Persistent change in cardiac fibroblast physiology after transient ACE inhibition

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D’Souza KM, Biwer LA, Madhavpeddi L, Ramaiah P, Shahid W, Hale TM. Persistent change in cardiac fibroblast physiology after transient ACE inhibition. Am J Physiol Heart Circ Physiol 309: H1346–H1353, 2015. First published September 14, 2015; doi:10.1152/ajpheart.00615.2015.—Transient angiotensin-converting enzyme (ACE) inhibition induces persistent changes that protect against future nitric oxide synthase (NOS) inhibitor-induced cardiac fibrosis and inflammation. Given the role of fibroblasts in mediating these effects, the present study investigates whether prior ACE inhibition produced persistent changes in cardiac fibroblast physiology. Adult male spontaneously hypertensive rats (SHRs) were treated with vehicle (C+L) or the ACE inhibitor, enalapril (E+L) for 2 wk followed by a 2-wk washout period and a subsequent 7-day challenge with the NOS inhibitor N\textsuperscript{\textsubscript{o}}-nitro-L-arginine methyl ester. A third set of untreated SHRs served as controls. At the end of the study period, cardiac fibroblasts were isolated from control, C+L, and E+L left ventricles to assess proliferation rate, collagen expression, and chemokine release in vitro. After 7 days of NOS inhibition, there were areas of myocardial injury but no significant change in collagen deposition in E+L and C+L hearts in vivo. In vitro, cardiac fibroblasts isolated from C+L but not E+L hearts were hyperproliferative, demonstrated increased collagen type I gene expression, and an elevated secretion of the macrophage-recruiting chemokines monocyte chemotactic protein-1 and granulocyte macrophage-colony stimulating factor. These findings demonstrate that in vivo N\textsuperscript{\textsubscript{o}}-nitro-L-arginine methyl ester treatment produces phenotypic changes in fibroblasts that persist in vitro. Moreover, this is the first demonstration that transient ACE inhibition can produce a persistent modification of the cardiac fibroblast phenotype to one that is less inflammatory and fibrogenic. It may be that the cardioprotective effects of ACE inhibition are related in part to beneficial changes in cardiac fibroblast physiology.

angiotensin-converting enzyme inhibitor; cardiac fibroblast; proliferation; collagen; inflammation

NEW & NOTEWORTHY

The present study demonstrates that prior transient angiotensin-converting enzyme inhibition produces a persistent change in the cardiac fibroblast phenotype characterized by a reduced inflammatory and fibrogenic response to myocardial injury. This is the first demonstration that angiotensin-converting enzyme inhibitors can produce a change in cellular physiology that persists after cessation of treatment.

HYPERTENSIVE HEART DISEASE is one of the leading causes of heart failure, a debilitating illness that affects 5.2 million Americans (2, 28). While hypertensive heart disease is defined by the presence of left ventricular (LV) hypertrophy, there is increasing recognition that cardiac fibrosis may mediate some of the long-term functional consequences of this condition (9). Cardiac fibrosis is characterized by uncontrolled fibroblast proliferation leading to an accumulation of the extracellular matrix (2, 9, 26, 34). In addition, inflammatory cells, including macrophages, play an important role in the development and progression of fibrotic remodeling in response to a variety of stimuli including nitric oxide synthase (NOS) inhibition (13, 18, 33). There is growing evidence that fibroblasts secrete cytokines and chemokines involved in recruiting macrophages and other inflammatory cells to sites of injury (17, 27). In addition to clearing cellular debris, macrophages secrete cytokines, proteases, growth factors, and superoxide to promote tissue destruction, fibroblast proliferation, and collagen deposition; this creates a vicious cycle of ongoing and expansive fibrotic remodeling (6, 10). Therefore, targeting fibroblasts may be a novel strategy to reduce both collagen deposition and limit the recruitment, prolonged survival, and activation of macrophages in the remodeling heart.

Angiotensin-converting enzyme (ACE) inhibitors have been widely demonstrated to be cardioprotective, both clinically and experimentally (4, 5, 14, 16, 20, 25, 29, 35). We and others have previously shown that ACE inhibitors induce apoptosis of cardiac fibroblasts in adult spontaneously hypertensive rats (SHRs) within the first 2 wk of treatment (7, 8, 32). In addition, ACE inhibitors have been shown to reduce fibroblast proliferation in vivo (25) and in vitro (38). It has thus been suggested that targeting fibroblast survival may be a mechanism by which these drugs are effective in limiting cardiac fibrosis. We have shown that prior transient (i.e., 2-wk treatment followed by a 2-wk washout period) ACE inhibition in young adult male SHRs to be protected against a future cardiac insult induced by the nonspecific NOS inhibitor N\textsuperscript{\textsubscript{o}}-nitro-L-arginine methyl ester (L-NAME) (3, 13). This protection was characterized by a preservation of cardiac output (3), a reduction in outer wall collagen deposition, and decreased densities of proliferating cells and macrophages in the LVs of rats that had been previously treated with an ACE inhibitor (13). That is, a transient ACE inhibitor treatment induced protective effects that persisted even after stopping treatment and restoration of the renin-angiotensin system. Importantly, NOS inhibition induced focal regions of cardiomyocyte loss as well as coronary artery injury in both naïve and previously treated rats (3). Thus, the reduction in inflammation and fibrosis in rats previously treated with an ACE inhibitor appears to reflect a modified response to myocardial injury. Given that fibroblasts play a role in both collagen deposition as well as inflammatory responses, it may be that ACE inhibition produces a persistent change in the cardiac fibroblast phenotype such that these cells display an altered physiological response to myocardial damage induced by NOS inhibition. Given that by 10 days of L-NAME treatment we have shown that there is a significant increase in collagen deposition, macrophage infiltration, and cellular proliferation only in naïve NOS-inhibited rats, we...
Drinking Water

Drinking Water

Drinking Water

L-NAME (15mg/kg/day)

If prior ACE inhibition impacted the NOS inhibitor-induced production of a change in fibroblasts that persisted in vitro and 

/h9251
Collagen type I (5)

Table 1. Primer sequences for real-time PCR assessments

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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</thead>
<tbody>
<tr>
<td>Collagen type I (α1)</td>
<td>5'-TGTTCTTGGAGAAAATTTGC-3'</td>
<td>5'-CTGTGCCTGCTTGTTCCGG-3'</td>
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<tr>
<td>Collagen type III (α1)</td>
<td>5'-ACCTGAAATCTCTGCCACCT-3'</td>
<td>5'-GCTGTGAATCTCTCCTACATGG-3'</td>
</tr>
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<td>Monocyte chemotactant protein-1</td>
<td>5'-CAGGTCTCTTGAGACTTCTC-3'</td>
<td>5'-AGTTCAGTGGAGAGAAATG-3'</td>
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<tr>
<td>Granulocyte macrophage-colony stimulating factor</td>
<td>5'-TCGAGAAGCTTGGATGAC-3'</td>
<td>5'-GATTGAGCCGGGCTATAG-3'</td>
</tr>
<tr>
<td>β-Actin</td>
<td>5'-GAAGCTGCTGCTATGTTGCGCT-3'</td>
<td>5'-TTGCGATGCCGCAAGGAAATG-3'</td>
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Fig. 1. Schematic representation of the study design. Rats were divided into one of three following treatment groups: control (Con), Con + N-nitro-l-arginine methyl ester (l-NAME) (C+L), and enalapril + l-NAME (E+L). At the end of the treatment period, hearts were either harvested for histology or fibroblast isolation.

performed an in vitro study to test the hypothesis that the protection against NOS inhibitor-induced pathological remodeling was related to a phenotypic difference in cardiac fibroblasts after the cessation of ACE inhibitor treatment. To evaluate phenotypic differences, we isolated fibroblasts from LVs of control and NOS inhibitor-treated SHRs that had been previously treated with vehicle or an ACE inhibitor. Fibroblasts were isolated from NOS-inhibited rats at a time point in which the injured myocardium displays inflammatory and early fibrogenic characteristics based on the time course of Tomita et al. (33). This allows for the evaluation of each of these roles in isolated cardiac fibroblasts. Specifically, in this in vitro portion of the study, we evaluated cardiac fibroblast physiology with respect to proliferation, collagen gene expression, and chemokine production to determine I) if in vivo NOS inhibition produced a change in fibroblasts that persisted in vitro and 2) if prior ACE inhibition impacted the NOS inhibitor-induced change in the fibroblast phenotype.

MATERIALS AND METHODS

Animals and treatments. Young adult (10 wk old) male SHRs were purchased from Charles River (Portage, MI) and allowed to acclimate for a minimum of 48 h before the initiation of treatments. All rats were provided food and water ad libitum throughout the study period. At 11 wk of age, rats were divided into the following three treatment groups (Fig. 1): control, control + l-NAME (C+L), and enalapril + l-NAME (E+L). E+L rats were treated for 2 wk with the ACE inhibitor enalapril (30 mg·kg⁻¹·day⁻¹ po) followed by a 2-wk washout period. At 15 wk of age, C+L and E+L rats were treated for 7 days with the NOS inhibitor l-NAME (15 mg·kg⁻¹·day⁻¹ po). The concentration of drug in the drinking water was adjusted every 3–5 days to account for changes in body weight and water consumption. The 7-day time point was selected in an effort to evaluate fibroblasts during a dynamic phase in which there are inflammatory changes and the initiation of scar tissue formation. A previous time-course analysis by Tomita et al. revealed that day 7 represented a time point in which there was still an increased macrophage density and chemokine level, cellular proliferation, and expression of α-smooth muscle actin in fibroblasts (33). To characterize the state of injury and collagen deposition, a subset of rats (n = 19) was euthanized for histological analysis while LV fibroblasts were isolated and cultured from the remaining rats (n = 38). All procedures were approved and in accordance with Arizona State University and University of Arizona institutional guidelines. All animals used in this study were cared for in accordance with recommendations in the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1986).

Histology and immunohistochemistry. Hearts were excised, and a transverse slice was taken from the middle of the heart and immersion fixed overnight in HistoChoice tissue fixative (Amresco, Solon, OH) before being embedded in paraffin. Five-micrometer sections were stained with picrosirius red (Sirius Red F3BA, Fisher Scientific, Pittsburgh, PA, in picric acid, Sigma-Aldrich, St. Louis, MO) to identify collagen fibers and counterstained with fast green FCF (Fisher Scientific). The evaluation of fibrosis in stained LVs was determined at a magnification of ×200. A blinded observer determined the percent area stained for collagen. Specifically, a 264-point grid was overlaid on an image, and the points touching the red-stained collagen were counted. Every other field was counted until the entire LV was scanned (i.e., 50% of the section was analyzed). The number of points of intersection with collagen stain was divided by the total number of potential intersection sites (e.g., 264 × the number of fields evaluated). Fields that contained large arteries with perivascular fibrosis were not evaluated.

Isolation and culture of cardiac fibroblasts. Cardiac fibroblasts were isolated from the LVs of control (n = 16), C+L (n = 16), and E+L (n = 12) rats. One rat from each group was euthanized on a given day for fibroblast isolation. Fibroblasts were isolated using a digestion buffer containing 5.4 mg (per LV) Liberase (Roche, Indianapolis IN) in 1× ADS. Briefly, the LV was minced and subjected to five digestions of 10 min each in a shaking water bath at 37°C. The supernatants from each digestion were pooled together and centrifuged at 1,200 rpm for 15 min to pellet the suspended fibroblasts, which were subsequently resuspended in complete medium comprising DMEM-F-12, 15% FBS, and 1% penicillin-streptomycin and plated in 60-mm dishes in a humidified 5% CO₂ incubator. Dissociated cells were allowed to adhere for 1 h, after which the medium was replaced and cells were grown to 90% confluence for a period of 1 wk to 10 days, after which they were suitably split. Experiments were performed at passage 1 to minimize any phenotypic drift while in culture. To minimize spontaneous differentiation of fibroblasts to myofibroblasts, cells were plated at a density of ~200 cells/mm² (31).

Real-time quantitative PCR. RNA was isolated from LV cardiac fibroblasts from control, C+L, and E+L rats using Ribozol (Amresco, Solon, OH) as per the manufacturer’s instructions. cDNA was synthesized using reverse transcriptase (New England Biolabs, Ipswich, MA) with oligo-dT. Real-time PCR was performed with an Applied Biosystems 7500 Real Time Thermocycler with AB SYBR green (Life Technologies, Calsbad, CA). Suitably designed primers were ordered from IDT (Coralville, IA; Table 1). The PCR conditions were as follows: 95°C for 5 min followed by 40 cycles of melting at 95°C for 15 s and annealing and elongation at 60°C for 1 min. Products
specificity was verified with a melt curve and by running real-time PCR products on a 1% agarose gel containing ethidium bromide.

Protein assessments. In cardiac fibroblasts, phosphorylated Erk42/44, total Erk42/44, phosphorylated p38, and total p38 (all from Cell Signaling) protein expression was determined via immunoblot analysis. Protein was extracted and separated via SDS-PAGE. Densitometry was performed using ImageJ software (phosphorylated p38 and p38) or LiCor software (phosphorylated Erk and Erk). Membranes were probed with the phosphospecific antibody and then stripped and reprobed for the total form. Analyses were based on the ratio of phosphorylated to total protein.

Media were collected at the end of the culture period for assessments of chemokine levels. Specifically, soluble monocyte chemoattractant protein (MCP)-1 and granulocyte macrophage-colony stimulating factor (GM-CSF) levels were quantified using commercially available rat-specific ELISA kits from Peprotech (Rocky Hill, NJ) as per the manufacturer’s instructions.

Proliferation assays. Proliferation was evaluated by flow cytometry and via population doubling assays. Washed samples of cardiac fibroblasts in 300-μl aliquots containing ~10^6 cells were incubated at 37°C for 24 h in the dark. Cell cycle analysis was carried out in a BD Flow Cytometry Analyzer and analyzed using FACS DIVA software. Both test and negative control samples (without propidium iodide) were run in duplicates. For population doubling assessments, cardiac fibroblasts were seeded at a concentration of 50,000 cells/well of a 12-well plate. Cells were trypsinized after 24, 48, and 72 h and counted using the trypan blue exclusion method to distinguish dead cells from live cells.

Statistical analysis. For cell culture experiments, comparisons were made against control cells isolated on the same day as C+L and E+L cells. As such, all data are presented as fold changes versus control. Statistical analysis was based on a one-sample t-test to evaluate significant differences from the value of 1 (i.e., data were normalized to control, which, as a result, has a value of 1) for data that passed the D’Agostino and Pearson omnibus normality test. Data that failed the normality test were analyzed using the nonparametric Wilcoxon signed rank test. Comparisons between C+L and E+L groups were based on either a Student’s t-Test or a Mann-Whitney test for parametric and nonparametric evaluations, respectively. In all cases, data are presented as means ± SE, and statistical significance was based on P < 0.05, as adjusted with the Bonferroni correction. For example, given that three comparisons were made (control vs. C+L, control vs. E+L, and C+L vs. E+L), statistical significance was based on 0.05/3 or P < 0.017.

RESULTS

Seven-day NOS inhibition did not significantly induce cardiac fibrosis. We have previously shown that after 10 days of L-NAME, there was a significant increase in collagen deposition only in C+L rats. In the present study, the degree of fibrosis was determined semiquantitatively in control SHRs or after 7 days of L-NAME in rats previously treated with vehicle (C+L) or enalapril (E+L). After 1 wk of L-NAME treatment, there was no significant increase in collagen accumulation in the hearts of C+L or E+L rats (Fig. 2).

In vivo NOS inhibition produced an increase in proliferation rate only in fibroblasts isolated from C+L rats. Fibroblasts isolated from C+L LVs were found to be hyperproliferative in vitro compared with those isolated from E+L LVs (Fig. 3). Population doubling experiments revealed that the rate of growth was significantly different across the three treatment groups. Analyses of the slopes of the population doubling lines showed a significant difference between C+L and E+L fibroblasts (control: 0.15 ± 0.0213, C+L: 0.22 ± 0.0391, and E+L: 0.12 ± 0.0158, P < 0.0167). At 48 h, there were significantly fewer E+L cells relative to control (Fig. 3). There were no differences in the percent survival or number of dead cells across treatment groups (not shown). Cell sorting analysis...
revealed an ~25% increase in the proportion of cells in the S phase from control for both C+L (P = 0.044 vs. control) and E+L (P = 0.068 vs. control) groups.

In vivo NOS inhibition produced an increase in collagen type I gene expression only in fibroblasts isolated from C+L rats. The present findings revealed that at 7 days, there was not yet an overall significant difference in LV collagen deposition. However, we previously demonstrated that collagen deposition was significantly increased in the outer LV wall of C+L but not E+L SHRs after 10 days of L-NAME treatment (13). Thus, we investigated whether enhanced collagen deposition with longer-term NOS inhibition in C+L hearts may be related to differences in collagen gene expression in fibroblasts isolated from the LVs from SHRs in these two treatment groups. The present study demonstrated that collagen type Ia1 gene expression is markedly and significantly increased in C+L fibroblasts relative to control (Fig. 4). In contrast, there was a tendency toward reduced (>30%) collagen type I gene expression in E+L fibroblasts relative to control that did not reach statistical significance (P = 0.02) with the Bonferroni correction. There were no statistically significant changes in collagen type III gene expression in either treatment group (Fig. 4).

Expression of Erk42/44 and p38 in cardiac fibroblasts was not altered by in vivo NOS inhibition. Erk42/44 and p38 have both been implicated in NOS inhibitor-induced fibroblast activation and pathological remodeling (21, 24, 38). However, in the present study, neither phosphorylated Erk42/44 relative to total Erk42/44 nor phosphorylated p38 relative to total p38 were significantly different across the three experimental groups (Fig. 5).

In vivo NOS inhibition produced an increase in chemokine release only from C+L fibroblasts. MCP-1 and GM-CSF are two chemokines that play important roles in recruiting, activating, and promoting the survival of macrophages (6, 10). Fibroblasts isolated from C+L hearts secreted increased levels of these chemokines in vitro (Fig. 6). In contrast, secretion of MCP-1 and GM-CSF was not different in fibroblasts isolated from rats that had been treated with the ACE inhibitor before NOS inhibition (E+L) compared with fibroblasts isolated from control SHRs (Fig. 6).

Given that the proliferation rate and chemokine levels were both elevated in C+L fibroblasts and that MCP-1 has been implicated in promoting fibroblast proliferation (11, 18), we investigated whether there was a relationship between these two parameters. However, analysis revealed that neither MCP-1 nor GM-CSF levels significantly correlated with cellular proliferation in either treatment group (Fig. 6).

DISCUSSION

The present study demonstrates, for the first time, that prior transient in vivo treatment with an ACE inhibitor can prevent NOS inhibitor-induced changes in the cardiac fibroblast phenotype. Specifically, fibroblasts isolated from the LVs of naïve...
SHRs treated for 7 days with a NOS inhibitor (i.e., C/H11001L) displayed significant increase in collagen type I gene expression as well as MCP-1 and GM-CSF secretion compared with cells isolated from untreated SHRs and an increased proliferation rate compared with cells from E/H11001L rats. In contrast, cardiac fibroblasts isolated from rats that had been transiently treated with an ACE inhibitor before NOS inhibition were not different from those from control SHRs. Specifically, fibroblasts isolated from the LVs of naïve SHRs treated for 7 days with a NOS inhibitor (i.e., C/H11001L) displayed significant increases in collagen type I gene expression as well as MCP-1 and GM-CSF secretion. In contrast, cardiac fibroblasts isolated from rats that had been transiently treated with enalapril tended to proliferate more slowly and show reduced collagen type I gene expression compared with control fibroblasts. These differences in cardiac fibroblast phenotype may in part account for the protection against cardiac remodeling and inflammation induced by the NOS inhibitor in rats that were previously transiently treated with an ACE inhibitor that we have previously reported in vivo (13).

ACE inhibitors have been widely shown to inhibit the development of, and in some cases reverse, cardiac fibrosis in various experimental models of pathological cardiac remodel-

![Graph](image1)

**Fig. 4.** Quantification of collagen type Iα1 (Coll1A1; A) and collagen type III (Coll III; B) gene expression in cardiac fibroblasts isolated from Con and treated rats. *P < 0.0167 vs. the Con group based on the Bonferroni correction (3 comparisons: Con vs. C+L, Con vs. E+L, and C+L vs. E+L).

ACE inhibitors have been widely shown to inhibit the development of, and in some cases reverse, cardiac fibrosis in various experimental models of pathological cardiac remodel-

![Graph](image2)

**Fig. 5.** A: representative blots of phosphorylated (p-)Erk (42/44 kDa) and total Erk (42/44 kDa) and densometric analysis. B: representative blots of p-p38 (38 kDa) and total p38 (38 kDa) and densometric analysis.
could be related to a difference in cardiac fibroblast physiology that remained after the cessation of ACE inhibition. If the differences in NOS inhibitor-induced pathological remodeling were simply due to a decrease in the number of fibroblasts, we would have predicted that the cells isolated from naïve and previously treated rats would have similar characteristics in vitro. However, the present study shows that unlike cardiac fibroblasts isolated from C/H11001L rats, those from NOS inhibitor-treated rats that had been previously administered an ACE inhibitor do not display significant increases in the proliferation rate, collagen type I gene expression, or secretion of the macrophage-recruiting chemokines MCP-1 and GM-CSF. These findings suggest that the fibroblasts isolated from rats that had been previously treated with an ACE inhibitor are phenotypically different from those isolated from naïve NOS inhibitor-treated rats. It may be that the prior ACE inhibitor treatment removed a subpopulation of fibroblasts that, in response to injury, becomes hyperproliferative and promotes excessive production of collagen type I and inflammatory mediators. On the other hand, it is possible that the previously observed cardioprotection conferred by prior transient ACE inhibition is unrelated to the induction of apoptosis and instead results from phenotypic modulation of cardiac fibroblasts.

ACE inhibitors have been shown to reduce fibrosis and inflammation (19, 30). This has been proposed to be mediated in large part to a reduction in ANG II production. In the present study, in vivo NOS inhibitor treatment was initiated 2 wk after the cessation of ACE inhibition. Therefore, it may be that in addition to the previously described impact of ACE inhibitors on fibroblast activity resulting from an interruption in ANG II signaling, these drugs may also induce epigenetic changes in fibroblasts that result in an altered response to a pathogenic stimulus. Regardless of the mechanism, it appears that an altered fibroblast phenotype may be in part responsible for the long-term protection against L-NAME-induced cardiac fibrosis, cellular proliferation, and macrophage infiltration that we have previously demonstrated in vivo (13). Although Erk 42/44 and p38 have previously been shown to be involved in promoting fibrogenic fibroblast responses (21, 24), they do not appear to be implicated in the phenotypic differences observed in the present study.

In summary, the present study not only demonstrates that in vivo L-NAME treatment can induce a phenotypic change in fibroblasts that persists in vitro but also that prior transient ACE inhibition can prevent the L-NAME-induced alterations. Our previous observations (3) as well as the present findings demonstrate that the incidence and extent of myocardial injury does not tend to be different between C+L and E+L rats. However, the response to that injury is altered with respect to cardiac function (3) and pathological remodeling (13). The in vitro findings from the present study raise the possibility that an excessive response of fibroblasts to injury may underlie the greater pathology observed in C+L hearts. Understanding the mechanism by

![Fig. 6. Quantification of soluble monocyte chemoattractant protein (MCP)-1 (A) and granulocyte macrophage-colony stimulating factor (GM-CSF; B) concentrations in the media from cardiac fibroblasts isolated from Con and treated rats. Neither chemokine showed a significant correlation with proliferation in C+L (C) or E+L (D) fibroblasts. *P < 0.0167 vs. the Con group and †P < 0.0167 vs. the C+L group based on the Bonferroni correction (3 comparisons: Con vs. C+L, Con vs. E+L, and C+L vs. E+L).](http://ajpheart.physiology.org/)
ACE INHIBITION MODIFIES THE FIBROBLAST PHENOTYPE

which ACE inhibitors produce persistent changes in fibroblast responses may provide greater insight into the cardioprotective effects of these commonly used drugs and reveal novel targets for future therapeutic interventions to prevent or limit the pathological remodeling that underlies heart failure.

REFERENCES