HEART FAILURE

SUPPLEMENTAL FILE


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Running title: Statins normalize respiratory variability in CHF

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Methods

**Induction of Heart Failure.** Briefly, all rats were first anesthetized with isoflurane (0.5-2% in oxygen), intubated and mechanically ventilated. Under sterile conditions, a left thoracotomy was performed through the fifth intercostal space. The pericardium was then opened and the heart was exteriorized. Using 6-0 prolene suture, the left anterior descending coronary artery was ligated by passing through the superficial layers of myocardium between the pulmonary artery outflow tract and left atrium. Following ligation, the heart was replaced in its original position. Sham-operated rats were prepared in an identical manner described below but did not undergo coronary artery ligation or were considered sham if EF% was greater than 50%. Following closure of the thorax, any air within the cavity was evacuated. Rats were allowed to resume spontaneous respiration and recover from anesthesia. All animals were given postsurgical analgesia (Buprenorphine, Reckitte Benckiser, Hull, UK; 0.1 mg/kg, sc). Six weeks following surgery, left ventricular function was assessed using anatomic and hemodynamic criteria. Echocardiograms were performed (Vevo 770; Visualsonics, Inc., Toronto, Canada) before and after SIM administration. Rats with ejection fraction (EF) as determined by echocardiogram of less than 45% were considered to be in CHF. In addition, clinical signs of CHF including pulmonary congestion, ascites, and pleural fluid were noted at the time of euthanasia.

**Resting Breathing and Chemoreflex evaluation with minute ventilation (VE).** Rats were placed in a Plexiglas chamber (volume 1 liter) and the chamber was sealed, except for an inlet and outlet port that allowed a continuous flow of air through the chamber and an additional port for gas sampling. Movement artifacts and sniffing were excluded from analyses. Resting breathing pattern was quantified in terms of respiratory variability (RV) and apnea/hypopnea incidence. To illustrate and quantify RV we used Poincare plots and analysis of short and long term variability of interbreath intervals (SD1 and SD2) as previously described\(^1,2\). To quantify apnea/hypopnea incidence we calculated an apnea/hypopnea index (AHI) to indicate the number of these events occurring per hour. An event was tallied if there was a 50% or greater reduction in tidal volume (Vt) for a duration of three or more respiratory cycles. Apneas occurring immediately after a sigh were tallied separately. RV was calculated over a period of 300 consecutive breaths in which no sighs or apneas were noted. AHI was calculated over the course of the two hour resting breathing period.

Changes in ventilation in response to chemoreceptor stimulation were measured by unrestrained plethysmography and quantified as minute ventilation (VE), which was defined as tidal volume (Vt) \(\times\) respiratory rate (RR). Vt was measured by temporarily (30 s) sealing the air ports and measuring the pressure changes in the sealed chamber using an MP45 differential pressure transducer and amplifier (Validyne Engineering Corp., Northridge, CA, USA) connected to a PowerLab data acquisition system. The chamber was sealed only for short intervals (30 s) to prevent appreciable changes in the composition of air in it.
Peripheral chemoreceptors were stimulated preferentially by allowing the rats to breathe graded mixtures of hypoxic gas under poikilocapnic conditions. Different concentrations of O₂ with balance of N₂ were delivered into the chamber in the following sequence: 21% O₂ (normoxia), 15% O₂ (mild hypoxia), and 10% O₂ (moderate hypoxia). Additionally we tested the Vₑ response to the following levels of CO₂ (balance air): 5% CO₂ (mild hypercapnia), 7% CO₂ (moderate hypercapnia). Each stimulation was held for 2.5 minute until a steady response was achieved. Ten minutes of recovery time at 21% O₂ was allowed between stimuli to ensure that all variables returned to baseline levels. All resting breathing and chemoreflex measurements were made between 0900-1400 hours when the animals are normally inactive.

**Carotid Sinus Nerve Recording.** Briefly, rats were anesthetized with urethane (0.75 g kg⁻¹ IP) and α-chloralose (70 mg kg⁻¹, IP) and were placed in supine position and the body temperature was maintained at 38.0±0.5 °C with a heating pad. Additional doses were used when necessary to maintain a level of surgical anesthesia. The trachea was cannulated and the rat mechanically ventilated (Kent Scientific, USA). One carotid sinus nerve was dissected and placed on a pair of platinum electrodes, and covered with warm mineral oil. The neural signal was pre-amplified (Grass P511, USA), filtered (30 Hz–1 kHz) and fed to an electronic spike-amplitude discriminator, allowing the selection of action potentials of given amplitude above the noise to be counted with a frequency meter to measure the frequency of CB chemosensory discharge (ḟcsn), expressed in Hz (LabChart, AD Instruments). Carotid sinus barosensory fibers were eliminated by crushing the common carotid artery wall between the carotid sinus and the CB. The contra-lateral carotid sinus nerve was cut to prevent vascular and ventilatory reflexes evoked by the activation of the CB. The chemosensory discharge was measured at several FiO₂ (10-100%).

**Carotid Body Immunofluorescence and chemoreceptor cell immunocytochemistry**

Anesthetized rats were perfused intracardially with PBS (pH 7.4) for 10 min, followed by buffered paraformaldehyde (PFA 4%, Sigma) for 10 min. The carotid artery bifurcations including the CBs were harvested from the rats and post fixed by immersion in buffered-PFA 4% for 12 h at 4°C followed by three 15 min washes in PBS, sucrose gradient (5%, 10%, 20% in PBS), and then embedded in OCT. Cryostat sections (10 μm) of the CB were obtained and mounted on Superfrost Plus slides (Thermo Fisher Scientific). Sections were blocked/permeabilized in 0.5% Triton X-100, 2% fish skin gelatin (Sigma-Aldrich), 1%BSA in PBS for 1 h at RT. Sections were incubated overnight at RT with a mixture of an goat anti-KLF2 polyclonal antibody (1:100 in the same blocking media, Novus Biological) and an mouse anti-TH monoclonal antibody (1:250 in the same blocking media, Millipore) the latter used as a positive control for CB chemoreceptor cells recognition. After being washed with PBS, tissue sections were incubated for 1 h with a mixture of Alexa-Fluor 488 rabbit anti goat IgG (1:200, Molecular Probes) and Alexa-Fluor 546 rabbit anti-mouse IgG (1:200, Molecular Probes). Finally, sections were mounted in DAPI-containing media (Vectashield, Vector Laboratory) and visualized using a confocal laser microscope (Leica).

In another set of experiments, immunocytochemical detection of KLF2 in CB chemoreceptor cells was performed. Isolated glomus cells were plated onto poly-l-
lysine (0.1 mg/mL) coated 10 mm glass coverslips and fixed in 4% PFA for 10 min. Coverslips were then incubated for 12 h at 4 °C with a mixture of anti-KLF2 (1:100, Novus Biological) and anti-TH (1:250, Millipore). After washing with cold (4 °C) PBS, cells were sequentially incubated with a mixture of fluorescent conjugated secondary antibodies (Alexa-Fluor 488 rabbit anti goat IgG 1:200 and Alexa-Fluor 546 rabbit anti-mouse IgG (1:200) and mounted in DAPI-containing media (Vectashield, Vector Laboratory) and visualized using a confocal laser microscope (Leica).

Micropunch of the NTS and isolation of carotid bifurcation protein for Western blot measurements. After euthanizing the rats, brains were removed and quickly frozen on dry ice. 100 µm coronal sections were cut through the medulla at the level of the NTS using a cryostat. According to the technique of Palkovits and Brownstein, NTS nuclei were bilaterally punched using a diethylpyrocarbonate (DEPC)-treated blunt 18-gauge needle attached to a syringe. Tissue from the carotid bifurcation was removed and snap-frozen on dry ice and stored at -80°C. All tissue was lysed in 200 µL of RIPA buffer with fresh protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO) and total protein concentration was assessed using a Thermo Scientific BCA Assay kit (Waltham, MA) prior to Western blot analysis.

Western blot measurement of AT1R, eNOS and KLF2 proteins. Samples were adjusted to contain equivalent concentrations of total protein, and an equal volume of 2 X 4% SDS sample buffer was added to each sample. All samples were boiled for 3 min and then loaded onto a 7.5% SDS-PAGE gel (50 µg/20 µl per well). Gels were subjected to electrophoresis at 115 V/gel for 75-80 min. The fractionated proteins were electrophoretically transferred to a PVDF membrane (Millipore, Billerica, MA) at 50 V for 90 min. The membrane was probed with any of the primary antibodies overnight: rabbit anti-KLF2 (1:750, Novus Biological), goat or rabbit anti-AT1R, rabbit anti-eNOS, and/or mouse anti-GAPDH (1:500-1:1,000, Santa Cruz). Following washes with PBST, the appropriate secondary antibodies (Li-Cor Biosciences, Lincoln, NE) were added to each membrane. Blots were developed using a Li-Cor Odyssey scanner and quantitative analysis of band densitometry was performed using the Li-Cor Odyssey software. The relative amount of protein of interest was calculated as the ratio of intensity of the band relative to the intensity of GAPDH. Graphs summarizing individual experiments are shown as a fold change compared to the sham vehicle animals.
References


**Supplemental Table S1. Echocardiographic measurements, heart rate and body weight**

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<td>LVDd (mm)</td>
<td>7.4 ± 0.2</td>
<td>7.8 ± 0.1</td>
<td>9.1 ± 0.3*</td>
<td>9.5 ± 0.3*</td>
</tr>
<tr>
<td>LVDs (mm)</td>
<td>4.1 ± 0.1</td>
<td>4.5 ± 0.1</td>
<td>7.1 ± 0.3*</td>
<td>7.4 ± 0.2*</td>
</tr>
<tr>
<td>LVd Vol (µL)</td>
<td>303.2 ± 16.3</td>
<td>316.1 ± 12.6</td>
<td>483.8 ± 41.0</td>
<td>532.6 ± 32.8*</td>
</tr>
<tr>
<td>LVs Vol (µL)</td>
<td>80.5 ± 7.5</td>
<td>91.8 ± 5.4</td>
<td>268.5 ± 25.1*</td>
<td>307.3 ± 21.9*</td>
</tr>
<tr>
<td>EF%</td>
<td>73.6 ± 1.3</td>
<td>71.3 ± 1.3</td>
<td>42.6 ± 1.2*</td>
<td>42.8 ± 1.01*</td>
</tr>
<tr>
<td>FS%</td>
<td>44.6 ± 0.9</td>
<td>42.3 ± 1.1</td>
<td>22.1 ± 0.7*</td>
<td>22.3 ± 0.6*</td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>434.9 ± 7.6</td>
<td>433.1 ± 8.5</td>
<td>433.1 ± 9.8</td>
<td>428.1 ± 6.6</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>350.1 ± 5.9</td>
<td>371.0 ± 8.0</td>
<td>350 ± 6.0</td>
<td>343.6 ± 6.1</td>
</tr>
</tbody>
</table>

*p < 0.001 compared to sham vehicle. EF (ejection fraction); FS (fractional shortening); LVDd (left ventricular diameter at end diastole); LVDs (left ventricular diameter at end systole); LVd (left ventricular volume at end diastole); LVs (left ventricular volume at end systole).
Supplemental Figure S2. KLF2 is constitutively expressed in CB glomus cells. (A) Immunocytochemical detection of KLF2 in isolated CB glomus cells from one Sham vehicle rat, one CHF vehicle rat and one CHF statin rat. Double-labeling of immunofluorescence for KLF2 (green) and the chemoreceptor glomus cell marker TH (red), showing co-localization of the markers. Scale bar = 25 μm. (B) KLF2 fluorescence intensity integrated from the same images displayed in (A). Note that CHF glomus cells displayed a decrease in KLF2 fluorescence intensity. (C) Summary data of the effects of statin treatment on the mean fluorescence intensity of KLF2 obtained in isolated glomus cells. Sham vehicle (n=4); CHF vehicle (n=4); CHF statin (n=4).