Endothelin-1 critically influences cardiac function via superoxide-MMP9 cascade

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We have generated low-expressing and high-expressing endothelin-1 genes (L and H) and have bred mice with four levels of expression: L/L, ∼20%; L/+, ∼65%; +/+ (wild type), 100%; and H/+, ∼350%. The hypomorphic L allele can be spatiotemporally switched to the hypermorphic H allele by Cre-loxP recombination. Young adult L/L and L/+ mice have dilated cardiomyopathy, hypertension, and increased plasma volumes, together with increased ventricular superoxide levels, increased matrix metalloproteinase 9 (Mmp9) expression, and reduced ventricular stiffness. H/+ mice have decreased plasma volumes and significantly heavy stiff hearts. Global or cardiomyocyte-specific switching expression from L to H normalized the abnormalities already present in young adult L/L mice. An epithelial sodium channel antagonist normalized plasma volume and blood pressure, but only partially corrected the cardiomyopathy. A superoxide dismutase mimetic made superoxide levels subnormal, reduced Mmp9 overexpression, and substantially improved cardiac function. Genetic absence of Mmp9 also improved cardiac function, but increased superoxide remained. We conclude that endothelin-1 is critical for maintaining normal contractile function, for controlling superoxide and Mmp9 levels, and for ensuring that the myocardium has sufficient collagen to prevent overstretching. Even a modest (∼35%) decrease in endothelin-1 gene (*Edn1*) expression is sufficient to cause cardiac dysfunction.

amiloride | extracellular matrix | reactive oxygen species | sodium retention | tempol

Previous studies have demonstrated that individuals with dilated cardiomyopathy have increased plasma levels of endothelin-1 (1) and elevated endothelin-1 mRNA levels in the heart (2). In addition, two polymorphisms in the endothelin type A receptor gene (*EDNRA*), G231A and C1363T, are associated with differences in the risk for pathogenesis and mortality in patients with idiopathic dilated cardiomyopathy (3, 4). These findings suggest endothelin-1 plays a causative and/or compensatory role in dilated cardiomyopathy.

In animal studies, mice completely lacking endothelin-1 have severe anomalies in the heart and aorta with craniofacial abnormalities and die at age 10–12 d post coitum (5). Mice with a cardiomyocyte-specific deletion of endothelin-1 develop dilated cardiomyopathy as they age or if they are subjected to aortic banding when young (6). In the opposite direction, mice having a conditional ∼10-fold overexpression of endothelin-1 in the heart also develop dilated cardiomyopathy associated with increases in the expression of inflammatory cytokines (7).

To gain a better understanding of the physiological role of endothelin-1 in mammals, we have used a method of altering the 3′ untranslated region (UTR) of a gene of interest without changing its 5′ transcriptional regulatory elements (8) to generate mice having four step-wise levels of expression of endothelin-1 covering physiologically likely ranges (from ∼20% normal to ∼350%). We here report that plasma volumes and blood pressure increase progressively as the expression of endothelin-1 gene (*Edn1*) decreases, and that decreasing expression of *Edn1* by as little as 35% causes severely dilated cardiomyopathy. A threefold increase in expression of endothelin-1 causes slight cardiac hypertrophy. Using tissue-specific switching of *Edn1* expression from low to high, we show that increases in superoxide levels and expression of matrix metalloproteinase 9 (Mmp9) play important roles in the cardiac dysfunction of the endothelin-1 hypomorphs. Together, our results show that cardiac function is sensitive to even modest decreases in endothelin-1 levels.

Generation of Hypo/Hypermorphic Mice for Endothelin-1

As a first step in generating modified forms of the *Edn1* gene expressing low or high levels of endothelin-1 for use in studies of the effects of both loss of function and gain of function of endothelin-1, we first compared the relative effects on mRNA stability of the 3′ UTR of WT gene (*Edn1*) with a panel of other 3′ UTRs, as described previously (9) (*SI Appendix*, Fig. S1). This comparison showed that the 3′ UTR of *Edn1* reduces mRNA stability to the same extent as that caused by the 3′UTR of the *Fos* gene, which has one of the most short-lived mRNAs we have studied (*SI Appendix*, Figs. S2 and S3). This result indicates that the levels of *Edn1* mRNA can change rapidly in response to decreases or increases in demand, as previously described (10).

To generate a form of the *Edn1* gene (L) with lower-than-normal expression, we used gene targeting in mouse embryonic stem cells, as illustrated in Fig. 1*A*, to insert a copy of the AU/U-rich element of the human *FOS* gene into the mouse *Edn1* gene

Significance

Congestive heart failure develops in human patients and experimental animals when the left ventricle becomes dilated. In the present study, mice were generated having graded genetic levels of endothelin-1 from 20% normal to 350% normal by modifying the 3′ untranslated region of the endothelin-1 gene. The 20% and 65% hypomorphs develop dilated cardiomyopathy, whereas the 350% hypermorph has a hypertrophic heart. Increases in superoxide levels and overexpression of matrix metalloproteinase 9 (MMP9) are involved in the development of the dilated cardiomyopathy in the 20% hypomorph. Our results show that endothelin-1 is critical for maintaining normal cardiac contractile function, for controlling superoxide and Mmp9 levels, and for ensuring that the myocardium has sufficient collagen to prevent overstretching.

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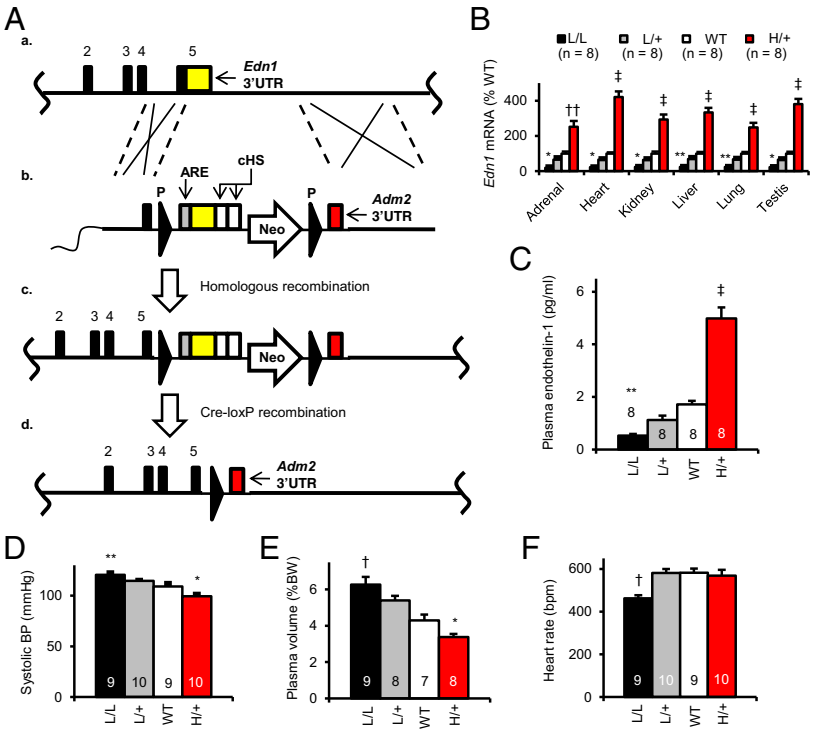

Fig. 1. Generation of hypo/hypermorphic mice for endothelin-1. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{\dagger}P < 0.001$; $^{\dagger\dagger}P < 0.0001$; $^{\ddagger}P < 10^{-5}$ versus WT. (*A*) The gene-targeting strategy. (*a*) The target locus, into which the exogenous 3′ UTR is introduced by homologous recombination, is *Edn1*. (*b*) The targeting vector contains the loxP sequence, the AU/U-rich element (ARE) of human *FOS* 3′ UTR, the natural *Edn1* 3′ UTR, two copies of transcriptional insulators (cHS) of chicken β-globin gene, the neomycin phosphotransferase gene with the MC1 promoter (Neo), loxP (P), and the 3′UTR of the adrenomedullin 2 (*Adm2*) gene. The insulators were inserted to minimize the transcriptional interference between the *Edn1* promoter and pMC1. (*c*) The resulting locus after homologous recombination. The endothelin-1 expression is controlled by ARE, which destabilizes *Edn1* mRNA and decreases endothelin-1 protein (L allele). Coding sequences of the *Edn1* gene are shown as black columns. (*d*) After Cre-loxP recombination, the floxed part is removed and the endothelin-1 expression is controlled by the 3′ UTR of *Adm2*, which stabilizes *Edn1* mRNA and increases endothelin-1 protein (H allele). (*B*) The mRNA levels of *Edn1* in different tissues in the four genotypes of 12-wk-old male mice. (*C*) Plasma concentrations of endothelin-1 in the four genotypes. (*D*) Systolic blood pressure (BP) on normal chow (0.26% [wt/wt] NaCl) determined with a tail-cuff method. (*E*) Plasma volume normalized by body weight (BW). (*F*) Heart rate on normal chow with a tail-cuff method.

just downstream of the stop codon, and therefore upstream of the normal 3′ UTR of *Edn1*. [Inserting this AU/U-rich element upstream of the 3′UTR of a test gene is known from our previous work to lower gene expression levels to approximately one-tenth (9).] To enable switching of the resulting L allele into a high-expressing form (H) by Cre-loxP recombination, the L allele also included loxP sites and the 3′UTR from a stable mRNA: that of the adrenomedullin 2 gene.

L/+ mice were generated from embryonic stem cells having the L allele, and H/+ mice were derived from them by breeding with a mouse carrying a globally expressed Cre-recombinase gene. By further breeding, mice were obtained that have the desired combinations of the mutated (L and H) and WT (+) alleles. Fig. 1*B* shows that the resulting mice have comparably graded levels of *Edn1* expression in the adrenal gland, heart, kidney, liver, lung, and skin. (In this and subsequent figures, the L/L level is shown as a black bar, L/+ level as a gray bar, WT level as a white bar, and H/+ level as a red bar.) The average mRNA levels relative to WT (100%) in the L/L, L/+, and H/+ mice were, respectively, ∼20%, ∼65%, and ∼350% WT. The L/L, L/+, and H/+ mice had 31%, 65%, and 291% WT levels of the mature peptide in their plasma (Fig. 1*C*). Thus, our study mice have progressively graded levels of *Edn1* expression ranging in all tissues from ∼1/5 to ∼3.5 times normal, along with parallel changes in plasma endothelin-1.

Survival and Baseline Characteristics of Endothelin-1 Hypo/Hypermorphic Mice

Homozygosity for the H allele proved to be embryonically lethal; no H/H embryos were found at 9.5 d post coitum. In contrast, the L/L, L/+, WT, and H/+ mice were born and reached adulthood in the expected Mendelian ratios and were fertile. The median survival age of the L/L and L/+ mice was 560 and 632 d, respectively (*SI Appendix*, Fig. S4). WT mice have a median survival of 841 d. The median survival age of the H/+ mice was 876 d, which is not different from WT.

The baseline characteristics of the L/L, L/+, WT, and H/+ mice were largely indistinguishable (*SI Appendix*, Table S1). These characteristics included body weight, kidney weight, heart weight, and their ratios; plasma glucose; insulin; urea nitrogen; creatinine; cholesterol; triglyceride; sodium; potassium; and chloride. However, the H/+ mice had significantly greater heart/body weights than WT mice.

Systolic blood pressures and plasma volumes increased stepwise and in parallel as *Edn1* expression decreased (Fig. 1 *D* and *E*). The pulse rate of the L/L mice, but not of the L/+ or H/+ mice, was significantly slower than that of WT mice (Fig. 1*F*). Thus, progressive changes in the level of expression of the *Edn1* gene do not affect the survival to adulthood and fertility of the study mice but do cause progressive changes in their plasma volumes and systolic blood pressures.

Dilated Cardiomyopathy in the Endothelin-1 L/L and L/+ Mice

Despite the normal survival of young adult endothelin-1 L/+ and L/L mice, the left and right ventricles of their hearts at 12 wk of age were markedly and progressively dilated as the expression of *Edn1* decreased (Fig. 2*A*). Remarkably, however, AZAN (azocarmine + aniline blue) trichrome staining showed no overt fibrosis in the dilated hearts. The hearts of the H/+ mice were slightly larger than WT but appeared to be compact and muscular, again without overt fibrosis. The changes in *Edn1* expression caused changes in the ultrastructure of the cardiomyocytes (Fig. 2*B*). Specifically, the L/L and L/+ cardiomyocytes contained autophagosomes, which were more prominent in the L/L mice than in the L /+ mice, which suggests the occurrence of progressively increasing damage to and turnover of mitochondria as the expression of *Edn1* decreases. This suggestion is supported by our finding that the ratio of processed to unprocessed microtubule-associated protein 1 light chain 3, an index of autophagy (11), is more than normal in the hearts of the L/+ and L/L mice and was less than normal in the H/+ mice (*SI Appendix*, Fig. S5). In contrast, the ultrastructure of the H/+ cardiomyocytes differed little from WT, except for the presence of intramyocellular lipid droplets. Previous studies have shown that an increase in intramyocellular lipid droplets is associated

with improvements in muscular function that result from training (12). We conclude that reduced *Edn1* expression results in mitochondrial damage, whereas modestly increased *Edn1* expression appears to have beneficial effects on metabolism.

The functional consequences of the different levels of *Edn1* expression and quantification of the changes in thickness of the heart walls were assessed by echocardiography (Fig. 2 *C* and *D* and *SI Appendix*, Figs. S6 and S7). Cardiomyocyte fractional shortening (FS) and ventricular ejection fractions (EF), clear indicators of cardiac function, were reduced in both the L/+ and L/L hearts but did not differ from WT in the H/+ mice (Fig. 2*D*). As an assay of function, we carried out in vivo pressure volume loop analysis with the study mice. The slope of end-systolic pressure volume relationship, an index of myocardial contractility, showed a progressive decrease as *Edn1* expression decreased below normal, indicating a decrease in myocardial function, but was unaffected in the H/+ mice (Fig. 2*E*). In contrast, the slope of end-diastolic pressure volume relationship, an index of ventricular stiffness, was more than twice normal in the H/+ mice, whereas the L/L and L/+ mice had progressively less-than-normal ventricular stiffness (Fig. 2*F*), thereby making their hearts susceptible to dilatation. We conclude that cardiac function and stiffness deteriorates as *Edn1* expression decreases, whereas increased *Edn1* expression has little effect on function except that it makes the cardiomyocytes stiffer than normal.

We investigated the ventricular expression in the study mice of several genes that are responsive to cardiovascular insults (Fig. 2*G*). Expression of the natriuretic peptide precursor genes (*Nppa* and *Nppc*) were not significantly different among the four genotypes; *Nppb* expression, increases of which are generally considered to be indicative of ventricular dysfunction, decreased rather than increased as ventricular function deteriorated (*SI Appendix*, Table S3). Expression of *Myh7*, the myosin beta heavy chain gene, showed a remarkably strong gradation in expression that paralleled that of *Edn1*. Expression of *Myh6*, the myosin alpha heavy chain gene, was not affected by *Edn1* expression.

MMPs play a key role in modulating fibrosis. Accordingly, we determined expression of *Mmp2* and *Mmp9* in the study mice (Fig. 2*H*). *Mmp2* gene expression was not affected by *Edn1* expression, but *Mmp9* mRNA levels more than doubled in the hearts of the L/+ mice, more than quadrupled in the L/L mice, and were less than normal in the H/+ mice. Mmp9 protein levels in the hearts changed in parallel with the mRNA levels (Fig. 3*G*). We conclude that changes in levels of *Edn1* cause marked progressive reciprocal changes in cardiac levels of Mmp9.

Effects on the Cardiovascular Abnormalities of Young Adult L/L Mice of Tissue-Specifically Switching Endothelin-1 Expression from L to H

We next determined whether any of the cardiac abnormalities caused by low expression of *Edn1* can be reversed if expression is switched to high in specific tissues. To do this, we used appropriate matings to generate L/L mice that also carried one of three Cre transgenes that cause *Edn1* expression to switch from low to high after induction, as illustrated in Fig. 1*A*, *c* and *d*. The first was a tamoxifen-inducible CAG promoter-driven transgene (CAG-cre/Esr1), which is globally expressed after induction (13). The second was a tamoxifen-inducible transgelin promoter-driven transgene (Tagln-cre/ERT2), expressed in adult cardiomyocytes and smooth muscle cells after tamoxifen induction (14, 15). The third was a doxycycline-inducible troponin T (Tnnt2) promoter-driven Cre transgene (Tnnt2-rtTA/TetO-cre), which is expressed in cardiomyocytes after induction (16).

At 12 wk of age, all three transgene-carrying mice had the increased blood pressure seen in L/L mice not carrying any transgenes (*SI Appendix*, Fig. S8), and echocardiography showed that they had already developed the abnormal cardiac function and dilated cardiomyopathy seen in L/L mice not carrying any

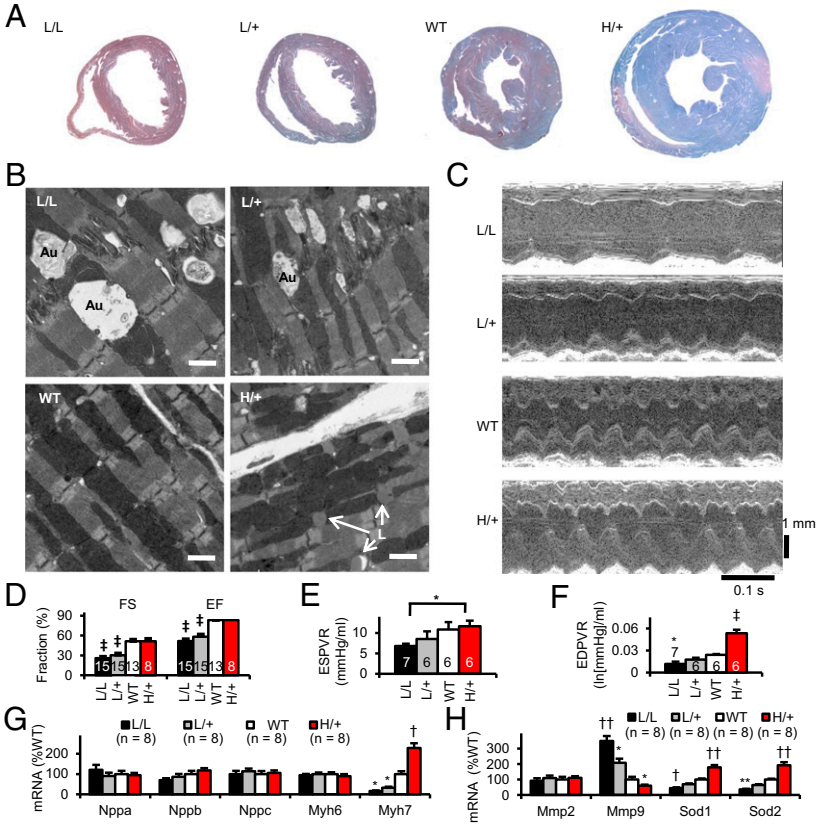

Fig. 2. Cardiac phenotype in male mice at age 12 wk. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{\dagger}P < 0.001$; $^{\dagger\dagger}P < 0.0001$; $^{\ddagger}P < 10^{-5}$ versus WT. (*A*) Representative cross-sections of the heart with Heidenhain's AZAN trichrome staining. (*B*) Transmission electron microscopy of the heart. Autophagosomes (Au) are frequently found in the L/L and L/+ mice. Lipid droplets (L) are prominent in the H/+ mice. (Scale bar, 1 μm.) (*C*) Representative M-mode images of echocardiography. (*D*) Echocardiographically determined systolic function of the left ventricle. (*E*) End-systolic pressure volume relationship (ESPVR) determined by in vivo pressure-volume loop analysis. (*F*) End-diastolic pressure volume relationship (EDPVR) determined by the pressure-volume loop analysis. (*G*) mRNA levels for natriuretic peptides and myosin heavy chains in the heart. (*H*) mRNA levels for Mmp2 and Mmp9 and Sod1 and Sod2 in the heart.

transgenes (*SI Appendix*, Figs. S9–12 *A*, *C*, and *E*). The mice were then treated with tamoxifen or doxycycline to induce Cre and cause switching of *Edn1* expression from low to high. Four weeks later, their cardiovascular status was reassessed.

The results show that switching from low to high had differing effects, depending on the tissue in which the switching occurred. Thus, in the mice having the tamoxifen-inducible ubiquitously expressed CAG-cre transgene (orange bars in Fig. 3), switching occurred in all tissues, and blood pressure was normalized (Fig. 3*A*). Echocardiography (*SI Appendix*, Fig. S12*B*) showed that myocyte FS and ventricular EF were normalized (Fig. 3*B*) and that the thinning of the interventricular septa and left ventricular posterior walls that occurs in untreated L/L mice was resolved (Fig. 3*C*). The left ventricular diameters also returned to normal (orange bars in *SI Appendix*, Fig. S13). Thus, the cardiovascular abnormalities already present in young adult mice with low expression of *Edn1* resolve if the expression is subsequently switched to high throughout the body.

In mice having the tamoxifen-inducible Tagln-cre transgene, switching from low to high in cardiomyocytes and smooth muscle cells (brown bars in Fig. 3) did not correct blood pressure. However, echocardiography (*SI Appendix*, Fig. S12*D*) showed that myocyte FS and ventricular EF were restored (Fig. 3*B*), although less well than with the ubiquitously expressed transgene. The thinning of the interventricular septa and left ventricular posterior walls was almost completely reversed (Fig. 3*C*). The left ventricular diameters were partially corrected (brown bars in *SI Appendix*, Fig. S13). Thus, although restoring expression of *Edn1* in cardiomyocytes and smooth muscle cells walls does not normalize blood pressure of the L/L mice, it restores cardiac function and substantially improves ventricular wall thickness.

The effects of switching from low to high only in cardiomyocytes (green bars in Fig. 3) were indistinguishable from those observed when switching also occurred in smooth muscle cells. We conclude that the cardiac abnormalities observed in L/L mice are not a result of low expression of *Edn1* in smooth muscle cells.

Both global and cardiomyocyte-specific overexpression of *Edn1* restored the lifespan of the L/L mice to near normal (*SI Appendix*, Fig. S14), suggesting that the shorter-than-normal lifespan in the L/L mice is largely caused by heart problems.

Effects of Pharmacological Agents on the Cardiovascular Abnormalities of Young Adult L/L Mice

Administration of amiloride, a diuretic inhibitor of the epithelial sodium channel ENaC (yellow bars in Fig. 3), normalized the blood pressure of the L/L mice (Fig. 3*D*) but incompletely corrected myocyte FS and ventricular EF (Fig. 3*E*). The diuretic had no effect on septal and ventricular wall thickness (Fig. 3*F*) or on cardiac metalloproteinase Mmp9 expression (Fig. 3*G*). Left ventricular dilatation was partially corrected (yellow bars in *SI Appendix*, Fig. S15). Thus, the effects of a diuretic on the status

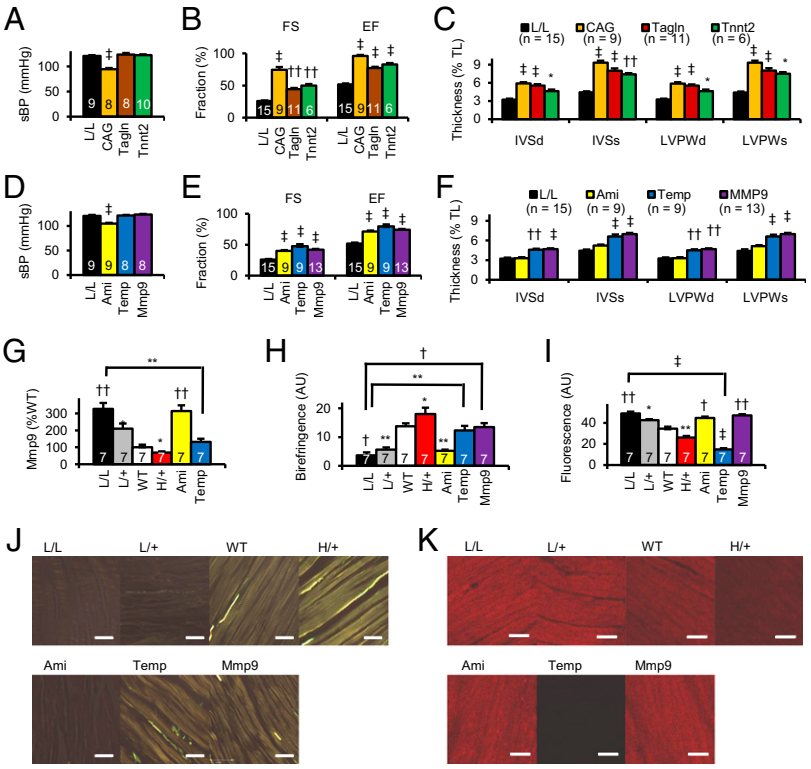

Fig. 3. Effects of postnatally global or tissue-specific overexpression of endothelin-1, pharmacological agents, and genetic deficiency of Mmp9 on the cardiac phenotype in the L/L mice. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{\dagger}P < 0.001$; $^{\dagger\dagger}P < 0.0001$; $^{\ddagger}P < 10^{-5}$ versus L/L. (*A*) Systolic blood pressure (sBP) in the L/L mice with postnatally global or tissue-specific overexpression of endothelin-1. CAG, L/L mice with CAG-cre; Tagln, L/L mice with Tagln-cre; Tnnt, L/L mice with Tnnt2-rtTA and TetO-cre. (*B*) Systolic function of the left ventricle. (*C*) Ultrasonographically determined thicknesses of the left ventricle (LV) of the heart in mice with postnatally global or tissue-specific overexpression of endothelin-1. TL, tibial length; IVSd and IVSs, interventricular septum thickness in diastole and systole; LVPWd and LVPWs, LV posterior wall thickness in diastole and systole. (*D*) sBP in the L/L mice with pharmacologic agents and genetic deficiency of Mmp9. Ami, L/L mice with amiloride; Temp, L/L mice with tempol; Mmp9, L/L mice with genetic deficiency of Mmp9. (*E*) Systolic function of the left ventricle. (*F*) Ultrasonographically determined thicknesses of the LV of the heart in mice with pharmacologic agents and genetic deficiency of Mmp9. (*G*) Protein levels of Mmp9 in the heart. (*H*) Comparison of Picrosirius Red birefringence. Tempol or Mmp9 deficiency significantly improved the birefringence, but amiloride did not, in the L/L mice. (*I*) Comparison of 2-dihydroethidium fluorescence in the heart. Amiloride and deficiency of Mmp9 did not significantly change the fluorescence in the L/L mice. (*J*) Collagen studied by Picrosirius Red birefringence in the heart. (Scale bar, 20 μm.) (*K*) Superoxide studied by 2-dihydroethidium fluorescence in the heart. (Scale bar, 20 μm.)

of the L/L mice are modest, suggesting they are probably the indirect effects of reducing plasma volume.

Tempol, a stable free radical that acts as a superoxide dismutase (SOD) mimetic (blue bars in Fig. 3), did not change blood pressure in the L/L mice (Fig. 3*D*), although it modestly improved myocyte FS and ventricular EF (Fig. 3*E*). However, in contrast to amiloride, tempol substantially increased interventricular septal thickness and left posterior ventricular wall thickness (Fig. 3*F*) and partially corrected ventricular dilatation (blue bars in *SI Appendix*, Fig. S15). In addition, tempol administration resolved the abnormally high cardiac Mmp9 levels of L/L mice (Fig. 3*G*).

Effects of Genetic Deficiency of Mmp9 on the Cardiac Function in the L/L Mice

In view of this observation that cardiac expression of Mmp9 is affected by tempol and is markedly and progressively increased in the L/+ and L/L mice (Fig. 2*H*), we asked whether genetic absence of Mmp9 would have any effects on the cardiac abnormalities of the L/L mice. To do this, we used breeding to obtain L/L mice that also have the *Mmp9* gene knocked out (17). Lack of Mmp9 (purple bars in Fig. 3) did not significantly affect blood pressure (Fig. 3*D*). However, it significantly improved myocyte FS and ventricular EF (Fig. 3*E*) and septal and ventricular wall thickness (Fig. 3*F*) and removed ventricular dilatation (purple bars in *SI Appendix*, Fig. S15) to the same extent as tempol. We conclude that Mmp9 overproduction in the L/L mice is an important contributor to their ventricular dilatation.

Cardiac Collagen in the Study Mice

Although none of our study mice develop overt fibrosis, with or without additional treatments or genetic modifications, the involvement of Mmp9 in their cardiac abnormalities raises the possibility that the amount of collagen in their hearts affects their phenotypes. Accordingly, as measured in Fig. 3*H* and illustrated in Fig. 3*J*, we determined the Picrosirius Red birefringence (a measure of collagen type/amount) of the hearts of untreated L/L, L/+, WT, and H/+ mice, as well as of L/L mice that had received amiloride or tempol or genetically lacked Mmp9. The results are clear: the birefringence in the L/L mice (black bar) and L/+ mice (gray bar) was less than 50% that of WT mice (white bar), whereas that in the H/+ mice (red bar) was increased to ∼125%. The low birefringence of the L/L mice was not changed by treatment with amiloride (yellow bar). In contrast, it was essentially normalized by treatment with tempol (blue bar), or when the production of Mmp9 was genetically knocked out (purple bar). We conclude that the collagen content of the hearts of the mice with even modestly less than normal *Edn1* expression is considerably reduced, whereas that of mice with higher expression is slightly above normal; both tempol and absence of Mmp9 increase the amount of collagen in the hearts of the L/L mice.

Cardiac Superoxide in the Study Mice

The marked improvements of the cardiac abnormalities in the L/L mice caused by the administration of tempol emphasize the importance of knowing the levels of superoxide in the study mice. Accordingly, as measured in Fig. 3*I* and illustrated in Fig. 3*K*, we determined the fluorescence of 2-hydoxyethidium generated from dihydroethidium by superoxide (18, 19) in sections of the hearts of our study mice. Fluorescence was higher in the L/+ hearts (gray bar) than in the WT hearts (white bar), and higher still in the hearts of the L/L mice (black bar). Fluorescence was lower in the H/+ hearts (red bar) than in WT. Administration of tempol reduced superoxide levels in the L/L mice to subnormal levels (blue bar). Fluorescence was not reduced significantly in the hearts of the L/L mice treated with amiloride (yellow bar) or with genetic absence of Mmp9 (purple bar).

Cardiac Expression of SODs in the Study Mice

The increased superoxide levels in the hearts of the L/L mice, and the beneficial effects of tempol, raised the possibility that changes in the expression of *Edn1* might be causing comparable changes in the expression of the SODs. This was confirmed by our finding a marked decrease in cardiac transcript and protein levels for *Sod1* and *Sod2* in the L/L mice (Fig. 2*H* and *SI Appendix*, Fig. S16). In the reverse direction, a significant increase in *Sod1* and *Sod2* expression was found in the H/+ mice. We conclude that endothelin-1 plays an important role in controlling the cardiac expression of SODs, and thence the levels of superoxide in the heart.

Discussion

The molecular pathway underlying the increases in plasma volumes and blood pressure that we find as *Edn1* expression is decreased from below normal in the L/+ and L/L mice is readily identified. Thus, previous studies have shown that renal-collecting duct-derived endothelin-1 acting via endothelin B (ETB) receptors promotes Na^+ excretion (20) and that stimulation of both the endothelin A (ETA) and ETB receptor inhibits ENaC activity (8, 21). We conclude that the increase in blood pressure that occurs as endothelin-1 levels decrease is a result of an increase in renal ENaC activity and the increase in plasma volume, whereas the H/+ mice have lower than normal blood pressure and plasma volume because of a decrease in renal ENaC activity.

Despite the increased plasma volumes in the L/L and L/+ mice, expression of two of the natriuretic peptide precursors, *Nppa* and *Nppc*, were not significantly different among the four genotypes, although *Nppb* expression tended to increase as *Edn1* expression increased (*SI Appendix*, Table S3; $P = 0.0116$ for significant slope). This result is in agreement with several previous findings. Thus, both ETA and ETA/ETB receptor antagonists decrease plasma atrial natriuretic peptide responses to volume overload (22); the nonselective endothelin receptor antagonist bosentan blocks the increase in *Nppb* mRNA levels produced by pressure overload in the left atria (23), and anti-endothelin-1 serum decreases the percentage of cells that secrete C-type natriuretic peptides in human vascular endothelial cells (24). Together, these studies suggest that below-normal expression of *Edn1* probably suppresses the increased expression of natriuretic peptides that is the normal response to volume expansion. Such a failure to properly induce natriuretic peptides would likely aggravate the fluid retention caused by the renal effects of decreased *Edn1* expression and would contribute to the increased plasma volumes of the L/L and L/+ hypomorphs.

Previous reports indicate that enhanced autophagy is associated with increased oxidative stress (25, 26). The decreased transcript levels for *Sod1* and *Sod2* that we have observed in the L/L and L/+ mice account for their increased levels in superoxide, which in turn contributes to their dilated cardiomyopathy. The effect is probably direct, as it is reversed by the administration of the stable free radical scavenger tempol. In support of these inferences, mice lacking SOD2 exhibit severe dilated cardiomyopathy with mitochondrial damage in their cardiomyocyte (27). In addition, a recent proteomic screening study has demonstrated that SOD2 protein levels are increased in transgenic mice expressing increased amounts of endothelin-1 (28).

Our L gene was designed to enable Cre recombinase-inducible switching of expression from L to H at will in a tissue-specific manner. With its use, we found that dilated cardiomyopathy already established in young adult L/L mice was almost completely reversed by switching expression from low to high throughout the body. Switching in cardiomyocytes only was equally effective. These findings are important in establishing that dilated cardiomyopathy caused by less-than-normal expression of *Edn1* is reversible.

Administration of the diuretic amiloride corrected the blood pressure and partially removed the ventricular dilatation that

occurs in the L/L mice but did not correct the increased Mmp9 levels. The SOD mimetic tempol, in contrast, failed to correct the increased blood pressure of the L/L mice but abolished the increased superoxide, which damages cardiomyocytes. It also reduced the increased level of the metalloproteinase Mmp9, which degrades ventricular collagen. Thus, our results suggest that pharmaceutical interventions affecting either superoxide and/or MMP9 levels should be considered for reversing the cardiovascular problems caused by decreased *Edn1* expression. The interplay between these two apparently unrelated factors has been recently described in relation to cardiac rupture after myocardial infarction (29) and neurodegeneration in amyotrophic lateral sclerosis (30). At what level and how the two systems interact remains to be determined.

The increased blood pressure in our L/L and L/+ mice probably contributes to their developing well-established dilatation by age 12 wk without the need to stress them, whereas mice with a cardiac-specific knock-out of the *Edn1* gene only developed dilatation at a much older age (≥8 mo) or after aortic banding to increase their intraventricular blood pressure (6).

In the opposite direction, transgenic mice with ∼15 times normal cardiomyocyte-specific expression of endothelin-1 peptide develop marked cardiac hypertrophy with dilatation and contractile dysfunction, and they die of congestive heart failure between 5 and 11 wk after the transgene induction (7), whereas our H/+ mice globally expressing ∼3.5 times normal levels of *Edn1* have only slightly hypertrophic hearts with good function. Thus, threefold higher than normal *Edn1* expression has small possibly even beneficial effects on cardiac function, but 15-fold higher expression is harmful.

An important question our study answers is whether there are any cardiac consequences of modest decreases in *Edn1* expression, such as might occur in humans either clinically or as a result of genetic differences. The answer is clear: even the 35% decrease in Edn1 expression we see in the L/+ mice causes dilated cardiomyopathy and severe cardiac dysfunction. Similar detrimental effects can be expected in patients treated with endothelin receptor antagonists. Indeed, it has been reported that adverse effects including fluid retention, congestive heart failure, and death resulted in discontinuation of trial medication of an endothelin ETA receptor antagonist (31).

In summary, we find that mice with progressively decreased expression of *Edn1*, the gene coding for endothelin-1, have increased plasma volumes and hypertension and spontaneously develop severe dilated cardiomyopathy together with increased superoxide levels and increased expression of matrix metalloproteinase Mmp9 in their hearts. The cardiovascular abnormalities already established in young adult hypomorphs were almost completely reversed by switching *Edn1*expression from low to high throughout the body or in cardiomyocytes only. The increased plasma volumes and blood pressure of the hypomorphs were normalized by an ENaC antagonist, although superoxide levels and Mmp9 expression remained high and the impaired cardiac function was only partially ameliorated. In contrast, a SOD mimetic decreased both superoxide levels and the expression of Mmp9 in the hypomorphs and greatly improved the condition of their hearts, although it did not change blood pressure. Nevertheless, these experiments show that the cardiovascular abnormalities caused by less-than-normal *Edn1* expression are amenable to correction by pharmacologic treatment. Genetic deficiency of Mmp9 also mitigated the dilated cardiomyopathy in the hypomorphs but did not change the superoxide levels. Together, our results show that endothelin-1 is critical for maintaining normal contractile function, for controlling superoxide and Mmp9 levels, and for ensuring that the myocardium has sufficient collagen to prevent overstretching during systole. Even a modest (∼35%) decrease in *Edn1* expression is sufficient to cause severe cardiovascular dysfunction.

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1. Hiroe M, et al. (1991) Plasma endothelin-1 levels in idiopathic dilated cardiomyopathy. *Am J Cardiol* 68(10):1114–1115.
2. Plumpton C, Ashby MJ, Kuc RE, O'Reilly G, Davenport AP (1996) Expression of endothelin peptides and mRNA in the human heart. *Clin Sci (Lond)* 90(1):37–46.
3. Telgmann R, et al. (2007) The G-231A polymorphism in the endothelin-A receptor gene is associated with lower aortic pressure in patients with dilated cardiomyopathy. *Am J Hypertens* 20(1):32–37.
4. Herrmann S, et al. (2001) A polymorphism in the endothelin-A receptor gene predicts survival in patients with idiopathic dilated cardiomyopathy. *Eur Heart J* 22(20):1948–1953.
5. Kurihara Y, et al. (1994) Elevated blood pressure and craniofacial abnormalities in mice deficient in endothelin-1. *Nature* 368(6473):703–710.
6. Zhao XS, Pan W, Bekeredjian R, Shohet RV (2006) Endogenous endothelin-1 is required for cardiomyocyte survival in vivo. *Circulation* 114(8):830–837.
7. Yang LL, et al. (2004) Conditional cardiac overexpression of endothelin-1 induces inflammation and dilated cardiomyopathy in mice. *Circulation* 109(2):255–261.
8. Lynch IJ, Welch AK, Kohan DE, Cain BD, Wingo CS (2013) Endothelin-1 inhibits sodium reabsorption by ET(A) and ET(B) receptors in the mouse cortical collecting duct. *Am J Physiol Renal Physiol* 305(4):F568–F573.
9. Kakoki M, et al. (2004) Altering the expression in mice of genes by modifying their 3′ regions. *Dev Cell* 6(4):597–606.
10. Mawji IA, Robb GB, Tai SC, Marsden PA (2004) Role of the 3′-untranslated region of human endothelin-1 in vascular endothelial cells. Contribution to transcript lability and the cellular heat shock response. *J Biol Chem* 279(10):8655–8667.
11. Kabeya Y, et al. (2000) LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *EMBO J* 19(21):5720–5728.
12. Tarnopolsky MA, et al. (2007) Influence of endurance exercise training and sex on intramyocellular lipid and mitochondrial ultrastructure, substrate use, and mitochondrial enzyme activity. *Am J Physiol Regul Integr Comp Physiol* 292(3):R1271–R1278.
13. Hayashi S, McMahon AP (2002) Efficient recombination in diverse tissues by a tamoxifen-inducible form of Cre: A tool for temporally regulated gene activation/inactivation in the mouse. *Dev Biol* 244(2):305–318.
14. Kühbandner S, et al. (2000) Temporally controlled somatic mutagenesis in smooth muscle. *Genesis* 28(1):15–22.
15. Li L, Miano JM, Mercer B, Olson EN (1996) Expression of the SM22alpha promoter in transgenic mice provides evidence for distinct transcriptional regulatory programs in vascular and visceral smooth muscle cells. *J Cell Biol* 132(5):849–859.
16. Wu B, et al. (2010) Inducible cardiomyocyte-specific gene disruption directed by the rat Tnnt2 promoter in the mouse. *Genesis* 48(1):63–72.
17. Vu TH, et al. (1998) MMP-9/gelatinase B is a key regulator of growth plate angiogenesis and apoptosis of hypertrophic chondrocytes. *Cell* 93(3):411–422.
18. Robinson KM, et al. (2006) Selective fluorescent imaging of superoxide in vivo using ethidium-based probes. *Proc Natl Acad Sci USA* 103(41):15038–15043.
19. Dugan LL, et al. (2013) AMPK dysregulation promotes diabetes-related reduction of superoxide and mitochondrial function. *J Clin Invest* 123(11):4888–4899.
20. Ge Y, et al. (2006) Collecting duct-specific knockout of the endothelin B receptor causes hypertension and sodium retention. *Am J Physiol Renal Physiol* 291(6):F1274–F1280.
21. Bugaj V, et al. (2008) Regulation of the epithelial Na+ channel by endothelin-1 in rat collecting duct. *Am J Physiol Renal Physiol* 295(4):F1063–F1070.
22. Leskinen H, Vuolteenaho O, Ruskoaho H (1997) Combined inhibition of endothelin and angiotensin II receptors blocks volume load-induced cardiac hormone release. *Circ Res* 80(1):114–123.
23. Magga J, Vuolteenaho O, Marttila M, Ruskoaho H (1997) Endothelin-1 is involved in stretch-induced early activation of B-type natriuretic peptide gene expression in atrial but not in ventricular myocytes: Acute effects of mixed ET(A)/ET(B) and AT1 receptor antagonists in vivo and in vitro. *Circulation* 96(9):3053–3062.
24. Evans JJ, Youssef AH, Yandle TG, Lewis LK, Nicholls MG (2002) Effects of endothelin-1 on release of adrenomedullin and C-type natriuretic peptide from individual human vascular endothelial cells. *J Endocrinol* 175(1):225–232.
25. Brunk UT, Jones CB, Sohal RS (1992) A novel hypothesis of lipofuscinogenesis and cellular aging based on interactions between oxidative stress and autophagocytosis. *Mutat Res* 275(3-6):395–403.
26. Marambio P, et al. (2010) Glucose deprivation causes oxidative stress and stimulates aggresome formation and autophagy in cultured cardiac myocytes. *Biochim Biophys Acta* 1802(6):509–518.
27. Li Y, et al. (1995) Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase. *Nat Genet* 11(4):376–381.
28. Vignon-Zellweger N, et al. (2014) Endothelin-1 overexpression and endothelial nitric oxide synthase knock-out induce different pathological responses in the heart of male and female mice. *Life Sci* 118(2):219–225.
29. He BJ, et al. (2011) Oxidation of CaMKII determines the cardiotoxic effects of aldosterone. *Nat Med* 17(12):1610–1618.
30. Kaplan A, et al. (2014) Neuronal matrix metalloproteinase-9 is a determinant of selective neurodegeneration. *Neuron* 81(2):333–348.
31. Mann JF, et al.; ASCEND Study Group (2010) Avosentan for overt diabetic nephropathy. *J Am Soc Nephrol* 21(3):527–535.