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Prevention and reversal of cardiac hypertrophy by soluble epoxide hydrolase inhibitors

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Sustained cardiac hypertrophy represents one of the most common causes leading to cardiac failure. There is emerging evidence to implicate the involvement of NF-κB in the development of cardiac hypertrophy. However, several critical questions remain unanswered. We tested the use of soluble epoxide hydrolase (sEH) inhibitors as a means to enhance the biological activities of epoxyeicosatrienoic acids (EETs) to treat cardiac hypertrophy. sEH catalyzes the conversion of EETs to form the corresponding dihydroxyeicosatrienoic acids. Previous data have suggested that EETs may inhibit the activation of NF-κB-mediated gene transcription. We directly demonstrate the beneficial effects of several potent sEH inhibitors (sEHIs) in cardiac hypertrophy. Specifically, we show that sEHIs can prevent the development of cardiac hypertrophy using a murine model of pressure-induced cardiac hypertrophy. In addition, sEHIs reverse the preestablished cardiac hypertrophy caused by chronic pressure overload. We further demonstrate that these compounds potently block the NF-κB activation in cardiac myocytes. Moreover, by using *in vivo* electrophysiologic recordings, our study shows a beneficial effect of the compounds in the prevention of cardiac arrhythmias that occur in association with cardiac hypertrophy. We conclude that the use of sEHIs to increase the level of the endogenous lipid epoxides such as EETs may represent a viable and completely unexplored avenue to reduce cardiac hypertrophy by blocking NF-κB activation.

epoxyeicosatrienoic acids | NF-κB

Cardiovascular disease represents an important and growing public health problem and is the cause of substantial morbidity and mortality. Cardiac hypertrophy is the heart's compensatory response to a variety of extrinsic and intrinsic stimuli including pressure or volume overload, mutations of sarcomeric proteins, or loss of contractile mass from previous myocardial infarction. Cardiac hypertrophy is believed to have a compensatory function by diminishing wall stress. Conversely, sustained cardiac hypertrophy represents one of the most common causes of cardiac failure (1, 2).

There is increasing evidence that prevention of cardiac hypertrophy may represent a new therapeutic target to improve patient survival (1, 3, 4). Inhibition of NF-κB has recently been investigated as one of the possible therapeutic approaches to treatment of cardiac hypertrophy. The results of the investigations may have important clinical implication (5–12). We surmised that the use of soluble inhibitors of NF-κB may present an attractive therapeutic means to treat cardiac hypertrophy. However, NF-κB can be both anti- and proapoptotic under certain conditions (13, 14). Therefore, the beneficial effects of NF-κB inhibitors in cardiac hypertrophy remain to be systematically investigated. Motivated by the uncertainties and the therapeutic potential of the inhibitors of this nuclear factor we tested the beneficial effects of several potent soluble epoxide hydrolase inhibitors (sEHIs) in cardiac hypertrophy. sEH catalyzes the conversion of epoxyeicosatrienoic acids (EETs) to form the corresponding dihydroxyeicosatrienoic acids (DHETs) (15, 16). EETs are products of cytochrome P450 epoxygenases that have vasodilatory properties similar to those of endothelium-derived hyperpolarizing factor (17). In addition, EETs can inhibit the activation of NF-κB-mediated gene transcription (18, 19). Because sEH converts EETs to DHETs, inhibition of sEH has been intensely investigated as a potential approach for enhancing the antihypertensive and antiinflammatory actions of EETs (17, 20–22). However, the potential benefits of sEHIs and EETs for cardiac hypertrophy have never been investigated.

Here, we tested the effects of sEHIs on the development and reversal of cardiac hypertrophy using a murine model of aortic banding. We show that there is an almost complete resolution of cardiac hypertrophy by sEHIs independent of the antihypertensive effects. We further demonstrate that these compounds potently block the NF-κB activation in cardiac myocytes. Moreover, our study shows a beneficial effect of sEHIs in the prevention of cardiac arrhythmias that occur in association with cardiac hypertrophy.

Results

sEHIs Prevent the Development of Cardiac Hypertrophy in a Murine Model with Thoracic Aortic Constriction (TAC). We created a TAC in 8-week-old male C57BL/6J mice (Charles River Laboratories, Wilmington, MA), resulting in a chronic pressure overload-induced cardiac hypertrophy. TAC mice were randomized to receive either vehicle ((2-hydroxypropyl)-β-cyclodextrin) or one of the two sEHIs: 1-adamantan-3-(5-(2-(2-ethylethoxy)ethoxy)pentyl)urea (AEPU; 0.1 mg/ml) or 12-(3-adamantan-1-yl-ureido)-dodecanoic acid (AUDA; 0.1 mg/ml) (Fig. 1*A*; and see Tables 1–3 and Fig. 7, which are published as supporting information on the PNAS web site; ref. 21) in drinking water after surgery for a period of 3 weeks. The synthesis of both sEHIs was scaled up from described procedures (21). AUDA has relatively low solubility in water compared with AEPU and requires (2-hydroxypropyl)-β-cyclodextrin to achieve the concentration required for administration in drinking water. Measurement of plasma drug levels is presented in *Supporting Text*, which is published as supporting information on the PNAS web site. Fig. 1*B* shows photomicrographs of examples of whole hearts from TAC mice treated with AEPU or AUDA in the

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Conflict of interest statement: N.C. and B.D.H. have filed patents for the University of California for sEH and cardiac hypertrophy therapy. B.D.H. founded Arête Therapeutics to move sEH inhibitors into clinical trials.

Abbreviations: Ang II, angiotensin II; ANF, atrial natriuretic factor; APEU, 1-adamantan-3-(5-(2-(2-ethylethoxy)ethoxy)pentyl)urea; AUDA, 12-(3-adamantan-1-yl-ureido)-dodecanoic acid; DHET, dihydroxyeicosatrienoic acid; EET, epoxyeicosatrienoic acid; LV-ESD, left ventricular end systolic dimension; MHC, myosin heavy chain; sEH, soluble epoxide hydrolase; sEHI, sEH inhibitor; TAC, thoracic aortic constriction.

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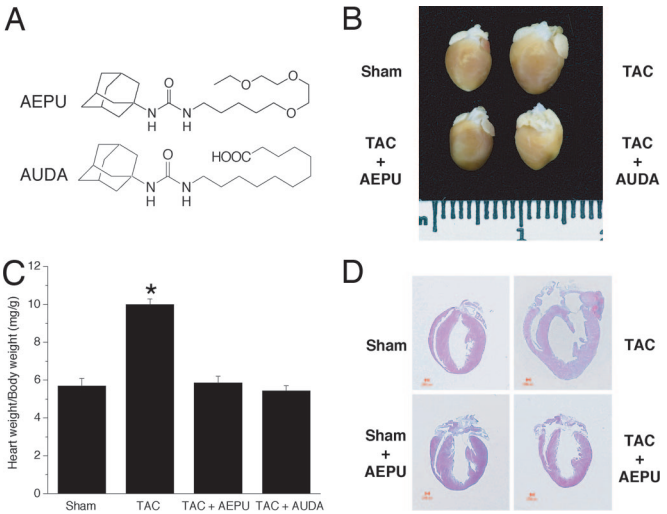

Fig. 1. Inhibition of cardiac hypertrophy in TAC mice by sEHIs (AEPU and AUDA). (*A*) Structure of the two sEHIs used in our studies: AEPU or AUDA. (*B*) Examples of whole hearts from TAC mice treated with AEPU or AUDA compared with TAC alone or sham-operated hearts. The mice were killed after 3 weeks of followup. (Scale, 1 cm.) (*C*) Analysis of heart/body weight ratios (mg/g) demonstrated that AEPU- and AUDA-free acid inhibited the increase in heart size in TAC mice compared with untreated TAC mice. Error bars are ±SEM of $n = 16$. (*D*) Histologic sections (H&E staining) of a sham-operated (control) and TAC mouse hearts, showing cardiac hypertrophy at 3 weeks in the TAC mouse. Treatment of TAC mice with AEPU (0.1 mg/ml) in drinking water prevented the development of cardiac hypertrophy (*, $P < 0.05$). All histologic sections are presented with the atria on top and the right ventricle to the left. (Scale bars, 200 μm.)

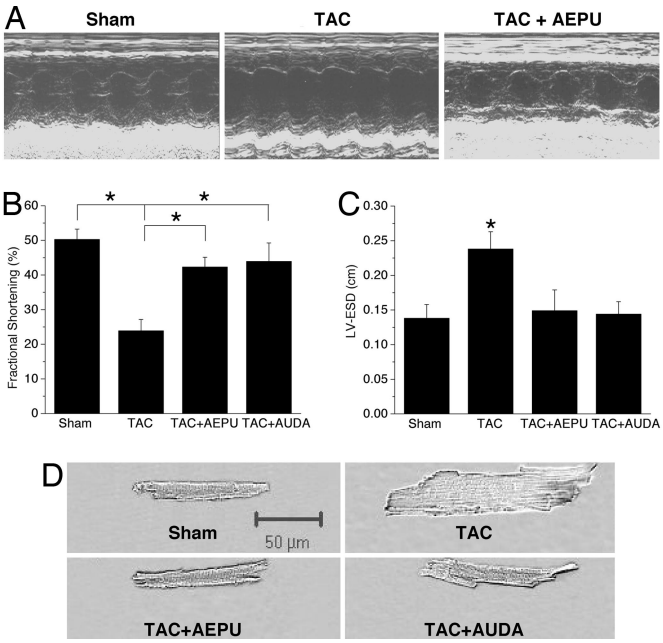

Fig. 2. Assessment of cardiac hypertrophy. (*A*) Examples of M-mode echocardiography in sham-operated, TAC, and TAC treated with AEPU mice after 3 weeks of treatment, showing evidence of cardiac failure with chamber dilatation in TAC mice. AEPU prevented the development of cardiac hypertrophy and failure in TAC mice. Summary data are shown in *B* and *C*. (*B*) Fractional shortening (FS), a surrogate of systolic function, was calculated from left ventricle dimensions as follows: FS = ((EDD − ESD)/EDD) × 100%. (*C*) LV-ESD. Data shown are mean ± SEM, $n = 10$–16 for each group; *, $P < 0.05$. (*D*) Photomicrographs of single isolated cardiac myocytes from left ventricular free wall. Treatment with compounds AEPU or AUDA prevented hypertrophy of the cardiac myocytes in TAC mice.

drinking water for 3 weeks compared with TAC alone or sham-operated hearts after 3 weeks of followup. All TAC mice showed the expected increase in heart size, with dilatation of all chambers as previously described in this model (23, 24). In contrast, treatment with sEHIs prevented development of cardiac hypertrophy. Summary data are shown in Fig. 1*C* illustrating a significant increase in the ratio of heart weight/body weight (HW/BW) in the TAC group but no significant changes in the HW/BW ratio in the TAC groups treated with AEPU or AUDA compared with sham-treated group were found. The H&E histologic sections in Fig. 1*D* show sections of a sham-operated and TAC hearts with or without the treatment. The TAC heart showed evidence of cardiac hypertrophy and chamber dilatation. The hypertrophic response was prevented by using either AEPU or AUDA in the drinking water. There were no changes in the sham-operated mice treated with AEPU. Both AEPU and AUDA are potent inhibitors of sEH; however, AUDA is far more lipophilic than AEPU and can be viewed as a possible mimic of 14,15 EET. The fact that these two inhibitors of sEH with radically different physical properties both yield similar biology suggests that the inhibition of sEH is involved.

Noninvasive Assessment of Cardiac Hypertrophy by Echocardiography. We assessed the chamber size and wall thickness in sham-operated and TAC mice compared with TAC mice treated with sEHIs by using echocardiography. As shown in Fig. 2*A* using M model echocardiography, AEPU prevented the development of cardiac hypertrophy in TAC mice after 3 weeks of followup. Fig. 2 *B* and *C* summarize the percentage of fractional shortening and left ventricular end systolic dimension (LV-ESD) from sham, TAC, and TAC mice treated with AEPU or AUDA. Treatment with sEHIs prevented the deterioration in fractional shortening and LV-ESD compared with TAC alone. We further analyzed the single isolated cardiac myocytes from the left ventricular free wall from the different groups of mice by using light microscopy. Fig. 2*D* shows photomicrographs of single ventricular myocytes isolated from the four groups of mice. Treatment with AEPU or AUDA prevented the hypertrophy of the cardiac myocytes in TAC mice. Furthermore, the disarray of myofibrils that characterized the hypertrophic hearts was also prevented by treatment with sEHIs (Fig. 3*A*).

Analysis of Hypertrophic Markers. To further test the hypothesis that sEHIs antagonize initiation of the hypertrophic response, we examined the induction of hypertrophic markers such as atrial natriuretic factor (ANF), skeletal α-actin, and α vs. β-myosin heavy chain (MHC) isoforms switch using total RNA isolated from hearts of sham-operated, TAC alone and TAC treated with the sEHI (AEPU). Expression of transcripts for ANF, skeletal α-actin, α- and β-MHC, and GAPDH were determined. The basal level of these mRNAs was detected in myocytes. Aortic banding resulted in the up-regulation of these hypertrophic markers as described (25). AEPU decreased the transcript expression of the hypertrophic genes including ANF, skeletal α-actin. and β-MHC as compared with TAC alone (Fig. 3*B*). These data further support our findings in Figs. 1 and 2 that sEHIs have beneficial effects in this *in vivo* model of cardiac hypertrophy. Additional data from microarray analysis are presented in Table 4, which is published as supporting information on the PNAS web site.

Expression of sEH in Murine Atrial and Ventricular Myocytes. sEH has been documented to be highly expressed in liver and kidney. To directly document the expression of the enzyme in the heart, we performed Western blot analysis using atrial and ventricular tissues from male mice (C57BL/6J) and polyclonal anti-sEH Ab (1:200 dilution) against human sEH that was raised from rabbits as described (26). As shown in Fig. 3*C*, both mouse atrial and ventricular tissues expressed a readily detectable level of sEH

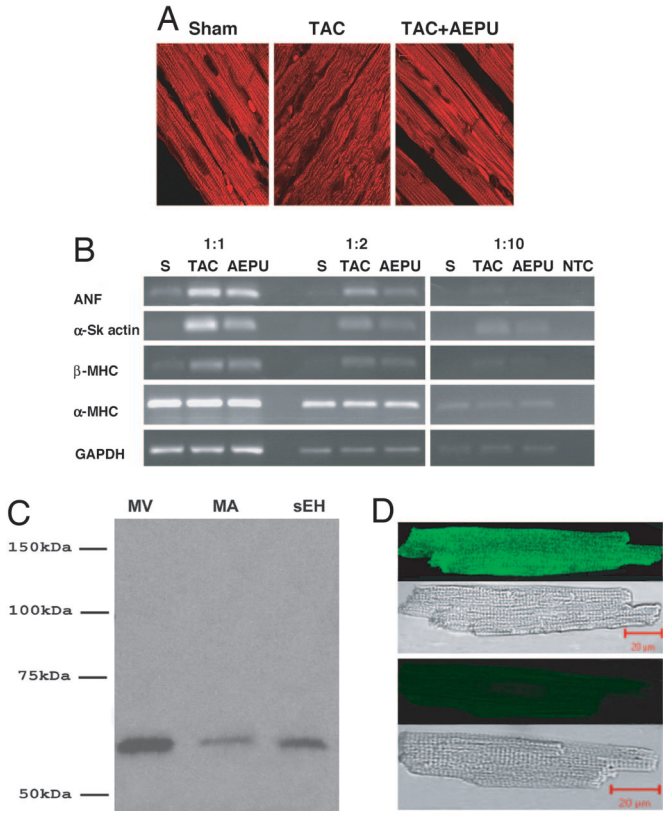

Fig. 3. Assessment of the expression of the hypertrophic markers and sEH. (*A*) Immunofluorescence confocal photomicrographs of sections of left ventricular myocytes stained with anti-α-actinin2 Abs with secondary Abs conjugated to Texas red. Sections were obtained from the left ventricles after 3 weeks from sham-operated, TAC alone, and TAC mice treated with AEPU. The sham-operated mice showed ordered myofibrils. In contrast, hearts from TAC mice showed loss of the organization of the myofibrils. The effects were prevented by the treatment with AEPU. (*B*) Semiquantitative RT-PCR analysis. Total RNA was isolated from left ventricular free wall from sham-operated, treated, and untreated TAC mice and subjected to RT-PCR using primers specific to skeletal α-actin, ANF, α-MHC, β-MHC, and GAPDH. S, sham. Serial dilutions of the template were loaded (1:1, 1:2, and 1:10). NTC, no-template controls. AEPU decreased the transcript expression of the hypertrophic genes including ANF, skeletal α-actin, and β-MHC. Transcript levels normalized to GAPDH (in arbitary units) for (*i*) ANF are 0.97 ± 0.05, 1.4 ± 0.05, and 1.2 ± 0.01; (*ii*) skeletal α-actin are 0.97 ± 0.2, 1.47 ± 0.05, and 1.1 ± 0.07; (*iii*) β-MHC are 0.82 ± 0.1, 1.05 ± 0.1, and 0.94 ± 0.08 in sham, TAC, and TAC mice treated with AEPU, respectively. (*C*) Western blot analysis using a polyclonal anti-sEH Ab against human sEH. Atrial and ventricular tissues from male mice were loaded (40 μg). MV, mouse ventricle, MA, mouse atrium. Purified sEH protein was used as a positive control (lane 3). (*D*) Confocal photomicrographs showing subcellular distribution of sEH in mouse ventricular myocytes by using same Ab as above. Immunofluorescence labeling was done by treatment with secondary Abs (fluorescein isothiocyanate-conjugated goat anti-rabbit Ab). *D Lower* show control experiments using secondary Ab only and corresponding differential interference contrast images are shown. (Scale bars, 20 μm.)

(detected as a single major band at a molecular mass of ≈60 kDa). Immunofluorescence confocal microscopy data showed specific reactivity to sEH Abs in isolated ventricular myocytes (Fig. 3*D*). Control experiments were performed by using secondary Abs only (Fig. 3*D*) and scanned by using the same settings. To directly test whether aortic banding may up-regulate the sEH expression level, we performed Western blot as well as microarray analysis using left ventricular free wall from sham, TAC, and TAC animals treated with AEPU at 3 weeks. There were no significant differences in the sEH mRNA or protein expression in sham, TAC, or TAC animals treated with AEPU (Fig. 8 and Table 4, which are published as supporting information on the PNAS web site).

Reversal of Cardiac Hypertrophy in TAC Mice. To further test whether sEHIs can reverse the established cardiac hypertrophy, TAC mice were randomized to receive the inhibitors after 3 weeks of aortic banding at which time, cardiac hypertrophy can be documented as shown in Fig. 1. The mice treated with sEHIs were compared with sham-operated or TAC mice at 6 weeks. Fig. 4 *A* and *B* shows a significant reversal in cardiac hypertrophy in the treated TAC mice compared with TAC alone at 6 weeks. Summary data for fractional shortening and LV-ESD using M-mode echocardiography are shown in Fig. 4 *C* and *D*. Moreover, treatment with sEHIs resulted in a significant decrease in cardiac fibrosis in the TAC hearts (see Fig. 9, which is published as supporting information on the PNAS web site).

Comparison of data obtained in Figs. 1 and 4 may be instructive. In Fig. 1, all endpoints were obtained at 3 weeks after TAC, whereas, in Fig. 4, all endpoints were at 6 weeks. Specifically, at 3 weeks, although chamber size was increased, there was progressive increase in LV size by 6 weeks. Future long-term experiments will be required to further assess whether sEH inhibition can reverse dilatation in addition to hypertrophy.

***In Vitro* Assessment of the Effects of sEHIs on the Development of Cardiac Hypertrophy.** sEHIs have been investigated for their antihypertensive activities (20, 21). Therefore, the question remains whether the antihypertrophic effects in our models are the results of the antihypertensive activities of these reagents. To directly examine the effects of the sEHIs on cardiac hypertrophy, we have adopted a well documented model of *in vitro* neonatal mouse cardiac myocytes and cardiac hypertrophy using phenylephrine or angiotensin II (Ang II).

Fig. 5*A* shows photomicrographs of primary culture of neonatal mouse cardiac myocytes in control compared with cells treated with Ang II, illustrating cardiac hypertrophy after 48 h of treatment with Ang II. Fig. 5*A Lower* shows the same set of cells treated with Ang II as well as AEPU (20 μM) or a mixture of regioisomers of EET (1 μM). There was a significant difference in the cell size in the neonatal cardiac myocytes treated with AEPU or EET compared with Ang II treatment alone. Cells were stained by using anti-α-actinin2 (Sigma, St. Louis, MO). These *in vitro* experiments further support our *in vivo* experiments that these compounds have a direct antihypertrophic effect independent of the antihypertensive activities of the drugs. Quantification of cardiomyocyte cell surface area was performed on digitized images by using NIH Image software by observers blinded to the treatment groups. A total of 250 cardiomyocytes in 15–20 fields were examined in three independent experiments (Fig. 5*B*). To further assess the specificity of the inhibitors on cardiac hypertrophy, we constructed a dose–response curve using different concentrations of AEPU. Fig. 5*C* shows results with a 19.1 μM half-blocking concentration.

Prevention of Nuclear Translocation of NF-κB by Treatment with sEHIs in Primary Mouse Neonatal Cardiomyocytes. EETs have been shown to exert the vasodilatory effects by membrane hyperpolarization through a membrane-associated G protein ($G_{\alpha s}$)-mediated activation of large conductance Ca^{2+}-activated K^+ (BK) channels (27). Evidence also indicates that EETs may interact with nuclear receptors to regulate gene expression (17) by maintaining NF-κB in the inactive state and inhibiting NF-κB-mediated gene transcription (18, 19, 28–30). Because sEH converts EETs to DHETs, we directly tested whether inhibition of sEH and a resultant increase in EETs can directly block the activation of NF-κB in the *in vitro* as well as *in vivo* models of cardiac hypertrophy. Fig. 6*A* shows the inhibition of nuclear translocation of NF-κB by treatment with AEPU in primary mouse neonatal cardiomyocytes. Cardiomyocytes grown in culture were serum-starved for 24 h before treatment with Ang II (50 μM) or phenylephrine (50 μM) for 45 min. The effect of sEHIs on nuclear translocation of NF-κB was assessed by using anti-p65

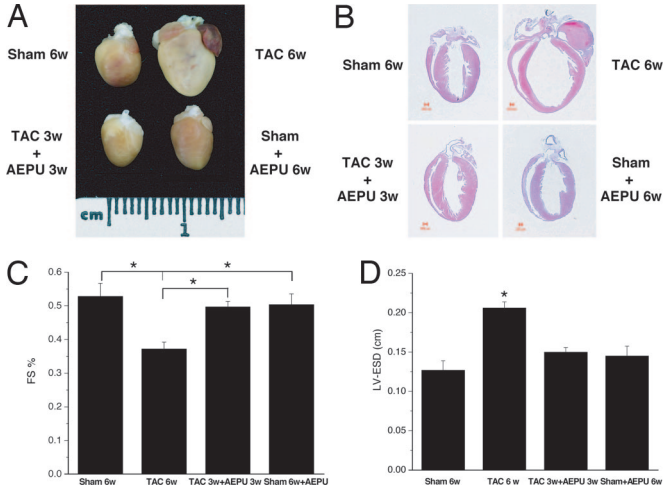

Fig. 4. Reversal of preestablished cardiac hypertrophy in TAC mice by sEHIs. (*A*) Examples of whole hearts from TAC mice at 6 weeks (TAC 6 week) compared with TAC mice started on the treatment with AEPU at 3 weeks and continued for 3 week (TAC 3W+AEPU 3W) or sham-operated hearts (with and without treatment). The mice were killed after 6 weeks of followup. (Scale, 1 cm.) (*B*) Photomicrograph of the corresponding histologic section (H&E). (Scale bars, 200 μm.) (*C* and *D*) Summary data for the fractional shortening (*C*) and LV-ESD (*D*) from TAC mice at 6 weeks (TAC 6w) compared with TAC mice started on the treatment with AEPU at 3 weeks and continued for 3 weeks (TAC 3w+AEPU 3w) or sham-operated hearts (with and without treatment). The mice were killed after 6 weeks of followup. AEPU significantly reversed the established cardiac hypertrophy in the TAC mice (n = 8–10 for each group) (*, $P < 0.05$).

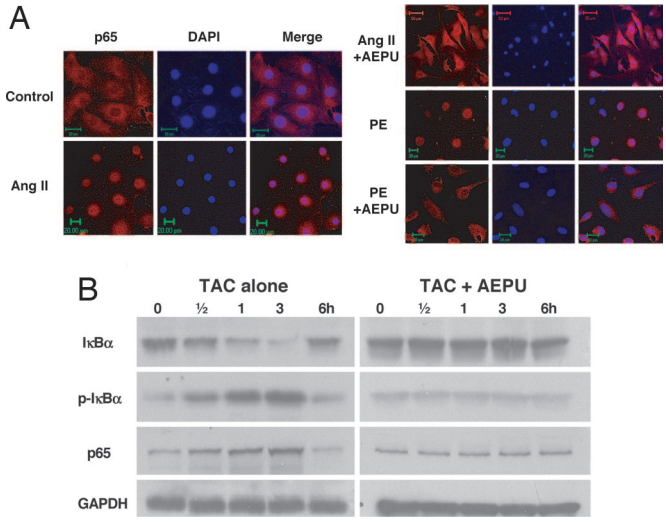

Fig. 6. Prevention of nuclear translocation of NF-κB by treatment with AEPU in primary mouse neonatal cardiomyocytes. (*A*) Cardiomyocytes grown on coverslips were serum-starved for 24 h before treatment with Ang II (50 μM) or phenylephrine (PE; 50 μM) for 45 min or left untreated (control) or Ang II + AEPU (20 μM) or PE + AEPU (20 μM). Cells were fixed, blocked, and stained with anti-p65 mAb, followed by anti-mouse Texas red-conjugated secondary Ab (red). The nuclear translocation of p65 was visualized by DAPI stain (blue) using a Zeiss confocal microscope. (*B*) AEPU inhibits NF-κB signaling in the hearts. Total IκBα and phospho-IκBα (*p*-IκBα)) levels after ascending aortic constriction (TAC). Proteins (50 μg per lane) from left ventricular free wall from banded mice compared with banded mice treated with AEPU at different time points (0, 1/2, 1, 3, and 6 h) were separated by SDS/PAGE. Total IκBα (top blot) and phospho-IκBα (second blot) were detected by immunoblotting. P65 level in the nuclear fraction is shown in the third blot. GADPH was used as internal loading control. AEPU prevented the increase in the p- IκBα level and the nuclear translocation of NF-κB.

mAb (1:200 dilution; Fig. 6*A*). Treatment with Ang II or PE resulted in nuclear translocation of NF-κB from the cytosol into the nucleus. The effects were inhibited by treatment of the cells with AEPU.

NF-κB Activation Is Inhibited in the Heart *in Vivo* by sEHIs. The inhibitory effect of sEHIs on the activation of NF-κB was further studied in the TAC model (Fig. 6*B*). Previous studies documented the activation of NF-κB in the murine model of cardiac hypertrophy with aortic banding (6, 8, 12). Here, we tested the hypothesis that sEHIs can block the activation of NF-κB in the *in vivo* model using Western blot analysis (Fig. 6*B*). Total IκBα and phospho-IκBα (*p*-IκBα) levels after TAC were assessed by using a total homogenate from the left ventricular free wall obtained from TAC mice compared with TAC mice treated with AEPU at different time points (0, 1/2, 1, 3, and 6 h after aortic banding). Mice received AEPU (10 mg/kg) vs. (2-hydroxypropyl)-β-cyclodextrin (vehicle only; 5 mg/kg) i.p. before surgery. Anti-IκBα and phosphor-IκBα mAbs were used (1:200 dilution). The level of NF-κB was further assessed in the nuclear fraction by using anti-p65 Ab (1:200 dilution). There was a progressive increase in the phospho-IκBα and a decrease in the total IκBα level, associated with nuclear translocation of NF-κB, which peaked at ≈3 h after aortic banding similar to a study documenting the activation of NF-κB in this *in vivo* model (8). Moreover, as shown in Fig. 6*B*, inhibition of sEH by AEPU prevented the increase in the *p*-IκBα level as well as the nuclear translocation of NF-κB induced by aortic banding.

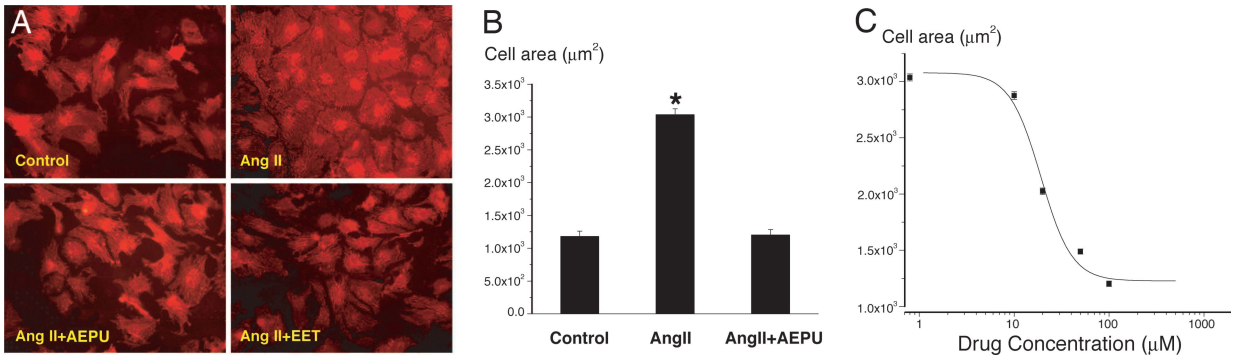

Fig. 5. *In vitro* assessment of the effects of sEHIs on the development of cardiac hypertrophy. (*A*) Photomicrographs of primary neonatal mouse cardiac myocytes treated with Ang II for 36 h to induce cardiac hypertrophy or AngII in the presence of AEPU or EET for 36 h. Cells were then fixed and treated with anti-α-actinin2 Abs and secondary Ab conjugated to Texas red. Shown are control neonatal cardiac myocytes and cells treated with Ag II alone (*Upper Left* and *Upper Right*, respectively). (*Lower Left* and *Lower Right*) Cells treated with Ang II plus AEPU or Ang II plus EET, respectively. (*B*) Quantification of cell surface area comparing control, AngII, and AngII treated with AEPU at 48 h (50 μM). *, $P < 0.05$. (*C*) Dose–response curve of cell surface area in various concentrations of AEPU.

Prevention of Cardiac Arrhythmias in TAC Mice. It is well documented that there is significant electrical remodeling in cardiac hypertrophy and failure leading to an increase in the susceptibility to cardiac arrhythmias. To further test whether sEHIs have salutary effects on cardiac arrhythmias in the setting of cardiac hypertrophy, we performed *in vivo* electrophysiologic studies (EPS) in treated and untreated TAC mice. *In vivo* electrophysiologic recordings were performed as described (31). Fig. 10, which is published as supporting information on the PNAS web site, shows examples of surface electrocardiogram and simultaneous intracardiac electrograms from atrium and ventricle from TAC mice, showing inducible ventricular and atrial arrhythmias by using programmed stimulation. The susceptibility to increased ventricular arrhythmias was significantly suppressed in TAC mice that had been randomized to treatment with sEHIs. Summary data are shown in Table 5, which is published as supporting information on the PNAS web site.

Discussion

Our study provides evidence that sEHIs can prevent and reverse cardiac hypertrophy in a well established murine model with aortic banding. To corroborate the *in vivo* data, we further show the direct antihypertrophic effects of the sEHIs using primary culture of neonatal mouse cardiac myocytes. We have observed that the antihypertrophic effect of the sEHIs is concentration-dependent. Moreover, the use of sEHIs prevents the occurrence of cardiac arrhythmias as assessed by *in vivo* EPS.

Signaling Pathways Involved in the Development of Cardiac Hypertrophy. The hypertrophic growth of cardiac myocytes can be initiated by endocrine, paracrine, and autocrine factors that stimulate a wide array of membrane-bound receptors (1, 2, 9, 14). Their activation results in the triggering of multiple cytoplasmic signal transduction cascades, which ultimately affects nuclear factors and the regulation of gene expression. No single intracellular transduction cascade regulates cardiomyocyte hypertrophy in isolation, but instead, each pathway operates as an integrated network with cross-talk at multiple levels e.g., calcineurin–NFAT signaling, phosphoinositide 3-kinases (PI3Ks)/Akt/GSK-3-dependent signaling, MEF2/histone deacetylases (HDACs) (1, 2, 9, 14). Blockade of specific intracellular signaling pathways in the heart can dramatically affect the entire hypertrophic response and effectively decrease cardiac hypertrophy. Furthermore, specific activation of a number of discrete signal transduction pathways may be sufficient to activate the entire hypertrophic response through effects on the interconnecting signaling networks (1, 2).

Recent observations from animal models and clinical trials have identified a number of signaling cascades that hold promise as potential targets for treatment of cardiac hypertrophy, such as the transcription factors MEF2, NFAT, and GATA4 (32–37). Chronic inhibition of cGMP phosphodiesterase 5A (PDE5A) has been shown to deactivate multiple hypertrophy signaling pathways triggered by pressure load and prevent and reverse cardiac hypertrophy in an animal model (23).

NF-κB Signaling Pathway. NF-κB is a transcription factor that can directly regulate the expression of immediate-early genes and genes involved in the stress response after a variety of physiological or pathological stimuli including cell growth and apoptosis (38). NF-κB transcription factors are homo- or heterodimers that translocate to the nucleus and bind DNA through a Rel-homology domain. In most resting cells, NF-κB is a p50/RelA dimer that is retained in the cytoplasm bound to IκBs, which function as cytoplasmic inhibitors of NF-κB (28, 38). Upon stimulation, IκB becomes phosphorylated through the action of the IκB kinase (IKK) complex (28). Phosphorylation of IκB triggers its ubiquitination and degradation, which permits NF-κB translocation to the nucleus, where it activates transcription of inflammatory and immune response target genes (28, 29).

Recently, several studies have implicated NF-κB activation as a causal event in the cardiac hypertrophic response, as modeled in cultured cardiac myocytes as well as with *in vivo* models (5–12). Purcell *et al.* (5) demonstrated that viral-mediated transfer of a "superrepressor" IκBα protein, a dominant-negative NF-κB approach, prevented cardiomyocyte hypertrophy in response to G protein-coupled receptor (GPCR) agonists such as phenylephrine, endothelin-1, and Ang II. This linkage between NF-κB and myocyte hypertrophy is especially intriguing given the long-standing hypothesis that inflammatory cytokines (e.g., TNF-α and IL1) are relevant mediators of cardiomyopathic disease states and that NF-κB itself is dominantly regulated by the cytokine TNF-α (39). Heart failure patients typically show elevated levels of circulating TNF-α in the blood (39) and have significant myocardial NF-κB activation (39, 40).

Our present data using both in vivo and in vitro models suggest an inhibitory effect of sEHIs on the NF-κB pathway; however, the study does not elucidate precisely where the inhibitory effects occur. Specifically, the inhibitory effects may occur at the level of NF-κB or more proximal in the signaling cascade. Future experiments are needed to further define the molecule responsible for the observed inhibitory effects in the NF-κB pathway.

Cytochrome P450 Metabolites and sEH. Cytochrome P450 (CYP450) metabolites of arachidonic acid (AA), including regio- and EETs and 20-hydroxyeicosatetraenoic acid (20-HETE), are important regulators of blood pressure (41). EETs function primarily as autocrine and paracrine effectors in the cardiovascular system and kidney (17, 22). They modulate ion transport and gene expression, producing vasorelaxation as well as antiinflammatory and profibrinolytic effects. All EET regioisomers show distinct vasodilatory actions and function as endogenous hypotensive agents (42). EETs are further metabolized by sEH to form the corresponding DHETs (15, 43). In the renal microcirculation, the vasoconstriction stimulated by 20-HETE (Ca^{2+}-activated K^+ channel antagonist) is attenuated by EETs (Ca^{2+}-activated K^+ channel agonists) (41).

Some functional effects of EETs occur through activation of either the guanine nucleotide-binding protein $G_{\alpha s}$ or the Src signal transduction pathways, suggesting that EETs act by binding to membrane receptors to initiate the signaling cascades (27). $G_{\alpha s}$, a membrane-associated protein, appears to be involved in EET-mediated activation of BK_{Ca} channels (27). However, other evidence indicates that the modulation of gene expression occurs through an intracellular action of EETs. EETs are incorporated into cell phospholipids, and EETs, or metabolites that are generated intracellularly from EETs, interact with nuclear receptors to regulate gene expression (17). It has been shown that EETs can inhibit IKK, preventing degradation of IκB, maintaining NF-κB in the inactive state and inhibiting NF-κB-mediated gene transcription (18, 19). Because sEH converts EETs to DHETs, inhibition of sEH has been intensely investigated as a potential approach for enhancing the biological activity of EETs and other epoxy lipids with antihypertensive and antiinflammatory actions (17, 21).

Here, we show that sEHIs can prevent and reverse cardiac hypertrophy. Even though treatment with sEHIs may result in a lack of normalization of wall tension, the treatment led to a preservation of cardiac contractility in the long-term by preventing progressive cardiac deterioration associated with cardiac hypertrophy. Indeed, previous studies using gene-targeted mouse models with attenuated hypertrophic response have shown that despite inadequate wall tension normalization in the transgenic models with chronic pressure overload, the transgenic animals showed little cardiac deterioration over time compared with WT animals (44, 45).

From our published work (20), we predict that sEHIs at doses used in this study will lead to a decrease in the systemic blood pressure in the mice. However, it is unlikely that blood pressure-lowering agents alone will prevent the development of cardiac

hypertrophy in this model because a persistent chronic increase in pressure is exerted on the heart in this model by the constriction of the thoracic aorta. Thus, future work is required to directly compare the effects of sEHIs and blood pressure-lowering agents that act through separate mechanisms on cardiac hypertrophy in this model.

Finally, we were able to show that use of sEHIs can prevent the development of ventricular arrhythmias that occur in association with cardiac hypertrophy. Indeed, cardiac hypertrophy and failure are associated with a significant increase in the risk of arrhythmias and sudden cardiac death (46). Moreover, treatment with conventional antiarrhythmic drugs generally carries a high risk of proarrhythmia and results in a decrease in patient survival (47). The most accepted form of therapy involves implantation of defibrillators, which is invasive and costly (48). Our study has important clinical significance in providing evidence for an alternative strategy in the treatment of cardiac hypertrophy and the prevention of ventricular arrhythmias associated with cardiac hypertrophy.

Materials and Methods

For more detail, see *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site.

Compensated Cardiac Hypertrophy Model in Mice. TACs were performed in 8-week-old male C57BL/6J mice as described (24). Animals with TAC were randomized to treatment with two different sEHIs: AEPU in drinking water (0.1 mg/ml), AUDA-free acid (0.1 mg/ml) in drinking water, or vehicle only for 3 weeks. Sham-operated animals received the same surgery except that the ligature was not tied. Echocardiograms to assess cardiac function were performed at the end of the experiments by using M-mode and 2D measurements. *In vivo* EPS were performed as described (31, 49). Standard pacing protocols were used. Each animal underwent an identical pacing and programmed stimulation protocol.

Semiquantitative RT-PCR Analysis. Total RNA from left ventricular free wall was reverse-transcribed. cDNA was then diluted with water to generate serial dilutions (1:1, 1:2, and 1:10) and used for PCR amplification with gene-specific primers.

Western Blot Analysis. Immunoblots were performed as described (50). The following primary Abs were used: (*i*) anti-IκBα and phosphor-IκBα mAbs (1:200 dilution, Santa Cruz Biotechnology, Santa Cruz, CA); (*ii*) anti-p65 (Santa Cruz Biotechnology, 1:200 dilution); (*iii*) polyclonal anti-sEH Ab (1:2,000) against human sEH was raised from rabbits as described (26); and (*iv*) anti-GAPDH Ab (Sigma) was used as an internal loading control.

Immunofluorescence Confocal Microscopy. Immunofluorescence labeling was performed as described and imaged using a confocal laser scanning microscopy (50) (Pascal Zeiss, Thornwood, NY). Control experiments performed with secondary Ab only did not show positive staining under the same experimental conditions used. Identical settings were used for all of the specimens.

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