Dose-dependent Blockade to Cardiomyocyte Hypertrophy by Histone Deacetylase Inhibitors*

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Postnatal cardiac myocytes respond to stress signals by hypertrophic growth and activation of a fetal gene program. Recently, we showed that class II histone deacetylases (HDACs) suppress cardiac hypertrophy, and mice lacking the class II HDAC, HDAC9, are sensitized to hypertrophic signals. To further define the roles of HDACs in cardiac hypertrophy, we analyzed the effects of HDAC inhibitors on the responsiveness of primary cardiomyocytes to hypertrophic agonists. Parasitically, HDAC inhibitors imposed a dose-dependent blockade to hypertrophy and fetal gene activation. We conclude that distinct HDACs play positive or negative roles in the control of cardiomyocyte hypertrophy. HDAC inhibitors are currently being tested in clinical trials as anti-cancer agents. Our results suggest that these inhibitors may also hold promising clinical value as therapeutics for cardiac hypertrophy and heart failure.

Postnatal cardiac myocytes undergo hypertrophic growth in response to a variety of stress signals (reviewed in Ref. 1). The hypertrophic response is characterized by increases in myocyte size and protein synthesis, assembly and organization of sarcomeres, and activation of a fetal gene program. Although traditionally considered an adaptive response to pathological signaling, chronic expression of fetal cardiac genes in the heart can result in maladaptive changes in cardiac contractility and calcium handling that culminate in dilated cardiomyopathy, heart failure, and sudden death from arrhythmias (2). Moreover, increasing evidence in rodent models indicates that cardiac function is preserved when hypertrophy is inhibited in the face of stress signaling, pointing to the potential importance of therapeutic strategies for modulating the hypertrophic process (3–9).

Recent studies have revealed key roles for chromatin-modifying enzymes in the control of cardiac hypertrophy (10–12). The structure of chromatin is governed by the acetylation state of nucleosomal histones (13, 14). Acetylation of histone tails by histone acetyltransferases (HATs)1 results in relaxation of nucleosomal structure and transcriptional activation. Acetylated histones also serve as targets for binding of bromo-domain proteins that possess HAT activity and act as transcriptional activators. The actions of HATs are opposed by histone deacetylases (HDACs), which deacetyl nuclear histones, thereby promoting chromatin condensation and transcriptional repression.

Mammalian HDACs can be divided into three classes based on their similarity with three yeast HDACs (reviewed in Refs. 15 and 16). Class I HDACs (HDACs 1, 2, 3, and 8) are expressed ubiquitously and consist mainly of a deacetylase domain. Members of class II (HDACs 4, 5, 7, and 9) are highly expressed in striated muscle and brain and have an extended N terminus in addition to the catalytic domain. Class III HDACs resemble the yeast HDAC Sir2, which is activated by nicotinamide adenine dinucleotide (17).

Class II HDACs interact with a variety of positive and negative cofactors as well as other HDACs through their N-terminal regions (reviewed in Ref. 18). These regions also contain two conserved phosphorylation sites for Ca2+/calmodulin-dependent protein kinase and other kinases that confer responsiveness to calcium signaling. Phosphorylation of these sites creates docking sites for 14-3-3 chaperone proteins, which escort the class II HDACs out of the nucleus, resulting in activation of genes that would otherwise be repressed by these HDACs (reviewed in Ref. 19). One of the important targets of HDACs in muscle cells is the myocyte enhancer factor-2 (MEF2) transcription factor (reviewed in Ref. 20). In the adult heart, MEF2 proteins exhibit only basal activity, which is dramatically enhanced by calcium-dependent stress signals that induce hypertrophy (21). The activation of MEF2 by hypertrophic signals can be explained, at least in part, by the phosphorylation-dependent dissociation of class II HDACs and subsequent recruitment of HATs by MEF2.

Several recent observations have implicated HDACs and HATs in the control of cardiac hypertrophy. First, expression of signal-resistant mutants of class II HDACs in primary cardiomyocytes silences the fetal gene program and renders myocytes insensitive to hypertrophic agonists (11). Overexpression of class II HDACs also blocks MEF2 activation by hypertrophic stimuli (10). Second, knock-out mice lacking HDAC9 are supersensitive to stress signals and develop massively hypertrophic

1 The abbreviations used are: HAT, histone acetyltransferase; HDAC, histone deacetylase; ANF, atrial natriuretic factor; MHC, myosin heavy chain; ET-1, endothelin-1; MEF2, myocyte enhancer factor-2; NaB, sodium butyrate; PE, phenylephrine; TSA, trichostatin A; PBS, phosphate-buffered saline; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; BSA, bovine serum albumin.
hearts when stressed (11). Third, the HAT p300 associates with the MEF2 and GATA4 transcription factors, which regulate fetal cardiac genes, and enhances their transcriptional activity (22, 23). Finally, overexpression of p300 induces hypertrophy of primary cardiomyocytes (12).

Numerous pharmacological inhibitors of HDAC activity have been identified including trichostatin A (TSA) (24), sodium butyrate (NaB) (25), HC-toxin (26), subercyl-anilide hydroxamic acid (27), and pyroxamide (28). Crystal structure analyses of HDAC-TSA and HDAC-subercyl-anilide hydroxamic acid complexes reveal how these inhibitors interact specifically with the catalytic core and abolish HDAC enzymatic activity (29). Inhibiting HDAC activity with these pharmacological agents alters gene expression; however, instead of simply activating gene transcription as would be expected to result from blocking the repressive activity of HDACs, these inhibitors result in increases as well as decreases in expression of specific genes (30–33). In several cell types, HDAC inhibitors have been...
shown to block growth and promote quiescence and differentiation (28, 33–36). HDAC inhibitors have also shown promise as anti-cancer agents, which may reflect their ability to selectively derepress expression of the cyclin-dependent protein kinase inhibitor p21 (reviewed in Ref. 37).

In light of the ability of class II HDACs to suppress cardiac hypertrophy, we sought to determine whether HDAC inhibitors would mimic the effect of genetic deletion of HDAC9 (11) and thereby promote cardiac hypertrophy. Paradoxically, we found that treatment of cultured cardiac myocytes with HDAC inhibitors prevented hypertrophy, sarcomere organization, and activation of the fetal gene program normally evoked by hypertrophic agonists. Taken together with our prior findings, these results suggest that HDACs play dual roles as repressors and activators of cardiac hypertrophy.

**EXPERIMENTAL PROCEDURES**

**Indirect Immunofluorescence**—Hearts were dissected from 2–3-day-old Sprague-Dawley rats (Harlan), minced in PBS, and digested with pancreatin (0.1% w/v, Sigma) in PBS. Cells were resuspended in DMEM:M199 (4:1) containing horse serum (10%), fetal bovine serum (FBS) (5%), l-glutamine (2 mM), and penicillin-streptomycin and plated for 2 h to separate adherent fibroblasts from cardiomyocytes. Cardiomyocytes were plated in 6-well dishes (1 × 10⁶ cells/well) containing laminin-coated glass coverslips. Following the indicated treatments, cells were fixed with formalin (10%) in PBS, permeabilized, and blocked with PBS containing Nonidet P-40 (0.1%) and bovine serum albumin (BSA) (5%) and incubated in the same solution but with primary antibodies for sarcomeric α-actinin (mouse monoclonal, 1:200 dilution, Sigma) or atrial natriuretic factor (ANF) (rabbit polyclonal, 1:200 dilution, Peninsula Laboratories). Coverslips were washed five times in PBS and incubated with fluorescein-conjugated secondary antibodies (1:200 dilution, Vector Laboratories) followed by a brief incubation with Hoechst dye 33542 (H-3570, Molecular Probes). Coverslips were washed five times in PBS, one time with water, and mounted on glass slides using Vectashield mounting medium (Vector Laboratories). Proteins were visualized with a fluorescence microscope, and images were captured using a digital camera (Hamamatsu Photonics).

**ANF and Ribosomal S6 Protein Cytoblot Assay**—Cytoblot analyses were performed as described previously (38) with modifications. Neonatal rat ventricular myocytes were prepared for indirect immunofluorescence as described above with the exception that cells were centrifuged through a Percoll gradient prior to pre-plating to further enrich for myocytes. Myocytes were plated on gelatin-coated 96-well dishes (1 × 10⁴ cells/well) in DMEM containing FBS (10%), l-glutamine (2 mM), and penicillin-streptomycin. Following overnight culture, growth medium was replaced with serum-free DMEM supplemented with Nutridoma-SP (which contains albumin, insulin, transferrin, and other defined organic and inorganic compounds) (0.1% v/v, Roche Applied Science) and the indicated agonists and inhibitors. After 48 h of treatment, cells were washed twice with PBS, fixed with paraformaldehyde (4%) in PBS (30 min), permeabilized with Triton X-100 (0.1%) in PBS (10 min), and blocked with BSA (1%) in PBS (1 h). Cells were incubated with PBS containing BSA (1%), normal goat serum (1%), and primary
antibodies against either ANF (mouse monoclonal, 10 μg/ml, Biodesign) or ribosomal S6 protein (rabbit polyclonal, 50 μg/ml, Cell Signaling Technologies) for 1 h. Cells were washed twice with PBS containing BSA (1%) and incubated in the same solution but containing HRP-conjugated anti-mouse or anti-rabbit secondary antibodies (1:1000 and 1:400, respectively, Jackson Laboratories). Cells were washed twice with PBS containing BSA (1%) and one time with PBS alone. Luminol (Pierce) was added to the cells, and luminescence was detected using a fusion plate reader (PerkinElmer Life Sciences). All of the manipulations were performed at room temperature.

**[3H]Leucine Incorporation and DNA Content**—Cardiomyocytes were incubated with [3H]leucine (1.0 mCi/ml, 172 Ci/mmol Sp. Activity, ICN/H9262 (100 nM). Treatment with TSA blocked the PE-induced increase in S6 protein expression. Results are graphed as the means ± S.D. from five independent samples. C, the graph depicts the amount of protein- incorporated [3H]leucine normalized to cellular DNA content. Measurements of [3H]leucine incorporation were taken from cardiomyocytes after the 24-h treatment as described in B. Treatment with TSA also blocked the PE-induced increase of [3H]leucine incorporation.

streptomycin. Following overnight culture, growth medium was replaced with serum-free DMEM containing Neuritodima (0.1%), phenylephrine (PE) (20 μM), and the indicated concentrations of TSA. After 48 h of treatment, cells were washed twice with PBS and whole cell protein extracts were prepared in Tris buffer (50 mM, pH 7.5) containing EDTA (10 mM), EDTA (5 mM), Triton X-100 (1%), and protease inhibitors (Roche Applied Science). Lysates were sonicated briefly and clarified by centrifugation. Total protein from each treatment group (15 μg) was resolved by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and immunoblotted with anti-acetyllhistone H4 primary antibody (06-846; 1:4000, Upstate Biotechnology) and an HRP-conjugated anti-rabbit secondary antibody (4050; 1:10,000, Southern Bio-technology) dilution. Protein was visualized using an enhanced chemiluminescence system (Pierce).

**Adenylate Kinase Release Assay**—Adenylate kinase was measured in culture supernatants employing the bioluminescent ToxiLight kit (Bio-Whittaker) according to the manufacturer’s instructions. Assays were run in a 96-well format, and values were measured using a fusion plate reader.

**RNA Analysis**—Neonatal rat ventricular myocytes were plated on gelatin-coated 10-cm dishes (2 × 10^5 cells/dish). Following the indicated treatments, RNA was isolated from cardiomyocytes using TrizOL Reagent (Invitrogen). Total RNA (1 μg) was vacuum-blotted onto nitrocel- lulose membranes (Bio-Rad) using a 96-well format dot blotter (Bio- Rad). Membranes were blocked in 4× SSC containing SDS (1%), 5× Denhardt’s reagent, sodium pyrophosphate (0.05%), and sonicated salmon sperm DNA (100 μg/ml; 4 h, 50 °C) and incubated with 32P-end-labeled oligonucleotide probes (1 × 10^6 cpm/ml; 14 h, 50 °C). Sequences of oligonucleotides were as follows: ANF, 5′-ATGTGACACTACACGACACAAAGGTTGAGATTTCAGGTGCCTGTCCTCAAGTGTCCATTTCATTCTTCCACACAGGG-3′; α-MHC, 5′-CGAAC- GTTATGGTTGTTAGGCAAGGCAGGCTGAGGGAGG-3′; β-MHC, 5′-CGCTTATTCGCTTCCACCTAAGGGGTCCGAG-3′; -MHC, 5′-CTTACCGTTTAAAGGCTGCTGAGGCTGTCCTGAGG-3′; and glyceraldehyde-3-phosphate dehydrogenase, 5′-GGAACATGTAAGGATGTTAGATTAGGCTGAG- GCTCAATGGAG-3′. Blots were washed twice with 0.5× SSC containing SDS (0.1%; 10 min, 50 °C) and analyzed by autoradiography. Hybridization signals were quantified using a Storm PhosphorImager (Amersham Biosciences).

**Promoter-Reporter Assays**—Neonatal rat ventricular myocytes were plated on gelatin-coated 6-well dishes (1 × 10^5 cells/dish) in DMEM: M199 (4:1), 10% horse serum, 5% FBS, 1-glutamine (2 mM), and penicillin-streptomycin. Twenty-four hours after plating, cells were co-transfected with a luciferase reporter under the control of 3 kilobases of
ANF promoter (39) and a CMV-lacZ reporter using LipofectAMINE Plus reagent (Invitrogen). Transfected cells were incubated in serum-free DMEM for 24 h prior to treatment for an additional 24 h with the indicated agonists and inhibitors. Cell lysates were prepared and assayed for luciferase activity using the luciferase assay system (Promega).

RESULTS

Inhibition of ANF Expression by HDAC Inhibitors

To begin to investigate the potential effects of HDAC inhibitors on cardiac hypertrophy, we assayed for expression of ANF, a secreted peptide and sensitive marker of hypertrophy, in primary neonatal rat cardiomyocytes treated with a series of hypertrophic agonists. As shown in Fig. 1A, the treatment of cardiomyocytes with FBS, PE, or endothelin-1 (ET-1) induced ANF expression. A, the ANF promoter is minimally active in unstimulated cardiomyocytes and activated ~3-fold by PE treatment. Each HDAC inhibitor completely abolished PE-induced ANF-reporter activity. B, the inhibitory effect HDAC inhibitors on the ANF promoter activity was specific as the CMV promoter activity in the same cells was either unaffected or enhanced by these inhibitors. Values represent the means ± S.D. from three independent experiments.

the mouse ANF promoter (39) and a CMV-lacZ reporter using LipofectAMINE Plus reagent (Invitrogen). Transfected cells were incubated in serum-free DMEM for 24 h prior to treatment for an additional 24 h with the indicated agonists and inhibitors. Cell lysates were prepared and assayed for luciferase activity using the luciferase assay system (Promega).

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Fig. 6. A model to account for the dual roles of HDACs in the control of cardiac hypertrophy. Class II HDACs block cardiac hypertrophy through interactions with the MEF2 transcription factor and possibly other transcription factors yet to be identified. Inhibition of gene transcription by class II HDACs is mediated in part by associated co-repressors. As such, catalytically inactive class II HDACs are capable of potently blocking MEF2 target gene expression and cardiac hypertrophy. Hypertrophic agonists stimulate the activity of a kinase that phosphorylates two serine residues conserved in class II HDACs (11). Upon phosphorylation, class II HDACs are bound by the 14-3-3 chaperone protein, resulting in their release from MEF2 and their export from the nucleus to the cytoplasm. The HDAC inhibitors used in this study target multiple HDACs that fall into classes I and II. We propose that select HDACs, presumably those in class I, serve positive roles in the control of cardiac hypertrophy, perhaps through inhibition of genes encoding anti-hypertrophic proteins. Accordingly, the products of these anti-hypertrophic genes are dominant over pro-hypertrophic genes suppressed by class II HDACs.

(Fig. 1C), demonstrating a direct effect of HDAC inhibitors on the fetal cardiac gene program.

To determine whether repression of ANF expression by HDAC inhibitors correlated with increases in cardiomyocyte histone acetylation, the effect of TSA treatment on histone H4 acetylation was monitored by immunoblot analysis. As shown in Fig. 1D, the ability of TSA to increase global histone H4 acetylation closely mirrored its capacity to block ANF expression (Fig. 1A).

Aagonist-dependent increases in ANF expression can also be monitored by immunostaining cardiomyocytes with ANF-specific antibodies. Following stimulation with PE and FBS, prominent ANF expression is observed within the secretory pathway as evidenced by its perinuclear localization (Fig. 1E). Consistent with the reduction in ANF protein and mRNA expression (Fig. 1, A and C), ANF immunostaining was significantly diminished in the presence of TSA, although residual expression was detectable.

Some reports have associated HDAC inhibitors with cell death (26, 29, 40, reviewed in Ref. 41). Therefore, we examined whether TSA and NaB were cytotoxic to cardiomyocytes by assaying for the release of adenylate kinase into the culture medium as a measurement of cell membrane integrity. As shown in Fig. 2, increasing doses of TSA (up to 100 nM) (A) and NaB (up to 25 mM) (B) did not increase adenylate kinase in the culture medium. These results suggest that inactivation of ANF expression is a specific effect of HDAC inhibition, not an indirect consequence of cytotoxicity. In subsequent experiments, we used 85 nM TSA or 5 mM NaB unless otherwise indicated.

Inhibition of Cardiomyocyte Hypertrophy by HDAC Inhibitors—To further examine the potential effects of HDAC inhibitors on cardiomyocyte hypertrophy, we stained myocytes for α-actinin, a component of the contractile apparatus. The sarcomeres of unstimulated primary neonatal cardiomyocytes are disorganized; however, stimulation with PE, FBS, or ET-1 results in the appearance of a highly organized sarcomeric structure and an increase in cell size (Fig. 3A). In the presence of TSA, agonist-mediated sarcomere organization was blocked.

A hallmark of cardiomyocyte hypertrophy is enhanced protein synthesis, resulting in increased total cellular protein. We assayed for changes in protein synthesis by measuring accumulation of the ribosomal subunit S6. As shown in Fig. 3B, PE stimulated S6 protein expression ~2-fold and TSA treatment totally abolished this increase. Comparable results were obtained when either FBS or ET-1 was employed to stimulate hypertrophy (data not shown). Similarly, TSA prevented the PE-induced increase in protein synthesis as measured by [3H]leucine incorporation (Fig. 3C). Together, these findings indicate that HDAC inhibitors specifically antagonize the program for cardiac hypertrophy without affecting cell viability.

Inhibition of the Fetal Gene Program by HDAC Inhibitors.—To further assess the ability of HDAC inhibitors to prevent myocyte hypertrophy, we examined the effect of TSA on expression of fetal cardiac genes. Hypertrophic growth is typically accompanied by a switch in MHC expression from the α- to β-isofoms. As shown in Fig. 4, A and B, TSA prevented the up-regulation of β-MHC mRNA expression in response to PE and instead provoked an increase in expression of α-MHC transcripts, which are normally down-regulated in response to hypertrophic agonists. TSA not only prevented the agonist-induced reduction in α-MHC expression but also increased α-MHC mRNA and protein levels in unstimulated cells as did NaB (Fig. 4, A–C, and data not shown). The up-regulation of α-MHC in the presence of HDAC inhibitors suggests that HDAC inhibition reverses the hypertrophic program and further indicates that the effects are not the result of cytotoxicity. The increase in expression of α-skeletal actin that typically accompanies hypertrophy was also blocked by TSA (Fig. 4, A and D).

Inhibition of the ANF Promoter by HDAC Inhibitors.—To begin to decipher the mechanism whereby HDAC inhibitors prevented expression of fetal cardiac genes, we assayed the effect of TSA, NaB, and HC-toxin on activity of the ANF promoter linked to a luciferase reporter. As shown in Fig. 5A, HDAC inhibitors interfered with the ability of PE to stimulate ANF promoter activity. This effect was specific because the inhibitors did not affect activity of the constitutive cytomegalovirus promoter (Fig. 5B). These results suggest that HDAC inhibitors selectively interfere with the expression or activity of an activator of the ANF promoter rather than the proteins involved in general transcription.

DISCUSSION

Postnatal cardiac myocytes activate a well defined program of hypertrophic growth and fetal gene expression in response to
a variety of stress signals and diverse agonists. Previous studies demonstrated that cardiac hypertrophy is suppressed by class II HDACs (11) and enhanced by p300 HAT (12). Thus, we expected that HDAC inhibitors would stimulate hypertrophy by relieving the repression imposed by class II HDACs. Unexpectedly, our results show that HDAC inhibitors are highly effective in suppressing hypertrophy and the fetal gene program in primary cardiomyocytes.

How can the seemingly paradoxical effects of HDAC inhibitors on cardiomyocyte hypertrophy be reconciled with our previous findings that class II HDACs block hypertrophy? One possibility is that the mechanism whereby class II HDACs suppress hypertrophy and the fetal gene program is independent of deacetylase activity. In this regard, the deacetylase domains of class II HDACs are not required for transcriptional repression because these HDACs recruit other corepressors to target genes (42–47). Consistent with this conclusion, MTR (MEF2-interacting transcriptional repressor), a splice variant of HDAC9 that lacks a deacetylase domain, is highly effective in repressing MEF2 activity and cardiomyocyte hypertrophy (11).

It is also conceivable that different classes of HDACs suppress distinct sets of genes that influence the hypertrophic program (Fig. 6). For example, while class II HDACs suppress pro-hypertrophic genes, class I HDACs might repress expression of anti-hypertrophic genes. If the anti-hypertrophic gene products are dominant over the genes suppressed by class II HDACs, one would expect HDAC inhibitors to block hypertrophy.

The HDAC inhibitors used in this study inhibit both class I and II HDACs (48). Little is known regarding possible selective effects of HDAC inhibitors on individual HDAC family members (49). It is unlikely that class III HDACs are involved in the TSA effect, because they are TSA-insensitive (50, 51). We hypothesize that the activity of one or more class I HDACs is dominant over the anti-hypertrophic effect of class II HDACs.

What might be the gene targets for pro-hypertrophic HDACs? As mentioned above, one possibility is that such HDACs are required for repression of one or more genes whose products repress hypertrophy. Accordingly, inhibition of these HDACs could result in derepression of such anti-hypertrophic genes and a consequent block to hypertrophy. Several such genes have been shown to repress hypertrophy including those encoding glycogen synthesis kinase-3 (52–54), inositol polyphosphate 1-phosphatase (55), modulatory calcineurin-interacting protein (6), and class II HDACs. We have assayed expression of glycogen synthase kinase-3 and various HDACs in the presence of TSA and have observed no effect. Whether the anti-hypertrophic effect of HDAC inhibitors involves other known or novel genes remains to be determined.

The present findings contrast with those of a previous study (57), which reported that ANP expression was induced by low doses of TSA. The effects of higher doses were not reported. In that study, the induction of ANP expression was shown to be dependent on the inactivation of a repressive element known as a neuron-restrictive silencer element in the β′-untranslated region of the gene. However, we have found that HDAC inhibitors can block activation of the ANP promoter by hypertrophic agonists (Fig. 5), which would be independent of such a mechanism.

HDAC inhibitors not only prevented hypertrophy but also stimulated expression of α-MHC, which is normally down-regulated during hypertrophy. These findings suggest that these inhibitors fully antagonize the hypertrophic program rather than selectively inhibiting only a subset of genes involved in this process. A previous study (58) reported that a MEF2 binding site upstream of the α-MHC gene was required for the repression of the gene. Given that class II HDACs repress the activity of MEF2, up-regulation of α-MHC in the presence of HDAC inhibitors could reflect derepression of MEF2 activity at this site or it could represent an indirect effect of these inhibitors on other transcriptional regulators.

HDAC inhibitors have been shown to block tumor cell proliferation, in part, by causing cell death (reviewed in Ref. 40). Although we cannot formally rule out the possibility that suppression of hypertrophy by HDAC inhibitors is a nonspecific consequence of cytotoxicity, our results argue against this interpretation because multiple inhibitors suppressed hypertrophy at concentrations at least 10-fold lower than those that caused cellular demise. Moreover, the concentrations in which these inhibitors suppressed hypertrophy are comparable to those reported in other studies to specifically inhibit histone deacetylation and other cellular processes.

HDAC inhibitors have shown efficacy as anti-cancer agents in humans and animal models (56, 59–61). It remains to be determined whether HDAC inhibitors will also prove to be efficacious in suppressing pathological cardiac signaling in vivo. Nevertheless, the results of this study point to the potential usefulness of such inhibitors in the treatment of cardiac hypertrophy, which frequently progresses to heart failure.

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