Essential role of sympathetic endothelin A receptors for adverse cardiac remodeling

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In preclinical studies, endothelin receptor A (ET_A) antagonists (ET_Ai) attenuated the progression of heart failure (HF). However, clinical HF trials failed to demonstrate beneficial effects of ET_Ai. These conflicting data may be explained by the possibility that established HF drugs such as adrenergic receptor blockers interfered with the mechanism of ET_Ai action in clinical trials. Here we report that mice lacking ET_A only in sympathetic neurons (SN-KO) showed less adverse structural remodeling and cardiac dysfunction in response to pathological pressure overload induced by transverse aortic constriction (TAC). In contrast, mice lacking ET_A only in cardiomyocytes (CM-KO) were not protected. TAC led to a disturbed sympathetic nerve function as measured by cardiac norepinephrine (NE) tissue levels and [^{124}I]-metaiodobenzylguanidine-PET, which was prevented in SN-KO. In a rat model of HF, ET_Ai improved cardiac and sympathetic nerve function. In cocultures of cardiomyocytes (CMs) and sympathetic neurons (SNs), endothelin-1 (ET1) led to a massive NE release and exaggerated CM hypertrophy compared with CM monocultures. ET_A-deficient CMs gained a hypertrophic response through wild-type SNs, but ET_A-deficient SNs failed to mediate exaggerated CM hypertrophy. Furthermore, ET1 mediated its effects indirectly via NE in CM-SN cocultures through adrenergic receptors and histone deacetylases, resulting in activation of the prohypertrophic transcription factor myocyte enhancer factor 2. In conclusion, sympathetic ET_A amplifies ET1 effects on CMs through adrenergic signaling pathways. Thus, antiadrenergic therapies may blunt potentially beneficial effects of ET_Ai. Taken together, this may indicate that patients with β blocker intolerance or disturbed sympathetic nerve function could be evaluated for a potential benefit from ET_Ai.

β adrenergic signaling | sympathetic nervous system | norepinephrine reuptake | epigenetic regulation | HDACs

Heart failure (HF) is common and, despite therapeutic advancements, is linked to a poor prognosis (1). Various neurohormones are elevated and correlate with the severity of HF (2, 3). Among them, the role of endothelin-1 (ET1) has been extensively studied (4, 5). ET1 was shown to be elevated in blood samples of HF patients, to predict outcome and to play a crucial role for adverse cardiac remodeling (6). In 1996, it was demonstrated that endothelin receptor inhibition is cardioprotective in a preclinical model of HF (7), which was supported by others (8–10). However, despite convincing preclinical data, unspecific ET1 receptor and specific ET_A inhibitors (ET_Ai) showed no beneficial effects in clinical HF trials (11, 12). In contrast to animal studies, HF patients in clinical trials are usually cotreated with other HF drugs such as angiotensin-converting enzyme inhibitors or adrenergic receptor antagonists, raising the possibility that these drugs interfere with the effects of ET_Ai. Conflicting data came also from genetic studies showing that mice lacking ET_A only in cardiomyocytes (CM-KO) were not protected when exposed to cardiac stress (13). Thus, the question has arisen of whether ET_A in nonmyocytes affects critical CM signaling pathways via cell–cell communication mechanisms.

ET_A is also expressed in neural crest derived tissue such as sympathetic neurons (SNs) that innervate the heart and release norepinephrine (NE) (14, 15), one of the neurotransmitters that regulate cardiac function and growth. The success of HF therapy with adrenergic receptor antagonists that inhibit the target receptor of NE in adult CMs underscores the pivotal role of NE in HF (16). Consequently, the mechanisms by which NE is released from sympathetic neurons impact on signaling cascades in CMs (17–19). We now hypothesized that ET_Ai mediates their

Significance

In failing hearts, norepinephrine (NE) net release and endothelin-1 (ET1) levels are increased. ET1 receptor antagonists were successfully used in preclinical heart failure (HF) studies, but clinical studies did not show beneficial effects in HF patients. We found that mice lacking endothelin receptor A (ET_A) only in sympathetic neurons but not in cardiomyocytes were protected from the development of HF. Mechanistically, the presynaptic reuptake of NE within the heart was preserved in mice lacking ET_A only in sympathetic neurons. These data provide an explanation of why patients in clinical studies do not benefit from ET1 receptor antagonists, because these patients are usually treated with β blockers, which interfere with the mechanism of action identified here.

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beneficial effects not through the inhibition of ET_A on CMs but on SNs and consequently indirectly through an attenuated activation of α-adrenergic receptors (αARs) and β-adrenergic receptors (βARs). In CMs, ET_A, and also αARs and βARs, signals to kinases such as protein kinase D (PKD) and calcium calmodulin dependent kinase II (CaMKII). These kinases induce phosphorylation-dependent nucleo-cytoplasmic shuttling of class II histone deacetylases (HDACs) (20, 21), resulting in the activation of the transcription factor myocyte enhancer factor 2 (MEF2), a crucial inductor of pathological cardiac remodeling (22).

In this study, we investigated two KO mouse lines, one lacking ET_A only in SNs (SN-KO) and another one lacking ET_A only in CMs (CM-KO). We show that SN-ET_A but not CM-ET_A contributes to adverse cardiac remodeling.

Results

Deficiency of ET_A Specifically in SNs but Not in CMs Protects from Pathological Cardiac Remodeling. To investigate the role of ET_A in specific cell types, we generated conditional mutant mice lacking ET_A selectively in SNs (SN-KO) vs. CMs (CM-KO) (Fig. S1*A*). Quantitative RT-PCR revealed that transgenic expression of Cre under the control of the dopamine-β-hydroxylase promoter (DBH-Cre) (23) led to a substantial loss of ET_A in sympathetic ganglia of SN-KO mice, whereas transgenic Cre expression under the control of α-myosin heavy chain promoter (αMHC-Cre) led to a comparable deletion of ET_A in the myocardium of CM-KO mice (Fig. S1*B*). Both lines developed normally without any obvious anatomical abnormalities. We then challenged these lines with pathological pressure overload induced by transverse aortic constriction (TAC) and compared them to control littermates (Ctrl. LM). The degree of TAC was similar in all experimental groups (Fig. S2). CM-KO mice did not show any signs of cardioprotection with regard to cardiac hypertrophy (Fig. S3 *A, D,* and *E*), interstitial fibrosis (Figs. S3 *B* and *F* and S4), function (Fig. S3 *C* and *G*), and pulmonary congestion (Fig. S3*H*) or gene expression (Fig. S3*I*). Strikingly, SN-KO mice did not develop massive cardiac hypertrophy (Fig. 1 *A, D,* and *E*), interstitial fibrosis (Fig. 1 *B* and *F* and Fig. S4), or cardiac dysfunction (Fig. 1 *C* and *G*). Pulmonary congestion as an additional sign of severe left ventricular dysfunction was diminished in SN-KO (Fig. 1*H*). Moreover, dysregulation of genes that are typically associated with adverse cardiac remodeling (including *nppa, myh7, Col3a*, and *Serca)* was attenuated in SN-KO after TAC compared with Ctrl. LM (Fig. 1*I*).

ET_A Impairs Sympathetic Nerve Function in SN-KO After TAC. To test whether sympathetic nerve function is preserved in SN-KO after TAC, sympathetic indices including NE tissue levels and meta-iodobenzylguanidine (MIBG) uptake were determined. Depleted myocardial NE stores indicated a substantial increase in NE net secretion as an estimate for overactivation of the local cardiac sympathetic nervous system (24, 25). Accordingly, we found left ventricular NE tissue levels to be decreased after TAC in Ctrl. LM but not in SN-KO (Fig. 2*A*). To address the question of whether depleted myocardial NE stores were mediated—at least in part—by a reduced NE reuptake via the NE transporter (NET), we used the [^{124}I]-MIBG-PET method to assess cardiac NET activity in vivo (17, 18, 26). This method does not directly measure cardiac sympathetic activity but serves as an index for cardiac sympathetic activity. We found that TAC leads to a reduction of MIBG uptake in Ctrl. LM but not SN-KO, strongly suggesting that ET1 impairs sympathetic nerve function in vivo via ET_A and NET-mediated NE reuptake (Fig. 2 *B* and *C*).

ET_A Inhibitors Improve Sympathetic Nerve Function in a Rat Model of HF. To investigate whether the findings in the mouse models translate to larger rodents, we first perfused isolated hearts of healthy rats with ET1 and found a significantly reduced NE uptake, which could be diminished by pretreatment with darusentan (ET_Ai) (Fig. 3*A*). To study whether ET_Ai normalizes sympathetic nerve function in a rat model of HF, we used salt-sensitive (DS) vs. salt-resistant (DR) Dahl rats, both fed on a high-salt diet (27). DS rats displayed an impaired NE uptake (Fig. 3*B*) and depleted cardiac NE stores (Fig. 3*C*). In accordance with the genetic deletion of ET_A in TAC mice, ET_Ai significantly improved NE uptake and NE stores (Fig. 3 *B* and *C*), confirming an improved

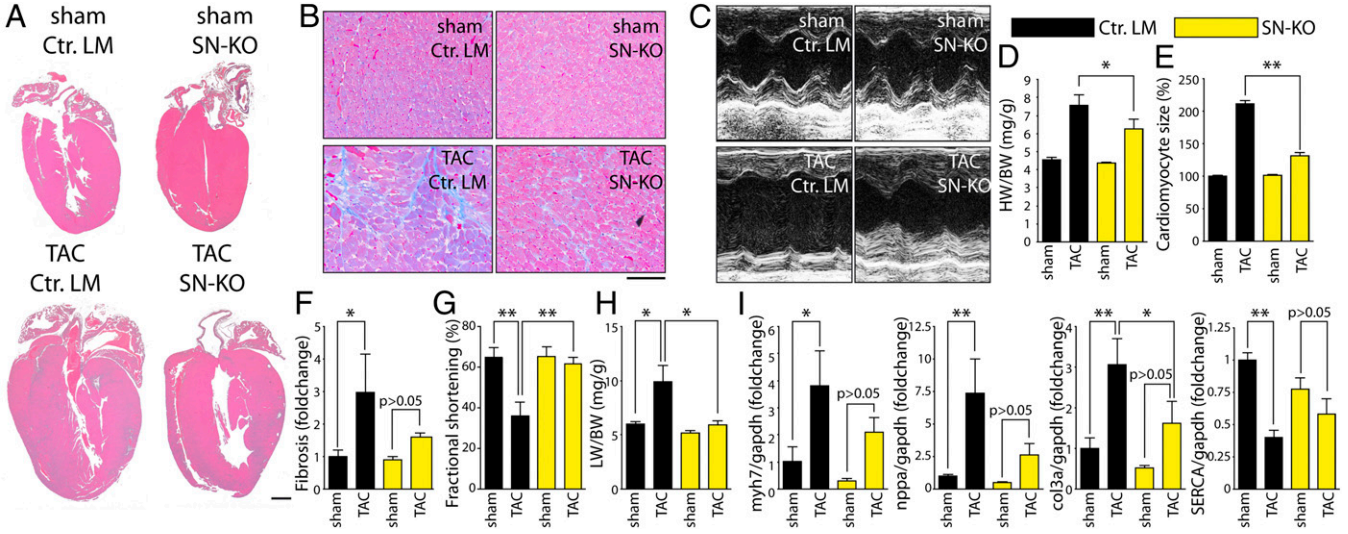


Fig. 1. Sympathetic ET_A is essential for the development of adverse cardiac remodeling. (*A*) Representative four-chamber view of mice lacking ET_A in sympathetic neurons (SN-KO) vs. control littermates with normal ET_A expression (Ctrl. LM) 7 wk after sham or TAC surgery. (Scale bar, 1 mm.) (*B*) Representative trichrome-stained sections. (Scale bar, 100 μm.) (*C*) Representative transthoracic echocardiography of the left ventricle (M-Mode, midventricular). (*D*) Heart weight/body weight (HW/BW) ratios (n = 5–7 per group). (*E*) Quantification of cardiomyocyte size (>80 cells per sample from more than five fields of view; $n \geq 3$). (*F*) Quantification of fibrosis in relation to Ctrl. LM ($n \geq 3$ per group). (*G*) Fractional shortening 3 wk after TAC (n = 5–7 per group). (*H*) Lung weight/body weight (LW/BW) ratios 7 wk after TAC surgery (n = 5–7 per group). (*I*) Transcript levels in hearts from Ctrl. LM and SN-KO 7 wk after TAC surgery from genes as indicated in relation to sham-operated Ctrl. LM (n = 5–7 per group). Values are presented as mean ± SEM. $^*P < 0.05$; $^{**}P < 0.01$.

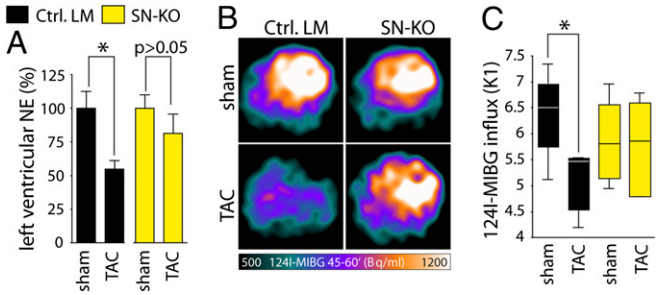


Fig. 2. Sympathetic ET_A impairs sympathetic nerve function. (*A*) ELISA-based quantification of NE within the left ventricle of SN-KO 7 wk after TAC in relation to sham-operated mice (n = 4–7 per group). (*B*) Representative PET scan images of [124I]-MIBG uptake in the frame 45–60 min after MIBG injection into sham- and TAC-operated Ctrl. LM vs. SN-KO. (*C*) Quantitative analysis of the influxes (K1) of [124I]-MIBG via PET scan 7 wk after TAC (controls, n = 4; TAC, n = 6). Values presented as mean ± SEM. *P < 0.05.

sympathetic nerve function. The improvement of sympathetic nerve function by ET_Ai was associated with an attenuation of cardiac hypertrophy, a prevention of pulmonary congestion, and a reduced mortality (Fig. 3 *D–F*). Moreover, we observed further improvements of hemodynamic characteristics and the HF biomarker atrial natriuretic peptide (ANP) (Fig. 3*G*). Because we focused in the present study on cardiac function, we cannot rule out that the reduced mortality is—at least in part—due to an improved function of the kidney or other organs that are innervated by sympathetic neurons.

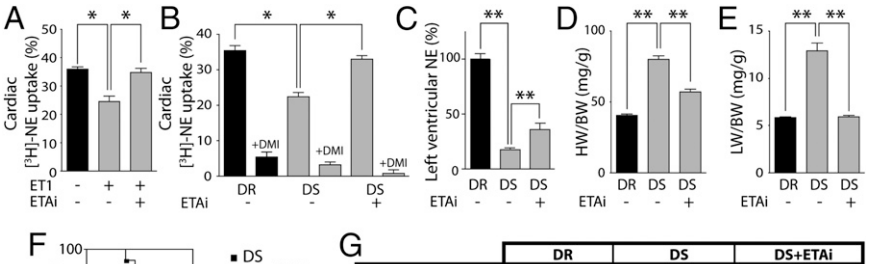
Sympathetic Neurons Regulate Cardiomyocyte Hypertrophy Through Sympathetic ET_A. Next, we aimed at recapitulating the in vivo findings in isolated cell culture systems. Treatment of rat sympathetic neuron-cardiomyocyte cocultures (SN-CM) with 10 nM ET1 led to a fourfold increase of NE net release into the culture medium, which was inhibited by ET_Ai (Fig. S5*A*). Although we cannot formally rule out that this effect was mediated by ET1-mediated exocytotic NE release, we propose based on previous experiments (28) that this effect is rather due to inhibition of NE reuptake. The presence of SNs in the SN-CM coculture was confirmed by Western blot analysis using an antibody against the SN-specific marker tyrosine hydroxylase (TH) (Fig. S5*B*). Remarkably, ET1 led to exaggerated CM hypertrophy in CM-SN cocultures (Fig. S5*C*). To further support the requirement of the sympathetic ET_A for CM hypertrophy, we also performed CM-SN coculture experiments with selective ET_A deletions either in CMs or SNs. First, we adenovirally expressed Cre in isolated murine SNs from $ET_A^{fl/fl}$ mice (SN^{KO}) and cocultured these cells with WT rat CMs (CM^{WT}). The deletion of SN-ET_A abolished exaggerated CM hypertrophy in SN^{KO}-CM^{WT} cocultures (Fig. S5 *D* and *E*). Immunocytochemistry with an antibody against ET_A illustrates not only a sufficient deletion of ET_A through Cre transduction but also that the concentration of ET_A on SNs is largely higher than on CMs (Fig. S5*D*). Second, we adenovirally expressed Cre in isolated murine CMs from $ET_A^{fl/fl}$ mice (CM^{KO}) and cocultured these cells with WT rat SNs (SN^{WT}). The deletion of CM-ET_A inhibited ET1-induced hypertrophy in monocultures (CM^{KO}) but the addition of SN^{WT} (CM^{KO}-SN^{WT} cocultures) restored the hypertrophic response of CM^{KO}, formally proving that ET_A on CMs is dispensable for ET1-induced CM hypertrophy in the presence of SNs (Fig. S5 *F* and *G*). Deletion of ET_A in CM^{KO} was confirmed by Western blot (Fig. S6).

Sympathetic ETA Regulates CM Hypertrophy Through Adrenergic Receptors. To test whether the SN-dependent increase in CM size depends on adrenergic receptors, we used pharmacological inhibitors for αARs and βARs (αARi and βARi). Strikingly, ET1-induced CM hypertrophy (Fig. 4 *A* and *B*) and protein synthesis (Fig. 4*C*) in CM-SN coculture, but not in CM monoculture, could be attenuated by βARi, whereas β1ARi seems to be sufficient to inhibit exaggerated hypertrophy (Fig. S7). However, αARi was ineffective in this regard. As a control, ET_Ai sufficiently blocked CM hypertrophy in both culture systems.

Sympathetic ET_A Activates the HDAC–MEF2 Axis in CMs. ET_A signaling via protein kinases was suggested to result in a phosphorylation-dependent nucleo-cytoplasmic shuttling of class II HDACs, which in turn results in activation of the transcription factor MEF2 and gene programs that lead to cardiomyocyte hypertrophy (20, 22). In SN-CM, ET1 led to a significant increased nuclear export of adenovirally expressed HDAC4 and HDAC5 (Fig. 5 *A–C*). HDAC4 nuclear export was significantly reduced by βARi, whereas HDAC5 nuclear export was significantly reduced by αARi. Moreover, βAR signaling leads specifically to 14-3-3 binding to HDAC4 but not HDAC5 (Fig. S8). ET_Ai prevented nuclear export of both HDAC4 and HDAC5. Using MEF2 reporter assays, we found that ET1 induced a higher increase in MEF2 activity in SN-CM coculture compared with CM monoculture (Fig. 5*D*). A hallmark of transcriptional remodeling is a switch in the expression of myosin heavy chain genes from *myh6* to *myh7* (29). ET1 caused a moderate *myh6*/*myh7* switch in CM monoculture but, remarkably, a dramatic switch in SN-CM coculture (Fig. 5*E*).

Discussion

In this study, we demonstrate that ET1 mediates adverse cardiac remodeling not through CM-ET_A but through SN-ET_A.



	DR	DS	DS+ETAi
EF (%)	79.02±1.9 (n=5)	70.14±1.8** (n=5)	75.28±2.9 (n=8)
LVEDP (mmHg)	4.93±0.59 (n=4)	8.64±2.32* (n=8)	4.81±1.17§ (n=11)
dP/dT+	13.51±2.3 (n=4)	7.73±2.1* (n=8)	12.04±2.6§ (n=11)
dP/dT-	7.94±1.8 (n=4)	4.57±2.1*(n=8)	5,93±1.3§ (n=11)
plasma ANP (pmol/l)	2.44±0.68 (n=5)	19.02±2.99**(n=5)	9.07±0.43§(n=8)

Fig. 3. ET_A inhibitors improve sympathetic nerve function in a rat model of HF. (*A*) [^{3}H]-NE uptake in isolated perfused hearts in the presence/absence of ET1 and ET_Ai. (*B*) [^{3}H]-NE uptake with/without ET_Ai (darusentan) treatment in salt-sensitive Dahl rats (DS) compared with salt-resistant rats (DR). Almost complete inhibition by desipramine (DMI) indicates that the majority of NE is taken up by the NE transporter (NET). (*C*) Left ventricular NE (tissue level) in DS vs. DR. ET_Ai treatment attenuates the depletion of left ventricular NE (n = 5–9 per group). (*D*) ET_Ai attenuates cardiac hypertrophy (HW/BW) and (*E*) pulmonary congestion (LW/BW) in DS (n > 10 per group). (*F*) Kaplan–Meier analysis of DS treated with/without ET_Ai (n = 10 per group). (*G*) Left ventricular ejection fraction (EF), left ventricular enddiastolic pressure (LVEDP), contractility index (dP/dT+), relaxation index (and dP/dT–), and plasma levels ANP in DS treated with/without ET_Ai. Values are presented as mean ± SEM. *P < 0.05; **P < 0.01; and §P < 0.05 vs. DS.

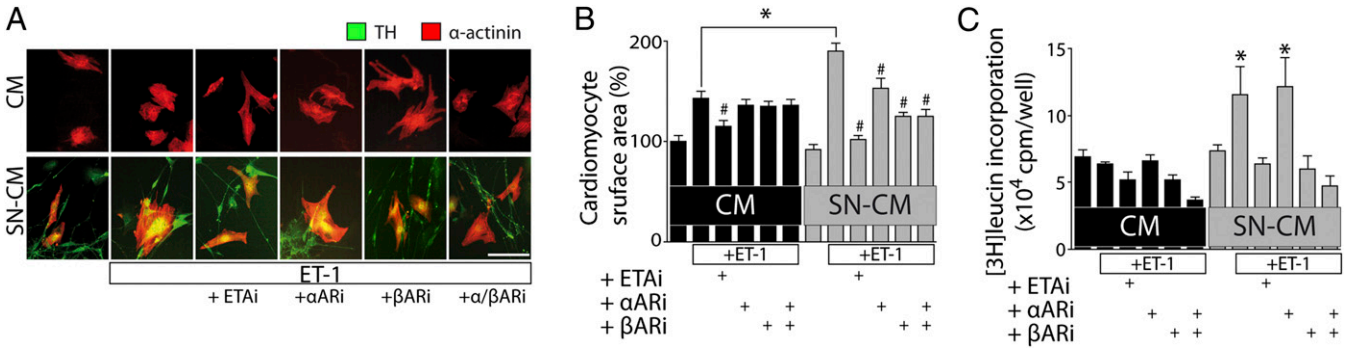


Fig. 4. Sympathetic ETA regulates CM hypertrophy through adrenergic receptors. (*A*) Immunocytochemistry of neonatal rat ventricular myocytes (NRVMs, CMs) and cocultures of CMs together with neonatal rat sympathetic neurons (SNs) was performed using antibodies recognizing sarcomeric α-actinin (red) and TH (green). Cultured cells were treated for 24 h with 10 nM ET1 in the absence or presence of the inhibitors darusentan (1 μM; ET_Ai), prazosin (1 μM; αARi), or propranolol (1 μM; βARi). (*B*) CM cell surface area was quantified. $^{\#}P < 0.05$ vs. ET1-treated; $^{*}P < 0.05$ ET1-treated SN-CM vs. CM (n = 100–150/per group). (*C*) [^{3}H]-leucin incorporation in CMs vs. SN-CM with treatment as indicated (ET1: 10 nM 18 h; αAR: 1 μM prazosin; βAR: 1 μM propranolol; 1 h before ET1 treatment). $n = 4$. $^{*}P < 0.05$ vs. nontreated SN-CM; $^{\#}P < 0.05$ vs. ET1-treated.

Sympathetic ET_A Is Required for Abnormal Sympathetic Nerve Function and Pathological Cardiac Remodeling. We have shown previously in isolated perfused hearts that ET1 impairs NET-dependent NE reuptake but not exocytotic NE release (28). However, isolated heart perfusion in combination with ET_Ai did not allow the determination of the underlying critical cell types or the investigation of disease-relevant remodeling processes. Now we demonstrate that SN-ET_A determines the cardiac vulnerability to pathological pressure overload. Based on our previous data (28) and on the MIBG uptake measurements, we conclude that inhibition of SN-ET_A leads to an attenuation of adrenergic neurotransmission and to cardioprotection. In contrast to SN-KOs, CM-KOs were not protected from pathological cardiac stress. The latter finding is consistent with the prior finding that CM-KO mice did not show an improvement of cardiac function after angiotensin or isoproterenol infusion (13) and highlights the critical role of ET_A in nonmyocytes. We cannot exclude that ET_B in CMs plays a critical role during pathological cardiac remodeling because the role of the ET_B has not been investigated in conditional KO models. However, some evidence is provided from mice lacking ET_B globally. These mice develop intestinal aganglionosis and megacolon with early death, which could be rescued by reexpression of the ET_B under the control of the dopamine-β-hydroxylase promoter, indicating that the SN-ET_B may also be important for sympathetic nerve function (15, 30). Previous pharmacological data indicate that sympathetic ET_B does not influence NE reuptake but inhibits exocytotic NE release in the heart (28). Through the use of CM-SN cocultures, we provide additional evidence that sympathetic ET_A is required for the transmission of ET1-dependent adrenergic and hypertrophic signals to CMs. These results explain how ET_Ai improves local NE homeostasis as also shown in DS rats on a high-salt diet. However, why did selective ET_Ai not result in reduced plasma NE levels in HF patients (11, 31)? This discrepancy might be explained by the idea that plasma NE levels are not ideal surrogates for the local cardiac adrenergic drive but rather serve as a marker for a generalized sympathetic activation. In fact, it was shown that the cardiac adrenergic drive independently alters from the generalized sympathetic tone (32).

Role of Nonmyocytes in the Regulation of ET1-Dependent Signal Transduction in CMs. Until now, it was assumed that ET1 mediates its hypertrophic effects through ET_A on CMs (33), which mainly is based on the observation that ET1 exerts hypertrophy of cultured CMs. However, CM primary cultures are heterogenous and contain—dependent on the type of isolation and duration of cultivation—variable amounts of nonmyocytes such as fibroblasts, endothelial cells, smooth muscle cells, and intrinsic

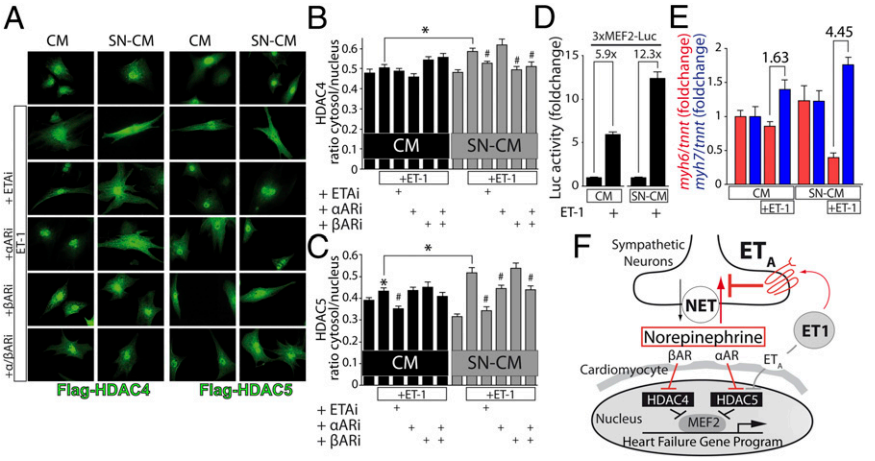


Fig. 5. Sympathetic ET_A activates the HDAC-MEF2 axis in CMs. (*A*) CMs were transduced with adenoviruses harboring Flag-HDAC4 or -HDAC5, starved for 4 h, and then treated for 12 h with 10 nM ET1 in the absence or presence of the inhibitors darusentan (1 μM; ET_Ai), prazosin (1 μM; αARi), or propranolol (1 μM; βARi). Immunocytochemistry was performed using antibodies recognizing Flag. (*B* and *C*) Quantitative analysis of cytosolic vs. nuclear localized HDACs. $^{*}P < 0.05$ ET1-treated vs. nontreated group and SN-CM vs. CM. $^{\#}P < 0.05$ inhibitor treated vs. ET1-treated group ($n \geq 40$ cells per group). (*D*) MEF2 activity was measured by MEF2 luciferase reporter assay ($n = 4$). $^{*}P < 0.05$. (*E*) Transcripts of myh6 and myh7 after 24-h treatment with10 nM ET1 ($n = 6$). $^{*}P < 0.05$. (*B–E*) Values are presented as mean ± SEM. (*F*) Working model: ET1 stimulates ET_A on CMs but to a larger extent on SNs. ET1 leads via SN-ET_A to a substantial increase of NE net release by inhibiting NET-dependent NE reuptake, which in turn leads to an activation of αARs and βARs and nuclear export of HDAC5 and HDAC4 through distinct signaling pathways (pathway highlighted in red). Thus, the involvement of adrenergic receptors amplifies ET1-driven activation of MEF2.

neurons (34). This heterogeneity is potentially one of the reasons for variable results from isolation to isolation as experienced by many investigators. The results of this study demonstrate that SN-ET_A is required for pathological remodeling of the heart and that CM-ET_A is dispensable in the presence of SNs. In this regard, previous studies on ET1-induced CM hypertrophy usually used relatively high concentrations of ET1 (10–100 nM). Accordingly, we observed robust CM hypertrophy in CM monocultures only with 100 nM ET1. In cocultures, by contrast, 10 nM ET1 was sufficient to induce robust CM hypertrophy, suggesting that the ET_A on SNs is more sensitive than on CMs. The reason might lie in the relative expression pattern. Fig. S5*D* implies that the ET_A is expressed to a larger extent in SNs as compared to CMs. Likewise, we reported previously that ET1 concentrations around 100 pM affect NE reuptake in isolated perfused hearts (28). Thus, the possibility exists that physiological ET1 levels that were reported to lie in the picomolar range predominantly act via SN-ET_A rather than CM-ET_A. These findings are not surprising from a developmental point of view: SNs are derived from neural crest cells and loss-of-function studies have unmasked that the deletion of ET_A in neural crest results in severe cardiac phenotypes (35), whereas the CM deletion of ET_A did not result in an obvious phenotype in classical pathological models (13).

Divergent and Convergent Signaling of ET1. ET_A and αAR signal via PKD toward HDAC5 to derepress MEF2 target genes that drive adverse cardiac remodeling (20, 36). We now show that ET1 signals via SN-ET_A indirectly to CM-αAR and then converges with CM-ET_A on HDAC5 signaling. However, our results also revealed that SN-ET_A transduces its signals via βARs to HDAC4, indicating the involvement of other kinases, because PKD is not a downstream kinase of βARs (37, 38). These data are consistent with our previous report that CaMKII selectively signals to HDAC4 (21, 39). In contrast to PKD, CaMKII is well known to be activated by βARs (40, 41). How βAR signaling acts on HDAC5 is under debate (42–44). In our hands, βAR activation leads to more 14-3-3 binding (as a measure of cytosolic accumulation of HDACs) of HDAC4 but to less 14-3-3 binding to HDAC5, suggesting that βARs lead to (*i*) cytosolic accumulation of HDAC4 possibly by CaMKII and (*ii*) nuclear accumulation of HDAC5. The latter might be explained by a previously reported inhibitory effect of protein kinase A (a major downstream mediator of βAR signaling) on PKD (44, 45). Based on our data, we propose a model (Fig. 5*F*), by which CM-ET_A signals specifically through PKD to HDAC5, whereas SN-ET_A signals indirectly via NE through αARs to HDAC5 but through βARs to HDAC4. Why does ET1 signaling diverge to adrenergic receptors that in turn converge on distinct class II HDAC family members? Both, HDAC4 and HDAC5 have been demonstrated to inhibit MEF2 (46). Thus, our findings provide a mechanistic basis by which SN-ET_A amplifies de-repression of MEF2 by removing more than one HDAC family member from the nucleus.

Potential Clinical Implications. An improved NE reuptake by ET_Ai leads to restoration of NE stores, which was reported to be associated with an increased exercise tolerance in patients (24). Thus, we hypothesize that exercise tolerance may be an additional clinical readout for future ET_Ai studies. Based on the findings of the present study, we hypothesized that individual patients not tolerating β blockers might benefit from ET_Ai. Likewise, studies in which only up to 56% of the patients received β blockers showed a significant benefit from ET_Ai or unspecific endothelin receptor inhibitors (Table S1). In studies in which more patients received antiadrenergic therapies, no significant benefit was observed, suggesting that β blockers indeed blunt the potential therapeutic effects of ET_Ai. We also aimed at retrieving supporting information from the largest ET_Ai trial on HF, namely the endothelin-a receptor antagonist trial in heart failure trial (11). However, the size of the subgroups (in particular patients without antiadrenergic therapy) did not allow a statistically reliable analysis. Thus, an ET_Ai trial with this small HF patient cohort might be an interesting approach to test the hypothesis that patients without antiadrenergic therapy might benefit from ET_Ai. Moreover, imaging techniques such as [^{123}I]-MIBG uptake (17, 18, 26) that allow an assessment of the sympathetic nerve function in patients may serve as a tool for the selection of patients that benefit in particular from ET_Ai.

In conclusion, we unmasked, by the use of cell type-specific in vivo and ex vivo gene deletion tools, a critical role for sympathetic ET_A in the development and progression of HF. ET1 amplifies its effect on CMs indirectly by the stimulation of adrenergic neurotransmission and the activation of αARs and βARs and subsequently distinct HDAC- and MEF2-dependent signaling pathways. Other than hypothesized before, ET_A on CMs does not seem to critically contribute to the development of HF.

Materials and Methods

Experimental Animals. All experiments were performed in accordance with relevant guidelines and regulations. This investigation followed the Principle of Laboratory Animal Care (NIH Publication No. 86-23, revised 1985) and was approved by the authorities of the Regierungspräsidium Karlsruhe. For detailed information, see *SI Materials and Methods*.

[^{124}I]MIBG-PET. The precursor resin for the production of ultratrace [^{124}I]MIBG was a kind gift from James F. Kronauge (Molecular Insight Pharmaceuticals, Cambridge, MA). To prevent eventual saturation of the norepinephrine transporter by MIBG in the animal model, MIBG of high specific activity was produced. For this purpose, the solid labeling approach was applied (47). For detailed information, see *SI Materials and Methods*.

Isolated Heart Perfusion. Hearts were perfused according to the Langendorff method as previously published (27). For detailed information, see *SI Materials and Methods*.

Determination of Neurohormones. Measurement of neurohormones followed the protocol as previously described (27). For detailed information, see *SI Materials and Methods*.

Transthoracic Echocardiography. Echocardiographic evaluation was performed using a VisualSonicsVevo 2100 echocardiograph. For detailed information, see *SI Materials and Methods*.

Isolation of Primary Cells. SNs were prepared and cultured from newborn Sprague–Dawley rats or $ETA^{fl/fl}$ mice as described previously (48). Briefly, freshly isolated superior cervical ganglia from 1- to 3-d-old pups were dissected, incubated for 45 min in 1.5 mg/mL collagenase, titrated, and then preplated on cultural plastic to permit the attachment of nonneuronal cells. After 1 h, SNs were plated with CM medium (*SI Materials and Methods*) and cocultured for 3–5 d with an additional 10 ng/mL NGF (rat recombinant). After 24 h in coculture, 1 μM cytosine arabinofuranoside was added to stop cell division of potential contaminating fibroblasts. The isolation of CMs is described in detail in *SI Materials and Methods*.

Plasmids and Reagents. For detailed information, see *SI Materials and Methods*.

Indirect Immunofluorescence, Western Blot Analysis and Coimmunoprecipitation, Histology, RNA Analysis, Reporter Assays, [^{3}H]-Leucine Incorporation, and Adenovirus Production. For detailed information, see *SI Materials and Methods*.

Statistics. The results are expressed as the mean ± SEM. Statistical analysis was performed using SPSS 12.0 software (SPSS). Differences between groups were tested by one-way ANOVA with post hoc comparisons by Bonferroni's multiple comparison test or unpaired Student *t* test where appropriate. Kaplan–Meier survival analysis was performed using the log-rank test. In the immunofluorescence experiments, the nucleocytoplasmatic distribution of HDAC4 and -5 was analyzed in >40 cells per experiment, whereas a ratio was built between fluorescence in nucleus vs. cytosol using ImageJ software. In all tests, $P < 0.05$ was considered statistically significant.

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