



A dipeptidyl peptidase-4 inhibitor ameliorates hypertensive cardiac remodeling via angiotensin-II/sodium-proton pump exchanger-1 axis



Haruya Kawase, Yasuko K. Bando*, Kazuyuki Nishimura, Morihiko Aoyama, Akio Monji, Toyoaki Murohara*

Department of Cardiology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, Aichi 466-8550, Japan

ARTICLE INFO

Article history:

Received 21 February 2016
Received in revised form 26 June 2016
Accepted 28 June 2016
Available online 30 June 2016

Keywords:

Dipeptidyl peptidase-4 inhibitor
Hypertension
Cardiac remodeling
Angiotensin-II
Sodium-proton pump exchanger

ABSTRACT

Background: To address the impact of antidiabetic drugs on cardiovascular safety is a matter of clinical concern. Preclinical studies revealed that various protective effects of dipeptidyl peptidase-4 inhibitor (DPP4i) on cardiovascular disease; however, its impact of on hypertension remains controversial.

Methods and results: Tenecligiptin (TEN; 10 mg/kg/day/p.o.) ameliorates hypertension and cardiac remodeling by normalizing a rise of angiotensin-II (AngII) that specifically observed in spontaneously hypertensive rats (SHR). TEN had no effects on vasculature and concentrations of the DPP4-related vasoactive peptides (bradykinin, neuropeptide Y, and atrial natriuretic peptide). The primary action of TEN on BP lowering was due to restoring the AngII-induced manifestation of congestive heart failure observed in SHR. Sodium-proton pump exchanger type 1 (NHE-1) is a regulator of intracellular acidity (pHi) and implicated pathophysiological role in cardiac remodeling occurred in diseased myocardium. Cardiac NHE-1 expression level was increased in SHR and this was restored in TEN-treated SHR. AngII directly augmented cardiac NHE-1 expression and its activity that contributed to hypertrophic response. TEN attenuated the AngII-induced cardiac hypertrophy with decline in pHi via suppression of NHE-1. Loss of NHE-1 activity by specific inhibitor or RNA silencing promoted intracellular acidification and consistently attenuated the AngII-mediated cardiac hypertrophy.

Conclusion: The present study revealed the protective actions of TEN on hypertension and comorbid cardiac remodeling via AngII/NHE-1 axis and the novel pathophysiological roles of intracellular acidification via NHE-1 in cardiac hypertrophy.

© 2016 Elsevier Ltd. All rights reserved.

1. Introduction

The higher the prevalence of diabetes worldwide, the more the prescription number of antidiabetic remedy is increasing [1]. Nowadays, to address the impact of antidiabetic drugs on cardiovascular system independently of its glucose-lowering effects is considered as essential for the safe prescription of these drug classes. Dipeptidyl peptidase-4 inhibitor (DPP4i) has dominated the new class of anti-diabetic remedies and their cardiovascular safety has been confirmed in terms of major cardiovascular events, namely macrovascular diseases [2–4] and heart failure [2,3,5,6]; however, the impact of DPP4i on hypertension clinically remains uncertain. Indeed, there are controversial preclinical evidences regarding the blood pressure (BP) lowering effects of DPP4i. Several reports demonstrated that DPP4i exerts anti-hypertensive property [7,8], however, a recent paper suggested this BP-lowering actions of DPP4i is the context-dependent [9]. To address the impact of DPP4i on BP precisely, the direct monitoring of central BP by using cardiac catheterization may provide more information than non-

invasive cuff procedure because BP is determined by the combination of peripheral vascular resistance and cardiac performance [10] and central BP is superior to assess organ damage [11].

It is unique characteristics of DPP4i that the chemical structure of this drug class is quite distinct from each other [12]. Tenecligiptin (TEN) is the one of the recent DPP4i and biochemical data showed that TEN exhibits more potent than other DPP4i because of its unique structure [12,13]. However, its effect(s) on cardiovascular system is little known.

Here we report the impact of TEN on central BP and cardiac remodeling of spontaneous hypertensive rats (SHR) and its normotensive counterpart WKY. Interestingly, TEN ameliorated hypertension and comorbid cardiac remodeling observed in SHR by attenuating a rise of circulating angiotensin-II (AngII). Furthermore, we found TEN attenuated clinical features of heart failure (mild pulmonary congestion, rises in left-ventricular end-diastolic pressure (EDP) and elevated brain natriuretic peptide (BNP)) observed in SHR independently of vasodilating action. In this context, we examined the pathophysiological role(s) of the sodium-proton pump exchanger-1 (NHE-1) in the TEN-mediated amelioration of hypertensive cardiac remodeling. NHE-1 plays a regulatory role(s) in maintenance of intracellular acidity (pHi) and in regulation of myocardial remodeling such as ischemic myocardium

* Corresponding authors.

E-mail addresses: ybando@med.nagoya-u.ac.jp (Y.K. Bando), murohara@med.nagoya-u.ac.jp (T. Murohara).

[14] and pressure-overload [15]. We found that cardiac NHE-1 expression and its activity were increased in an AngII-dependent fashion. NHE-1 inhibition decreased pHi and attenuated cardiomyocyte hypertrophy. TEN suppressed cardiac NHE-1 thereby ameliorated AngII-induced cardiac hypertrophy. Beyond the pharmacological evidences of TEN on hypertension, present study reinforces the role of NHE-1 in terms of modulating intracellular acidification in hypertensive cardiac remodeling, at least in part, via AngII.

2. Methods

2.1. Experimental design

Animal experiments were approved by the Committee on the Use of Live Animals for Teaching and Research of Nagoya University. Male SHR and WKY (10-week-old) were purchased from SLC Japan and randomly allocated to vehicle, TEN (teneligliptin hydrobromide, CAS Number 760937-92-6, generously gifted by Tanabe-Mitsubishi, Inc.), and SITA (sitagliptin phosphate monohydrate, CAS Number: 654671-77-9, Santa Cruz Biotechnology) group (10 mg/kg/day for 4 week; WKY-CON, WKY-TEN, SHR-CON, SHR-TEN, and SHR-SITA). An experimental timeline is displayed in Supplemental Fig. 1.

2.2. Echocardiography and cardiac catheterization

Cardiac function of each rat was assessed using echocardiography (SONOS 7500; Philips, Amsterdam, The Netherlands) under inhalation of isoflurane (2% in oxygen) just prior to cardiac catheterization. Rats were subjected to hemodynamic analysis by using a 2.0 Fr micromanometer-tipped catheter (SPR-320; Millar Instruments, Houston, TX, USA) that was inserted through the right carotid artery into left ventricle. Changes in hemodynamic parameters were monitored using the Power Lab™ system (AD Instruments, Colorado Springs, CO, USA).

2.3. Measurements of laboratory chemistry and circulating bioactive peptides

Casual blood glucose (BG) concentration was measured by dextrometer. Right after cardiac catheterization, blood and urine were collected from each rat by puncture of heart and bladder, respectively. Circulating vasoactive peptide concentrations were determined using immunoassay kits according to the manufacturer's protocol; Glucagon-like peptide-1 (GLP-1, EMD Millipore, Billerica, MA, USA), atrial natriuretic peptide (ANP, RayBiotech, Norcross, GA, USA), brain natriuretic peptide (BNP, RayBiotech), AngII (Sigma-Aldrich, St. Louis, MO, USA), neuropeptide Y (NPY, EMD Millipore), and bradykinin (BK, Phoenix pharmaceuticals, Burlingame, CA, USA).

2.4. Analysis of cardiac remodeling

Cardiomyocyte surface area (CSA) was detected by immunohistochemistry using anti-dystrophin antibody (Novus Biologicals, Littleton, CO, USA) for each heart section and by digital imaging obtained from bright-field microscope for cultured cardiomyocytes. Cardiac fibrosis was assessed by picosirius red with counterstain of Fast Green (Sigma-Aldrich). Quantitative analysis of each positive lesion was made using Image J software (National Institutes of Health, USA).

2.5. Enzymatic activities of cardiac and circulating DPP4, angiotensin converting enzymes, and renin activity

Cardiac DPP4 proteolytic activity was detected by in situ colorimetry as described previously [16,17]. Circulating DPP4 activity (DPP4-Glo™ protease assay; Promega, Madison, WI, USA), Plasma ACE (ACE color,

Fuji-rebio, Tokyo, Japan), and renin activity (Fuji-rebio) were measured by the commercially available kit.

2.6. Immunoblotting

Heart and aorta specimens were processed with frost shattering by using Cryopress™ (Microtech Nichion K.K., Chiba, Japan). Tissue lysates (20 µg/lane) were subjected to electrophoresis followed by immunoblotting. In case of in vitro assay, cardiomyocytes were plated onto 6-well dish at 1.0×10^5 cells/well (Supplemental Fig. 3A). List of antibodies used in the present study was displayed in Supplemental Table 1.

2.7. Intracellular acidity measurement

Cultured cardiomyocytes (plated on 96-well dish at 1.0×10^4 cells/well) were used for pHi detection with a fluorescence probe for intracellular proton level (pHrodo™ Green AM; Thermo Fisher Scientific, Waltham, MA, USA). Change in fluorescence intensity was monitored by a micro-plate reader (Tecan, Männedorf, Switzerland). Experimental timeline is illustrated in Supplemental Fig. 3B.

2.8. RNA silencing in cultured cardiomyocytes

Cultured cardiomyocytes were plated onto an 8-well chamber slide coverslip pre-coated with collagen type 1 (BD Biosciences) at a density of 1×10^5 cells/well. Efficacy-validated siRNAs (Sigma-Aldrich) for silencing NHE-1 (siPKA) and for a negative control (siCON) were introduced into cells using lipofectamine RNAiMAX (Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions.

2.9. Quantitation of gene expression

Changes in the NHE-1, BNP, β -myosin heavy chain (MYH7) gene expression levels were assessed by use of 2-step Real-time PCR. Total RNA was extracted cardiomyocyte using from using RNeasy Micro Kit (Qiagen, Valencia, CA, USA). cDNA was produced using a ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan). PCR procedure was performed with a Bio-Rad real-time PCR detection system using SYBR Green I as a double-standard DNA-specific dye. Bio-informatically validated primers specific for the BNP, MYH7, and Eukaryotic 18S ribosomal RNA were purchased from QIAGEN and Sigma-Aldrich.

2.10. Statistical analysis

Statistical analysis was performed by JMP Pro (ver 11, SAS Institute, Cary, NC, USA). Values are expressed as the mean \pm SEM. Comparison of 2 groups were performed by Student's *t*-test (parametric) or Mann-Whitney *U* test (non-parametric). For multiple groups, ANOVA (parametric) or Kruskal-Wallis test (non-parametric) were applied as appropriate. A 2-way ANOVA was conducted to compare 2 different categorical variables. Post-hoc comparisons of considered pairs were performed using the Tukey-Kramer test for differences between means. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Impact of TEN on clinical characteristics

We first assessed the impact of TEN on circulating and membrane DPP4 activities (Fig. 1) and its impact on physical and biochemical characteristics of each rodent (Table 1). Because DPP4 exists both in soluble and localized form [18] and the latter plays a crucial roles in cardiac remodeling [16,17], we measured both DPP4 activities in plasma and heart. TEN successfully inhibited circulating (Fig. 1A) and cardiac DPP4 activities (Fig. 1B and C). We then assessed the drug-class effect of

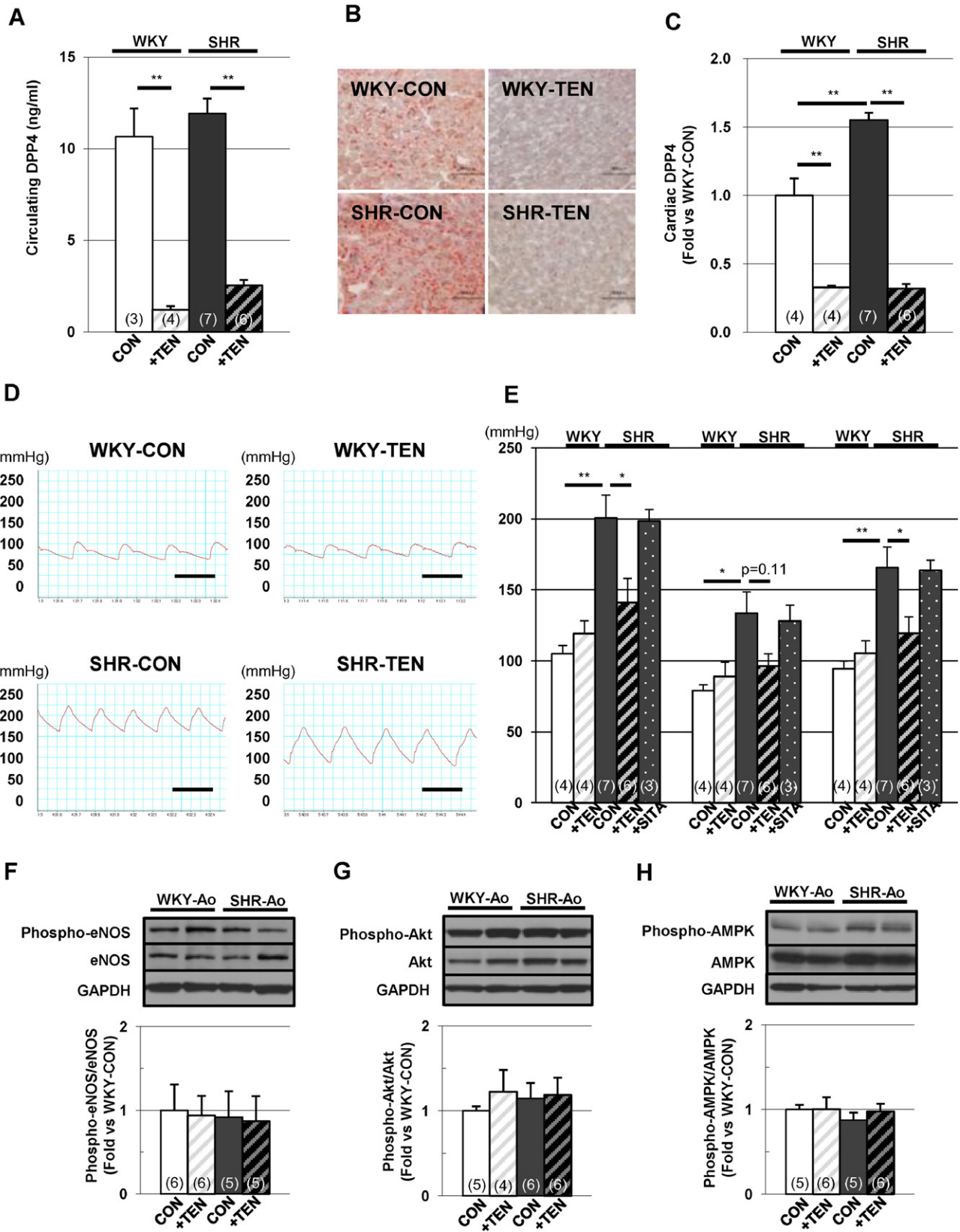


Fig. 1. Effects of telagliptin on DPP4 activities and arterial blood pressure of hypertensive and normotensive rats. **A:** Changes in circulating DPP4 activity and impact of TEN in hypertensive (SHR) and normotensive (WKY) rats. Inset of number in each bar indicates total sample count of the corresponding group. Representative images (**B**) and quantitative summary (**C**) of in situ colorimetric staining of cardiac DPP4 activity (red). Scale bar: 100 μ m. **D** and **E:** Effects of TEN on central BP of SHR and WKY. **D:** Original recordings of central BP of WKY (upper panels) and SHR (lower panels) measured by cardiac catheterization. Scale bar; 0.2 s. **E:** Summary of changes in BP. CON; vehicle-treated group. TEN; telagliptin-treated (10 mg/kg/day). SITA; sitagliptin-treated (10 mg/kg/day). **F–H:** Typical images of immunoblot analysis of each rat aorta demonstrating the changes in phosphorylated and total eNOS (**F**), Akt (**G**), AMPK (**H**) (upper panels). GAPDH was also measured as an internal control. Ratio of phosphorylated versus total protein was summarized in bar graph (lower). Data are shown as the mean \pm SEM. * $P < 0.05$ and ** $P < 0.01$.

TEN on BG level and found that TEN decreased casual BG levels both of SHR and WKY (Table 1).

3.2. TEN ameliorates blood pressure exclusively to SHR

We next measured the effect of TEN on hemodynamics by cardiac catheterization (Fig. 1D and E). Systolic and diastolic central BP (SBP and DBP, respectively) was significantly higher in SHR-CON (in mmHg; 201 ± 16 for SBP and 134 ± 15 for DBP) than WKY-CON (105 ± 6 for SBP and 79 ± 4 for DBP) (Fig. 1D and E). TEN reduced SBP and DBP exclusively of SHR (SHR-TEN; 141 ± 17 and 96 ± 6) (Lower panels of Fig. 1D and white shaded bars in Fig. 1E). In contrast, sitagliptin had no effect on SBP and DBP of SHR (dotted bars of Fig. 1E).

3.3. TEN lowers BP independently of vasodilatation exclusively for SHR

To address whether the BP lowering effects of TEN may be due to its direct action on vasculature, we measured changes in the Akt/AMPK/eNOS signal of each aorta [19] (Fig. 1F–H). However, we found that TEN had no effects on these signals. Because a recent report demonstrated that GLP-1 lowered BP by augmenting circulating ANP levels via arterial vasodilatation [20], we measured the circulating active GLP-1 (Supplementary Fig. 2A) and ANP (Supplementary Fig. 2B). TEN significantly increased the GLP-1 concentration of SHR, but it had no effect on their ANP levels under our experimental setting. We further measured other neurohormonal candidates of vasodilatation that exhibit specific amino acid sequence for protease cleavage by DPP4, however, TEN had no influence on the circulating levels of NPY and BK (Supplementary Fig. 2C and D).

3.4. TEN ameliorates hypertensive heart failure in SHR

Renin-angiotensin system (RAS) is a key mediator of both hypertension and heart failure progression, in which AngII plays a primary role in hypertensive cardiac remodeling [21,22]. Circulating AngII level was significantly elevated in SHR, which was restored by TEN (Fig. 2A) with consistent rise of plasma renin activity [23,24] (PRA; Fig. 2B). In contrast, angiotensin-converting enzyme (ACE) activity [25], that cleaves angiotensin I to AngII, remained unchanged (Fig. 2C).

We next evaluated the effect of TEN on cardiac performance by cardiac catheterization (Fig. 2D–F) and by echocardiography (Table 1).

Cardiac catheterization revealed that both indices of systole (maximum dp/dt, Fig. 2D) and diastole (minimum dp/dt, Fig. 2E) of SHR-CON were markedly augmented, which is consistent as hypertensive heart failure [26,27]. TEN reversed this hyperkinetic cardiac performance with concomitant amelioration of EDP (Fig. 2F) and plasma BNP observed in SHR (Fig. 2G). SHR-CON exhibited increases in heart rate (HR) and norepinephrine level that are hallmarks of sympatho-excitation in heart failure [28], which were unaffected by TEN (Fig. 2H). We then assessed clinical features of heart failure in each model (Table 1). Consistently with these hemodynamic data, SHR-CON exhibited significant increases in heart and body weight (BW) ratio, lung and BW ratio, which were ameliorated by TEN (Table 1).

3.5. TEN ameliorates hypertensive cardiac remodeling in SHR

To address whether TEN may affect hypertensive cardiac remodeling, we evaluated changes in cardiac hypertrophy and fibrosis (Fig. 3). TEN attenuated cardiac hypertrophy (Fig. 3A) and fibrosis (Fig. 3B) of SHR with concurrent changes in hypertrophic signaling (Fig. 3C–F).

3.6. Role of AngII and NHE-1 pathway in hypertensive cardiac hypertrophy

We next aimed to address the possible link(s) between the TEN-mediated normalization of circulating AngII concentration and its impact on the hypertensive cardiac remodeling (Fig. 4). NHE family plays regulatory roles in cardiovascular system and several reports demonstrated that NHE-1 modulates cardiac remodeling via neurohormonal pathway including AngII [29,30]. It remains unclear whether cardiac NHE-1 may regulate hypertensive myocardial remodeling. Expression of cardiac NHE-1 of SHR was elevated and this was restored in TEN-treated SHR heart (Fig. 4A). We next tested the direct impact of AngII on NHE-1 expression by using cultured cardiomyocyte (CMC) (Fig. 4B and C). We found that AngII augmented NHE-1 protein expression of CMC (middle lane in the upper panel) and this effect was abolished in the presence of TEN (right lane). The impact of AngII on NHE-1 mRNA expression was tested in CMC by QPCR in the combination of RNA silencing of NHE-1 (Fig. 4C). AngII consistently increased NHE-1 mRNA. Furthermore, we measured functional changes of NHE-1 by monitoring intracellular acidity (pHi) that is a surrogate of NHE-1 activity, because the pHi increases if NHE-1 becomes more active due to excretion of proton from intracellular space with simultaneous intake

Table 1

Anatomical parameters, laboratory chemistry and echocardiography analysis.

Parameter	WKY-CON	WKY-TEN	SHR-CON	SHR-TEN
Anatomical parameters (n = 4–10)				
BW (g)	398 ± 3	387 ± 4	$366 \pm 4^{**}$	354 ± 3
HW (mg)	1048 ± 28	994 ± 15	$1317 \pm 14^{**}$	$1231 \pm 21^{***}$
HW/BW	2.6 ± 0.1	2.6 ± 0.0	$3.7 \pm 0.0^{**}$	$3.4 \pm 0.1^{***}$
LW (mg)	1373 ± 33	1365 ± 6	1405 ± 29	1322 ± 23
LW/BW	3.4 ± 0.1	3.5 ± 0.0	$3.9 \pm 0.1^{**}$	$3.7 \pm 0.0^{***}$
Labo chemistry (n = 3–10)				
GLU (mg/dL)	143 ± 9	109 ± 4	144 ± 9	$105 \pm 11^{***}$
Na (mEq/L)	135 ± 3	134 ± 4	136 ± 2	135 ± 2
Urine Na (mEq/L)	19 ± 3	39 ± 9	69 ± 18	42 ± 12
Echocardiography (n = 4–7)				
IVST (mm)	1.98 ± 0.01	2.11 ± 0.02	$2.55 \pm 0.01^*$	$1.93 \pm 0.01^{***}$
PWT (mm)	1.98 ± 0.01	2.02 ± 0.02	$2.52 \pm 0.01^*$	$1.94 \pm 0.01^{****}$
LVdD (mm)	6.72 ± 0.28	6.45 ± 0.30	6.42 ± 0.12	6.63 ± 0.24
LVdS (mm)	4.11 ± 0.15	4.13 ± 0.26	4.02 ± 0.15	4.06 ± 0.1
EF (%)	74.9 ± 1.47	71.6 ± 1.69	71.5 ± 2.33	74.3 ± 2.05
FS (%)	38.8 ± 1.29	36.1 ± 1.30	37.7 ± 2.60	38.6 ± 1.83

BW; body weight, HW; heart weight, HW/BW; relative ratio of HW to BW, LW; lung weight, LW/BW; relative ratio of LW to BW, Na; sodium, GLU; casual blood glucose, IVST; interventricular septum thickness, PWT; posterior wall thickness, LVdD; left ventricular diastolic diameter, LVdS; left ventricular systolic diameter, EF; ejection fraction, FS; fractional shortening. Data were obtained from male WKY and SHR (14-week old) with or without TEN treatment (10 mg/kg/day). The value represents the mean \pm SEM.

* $P < 0.05$ versus WKY-CON.

** $P < 0.01$ versus WKY-CON.

*** $P < 0.05$ versus SHR-CON.

**** $P < 0.01$ versus SHR-CON.

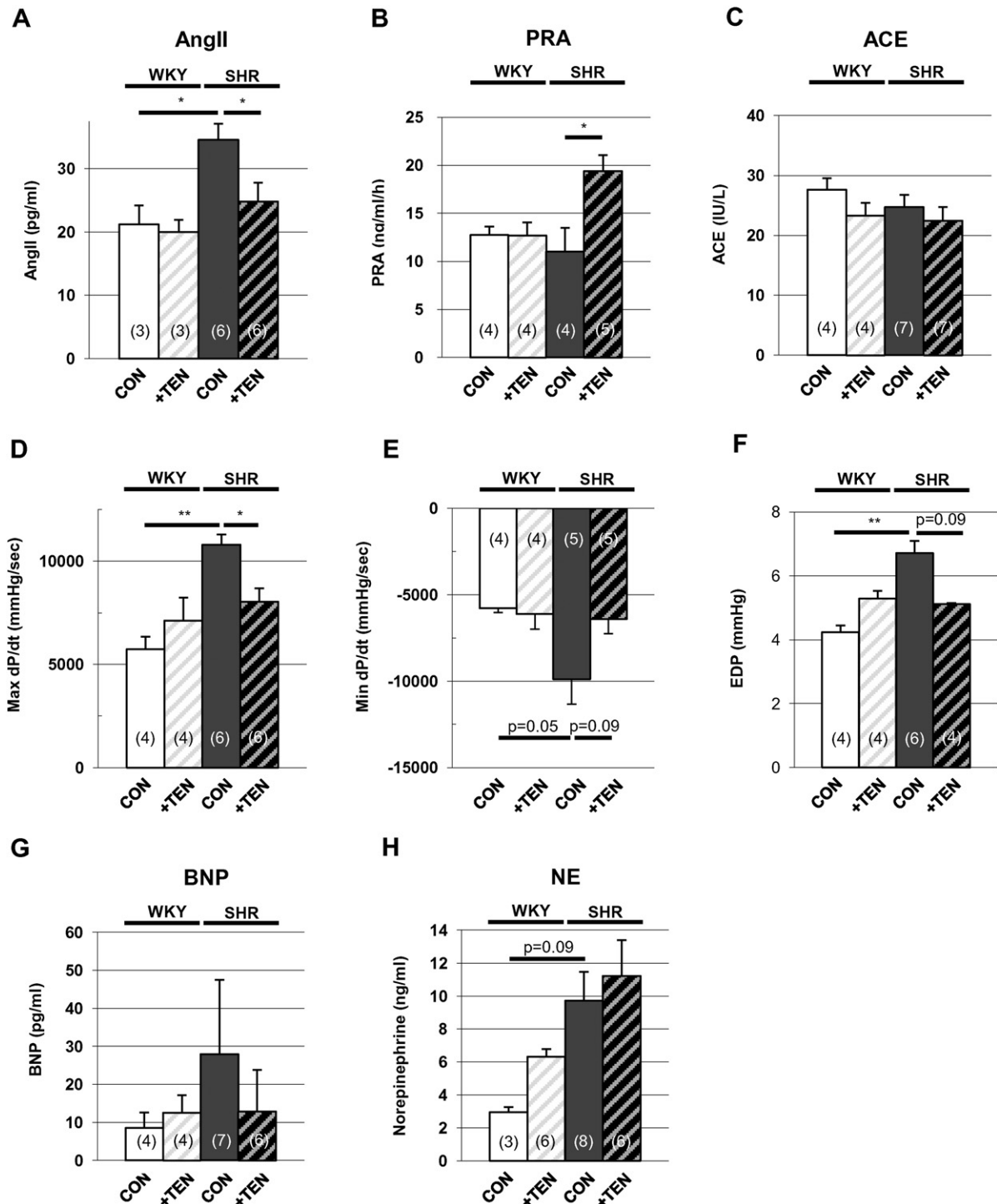


Fig. 2. Changes in neurohormonal factors and hemodynamic indices related to heart failure. Circulating levels of angiotensin II (AngII, A), plasma renin activity (PRA, B), angiotensin converting enzyme (ACE, C), brain-type natriuretic peptide (BNP, G), and norepinephrine (NE, H) in WKY and SHR with (TEN) or without (CON) treatment with teneligliptin. D-F: Changes in hemodynamic indices indicating systolic performance (the rate of left ventricle pressure (LVP) rise in early systole; Max dP/dt; D), diastolic performance (the rate of LVP decline during isovolumic relaxation period; Min dP/dt; E), and congestion (LVP at end-diastole, EDP; F) observed in WKY and SHR, respectively. Data are shown as the mean \pm SEM. * $P < 0.05$ and ** $P < 0.01$.

of sodium (Figs. 4D, E, and 7). TEN decreased pHi of CMC likewise EIPA, a specific inhibitor for NHE-1 (Fig. 4D) and the RNA silencing of NHE-1 (Fig. 4E).

3.7. Impact of NHE-1 inhibition on cardiomyocyte hypertrophy

We further compared the impacts of NHE-1 inhibition and TEN on cardiomyocyte hypertrophy in vitro by use of small molecule inhibitor

(Fig. 5) and RNA silencing (Fig. 6). AngII enhanced CMC surface area with increases in BNP and MYH7 mRNA levels (Figs. 5C, D, 6C, and D). TEN prevented pro-hypertrophic signaling induced by AngII (Fig. 5E–H). Lastly, we tested the impact of NHE-1 deletion by using RNA silencing (Fig. 6). The lack of NHE-1 induced intracellular acidification and concurrent decrease in cardiac hypertrophy that exhibits attenuation of hall mark of cardiac stress (Fig. 6C and D) and prohypertrophic signaling (Fig. 6E–H).

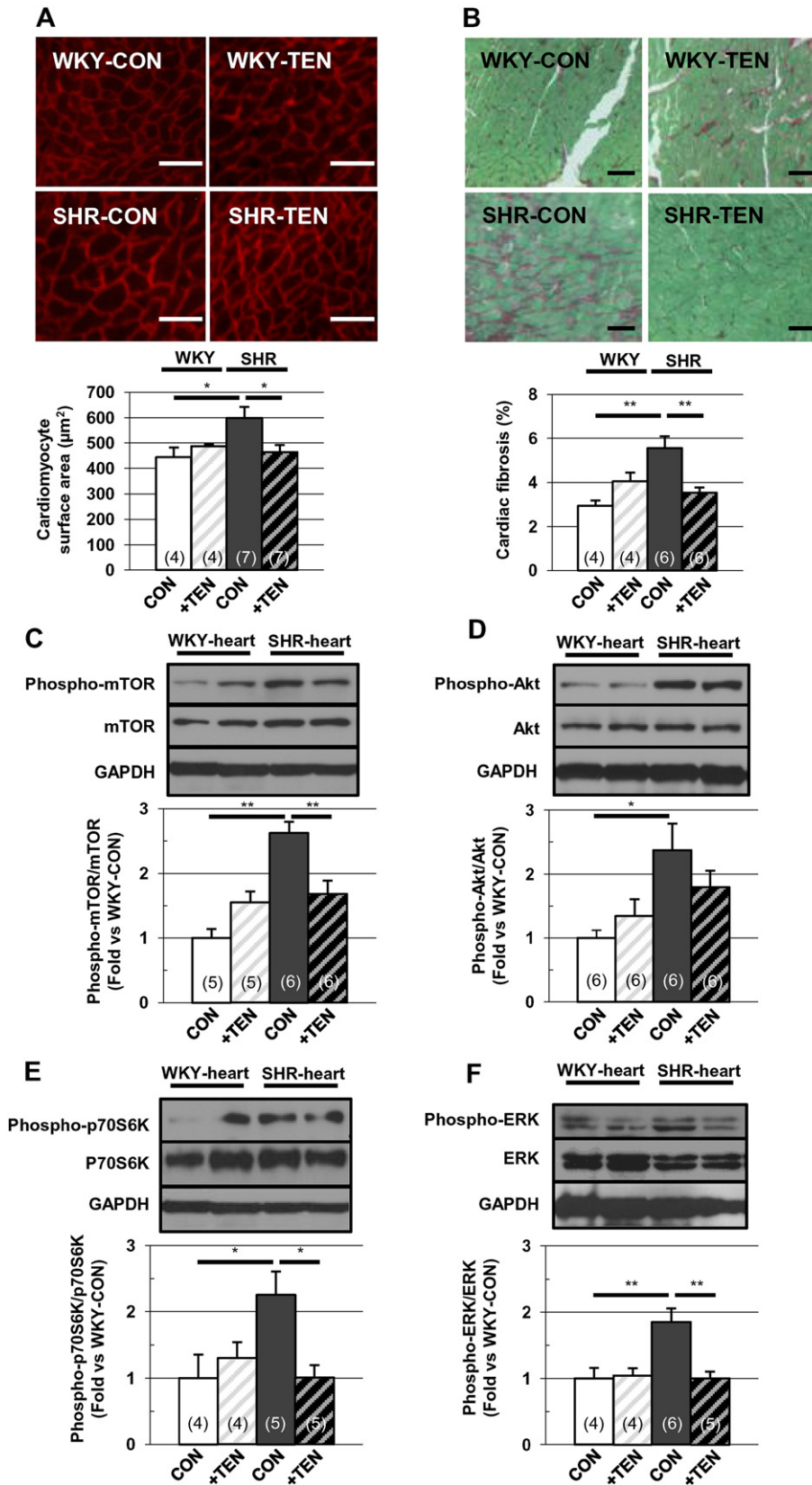


Fig. 3. Effects of teneligliptin on cardiac remodeling in SHR. **A.** Representative immunofluorescence images from heart section of WKY (upper panels) and SHR (lower) with or without TEN. Cardiomyocyte surface area (CSA) was measured by detecting a margin of each cardiomyocyte using anti-dystrophin antibody (red). Scale bar: 50 μ m. Bar graph is quantitative summary of CSA measurement. **B.** Representative images and quantitative summary of cardiac fibrosis in WKY and SHR detected by picrosirius red staining. Data are shown as the mean \pm SEM. Scale bar: 50 μ m. **C–F.** Effect of TEN on cardiac hypertrophy signaling. Each panel demonstrates typical image of mTOR (**C**), Akt (**D**), p70S6K (**E**) and ERK (**F**). Bar graph is quantitative summary by densitometry. * $P < 0.05$ and ** $P < 0.01$.

4. Discussion

The present study demonstrated the impact of TEN on the features of hypertension and revealed the novel pathophysiological roles of intracellular acidification via NHE-1 in the AngII-mediated cardiac hypertrophy. Prevalence of hypertensive left ventricular hypertrophy rises with severity of hypertension and the prevalence ranges from 20% in those mild-grade population to about 100% in those with severe grade [31,32]. Moreover, hypertensive patients with cardiac hypertrophy exhibits adverse prognostic outcome [33–35]. Interestingly, the effects of antihypertensive remedy that target on the renin-angiotensin system (RAS) ameliorates hypertensive cardiac hypertrophy independently of BP reduction [31,36]. AngII promotes vasoconstriction, cardiac hypertrophy, and fibrosis [31]. Furthermore, AngII stimulates a release of anti-diuretic hormones that increase the retention of sodium and consequential congestion [28]. These actions result in hypertension [37,38], congestive heart failure [28] and myocardial remodeling [39]. Because

of our experimental observations regarding the effect of TEN on hypertension (Fig. 1D and E) and mild but significant features of congestive heart failure (Fig. 2D–H and Table 1), we reasoned that the essential effect(s) of TEN on hypertension may be due to the restoration of the abnormal rise of AngII. Indeed, we found that TEN normalized AngII level of SHR with concurrent increase in PRA (Fig. 2). Regarding the elevation of PRA, a causative relationship to cardiovascular risk has been demonstrated [24], however, the reactive elevation of PRA by use of RAS inhibitors is identified [40]. In the present study, the reactive patterns of AngII and PRA to the treatment of TEN may be consistent with the RAS-inhibitory action of TEN.

ACE is the most elementary generator of AngII. Devin et al. demonstrated the possible cross-reaction of DPP4 and ACE activities that augmented the concentration of BK, a vasodilator peptide [8]. However, we found that TEN had no influence on circulating ACE activity (Fig. 3H) and BK level (Fig. 3G). Furthermore, another report suggested the possible link between DPP4 and NPY [41], which is either cleavable by ACE and by DPP4 (Fig. 3F); however, consistently with previous report

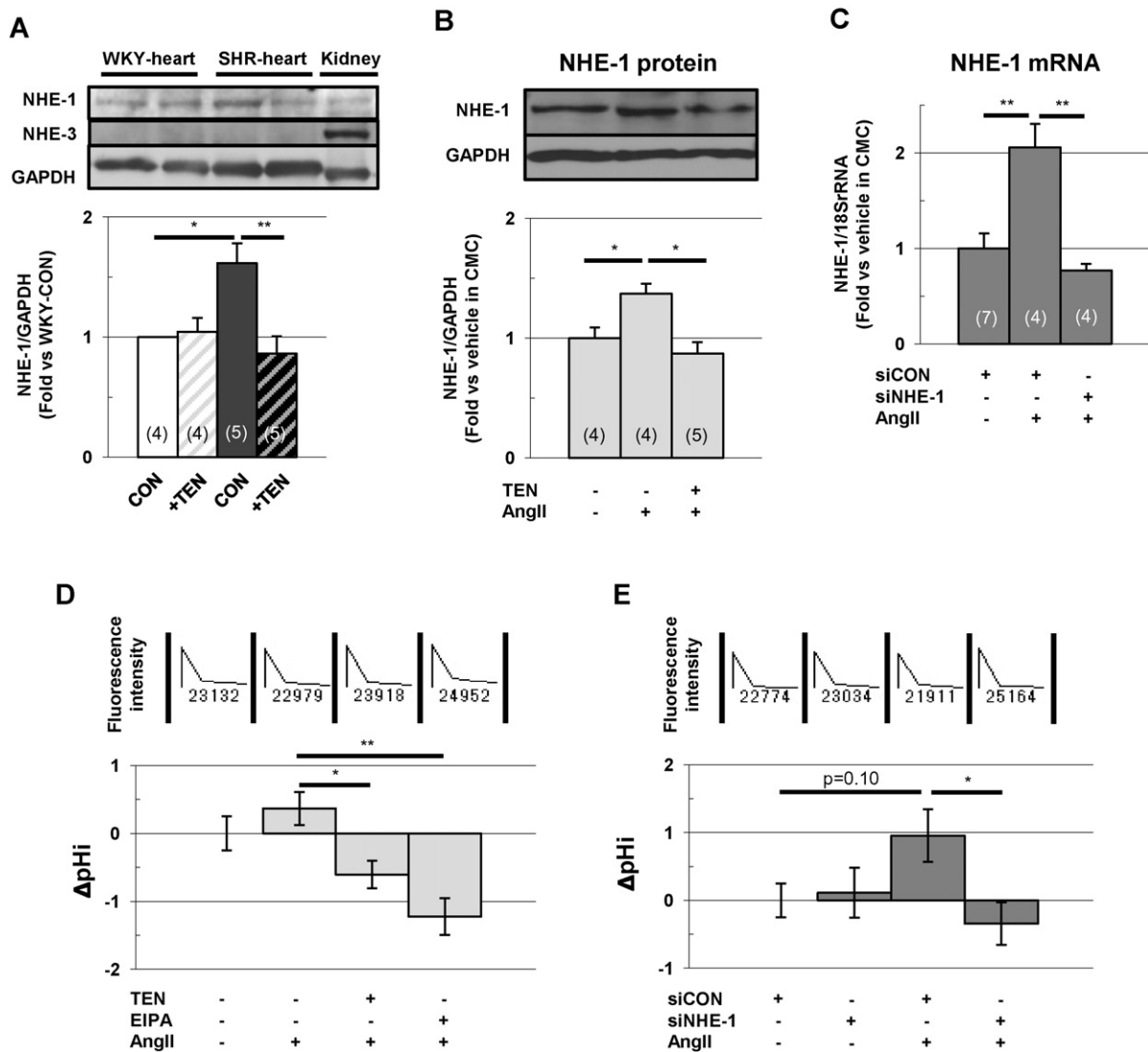


Fig. 4. Role of NHE-1 in hypertensive cardiac hypertrophy in SHR and cultured cardiomyocytes. A. Cardiac expression of NHE-1 was compared between WKY and SHR. Distinct subtype of NHE (NHE-3, renal subtype) was examined as a counterpart of NHE-1. Data were summarized by densitometry and displayed in bar graph below. B and C. Effect of angiotensin-II (AngII) and TEN on NHE-1 protein (B) and mRNA (C) expressions of cultured cardiomyocytes. Both protein and mRNA of NHE-1 were upregulated by AngII. D and E. Effect of teneligliptin and angiotensin-II on intracellular acidity of cardiomyocytes. Effect of pharmacological intervention (D) with TEN (1 μM) and EIPA (2 μM) and RNA silencing of NHE-1 (E) on the changes in intracellular acidity (pHi) in the presence and absence of AngII (100 nM). Representative scan recording of intracellular fluorescence intensity monitored by pHrodo. Changes in fluorescence intensity of pHrodo were converted into pHi by use of the calibration curve determined by measurement of pH standard buffers. Quantitative summary of relative changes in pHi as a subtraction of baseline pHi from those obtained after application of TEN, AngII, EIPA (a specific inhibitor of NHE-1), and RNA silencing of NHE-1 (ΔpHi). Data are shown as the mean ± SEM. *P < 0.05 and **P < 0.01.

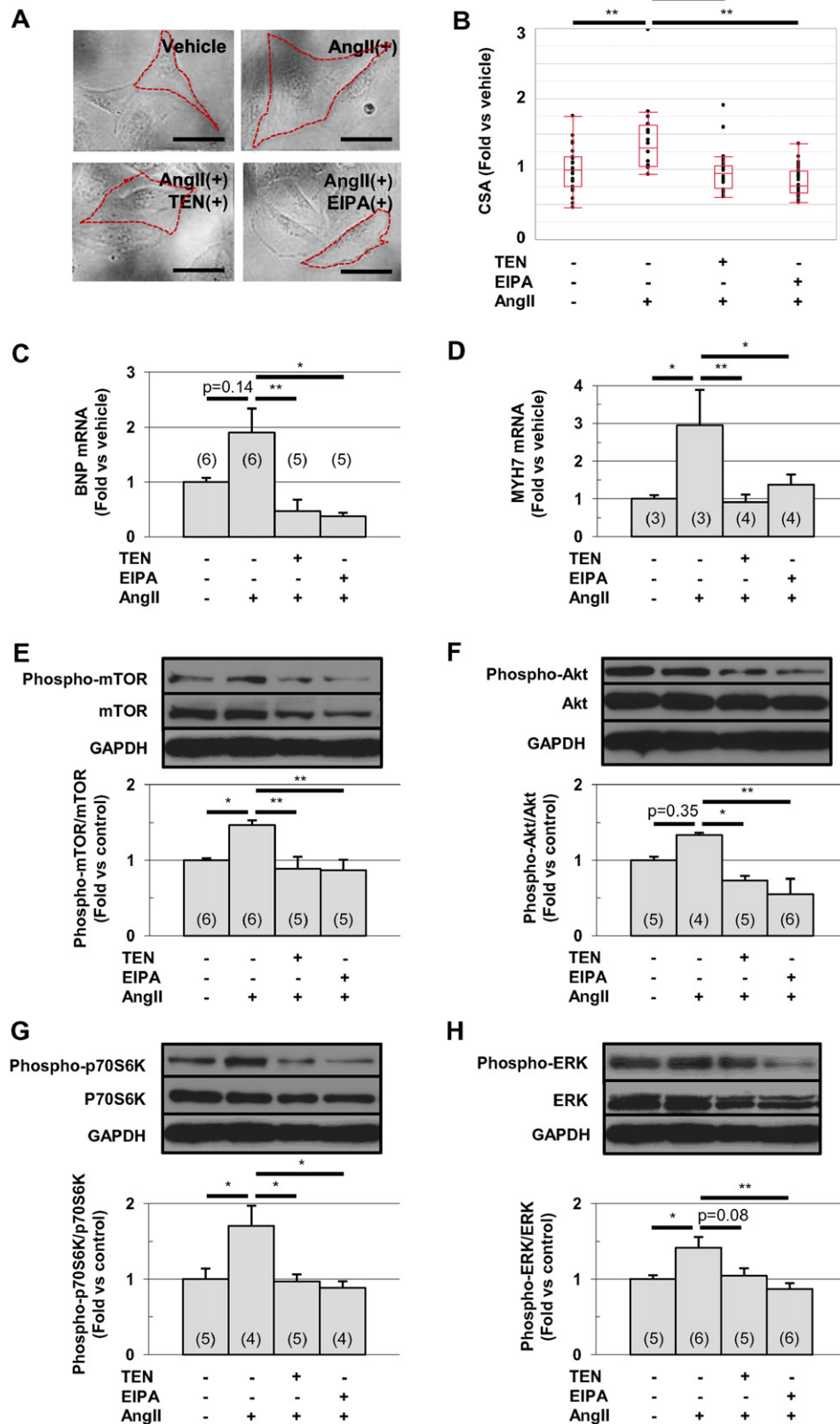


Fig. 5. Impact of pharmacological inhibition of NHE-1 on cardiomyocyte hypertrophy in vitro. Effects of NHE-1 inhibition on cardiomyocyte hypertrophy were assessed by the distinct observations in terms of morphological changes (cardiomyocyte surface area, CSA; A and B. Scale bar: 100 μ m), prohypertrophic markers (BNP and MYH7 mRNA level detected by QPCR; C and D), and hypertrophic cell signaling (changes in mTOR, Akt, p70S6K, and ERK activities monitored by immunoblot; E–H) by use of small molecule inhibitor (EIPA) and TEN. A. Representative images of cardiomyocytes treated with AngII (100 nM), TEN (1 μ M), and EIPA (2 μ M) observed under bright-field microscopy. CSA was measured and summarized as relative changes obtained from the comparison with the CSA value of unstimulated counterpart (fold versus CSA value of each vehicle-treated cardiomyocyte). Data distribution and statistical analysis are displayed in the box plot (B). * P < 0.05, ** P < 0.01.

[42], we found the unexpected increase of NPY in SHR (in ng/ml; 4.19 ± 0.38 for SHR-CON and 0.82 ± 0.02 for WKY-CON; unpublished data) that was insensitive to TEN (Fig. 3F).

Alternatively, there are ACE-independent and noncanonical pathway that generates AngII [37,43,44]. Several proteases such as chimase [45] or cathepsin [46] are known as possible regulators for

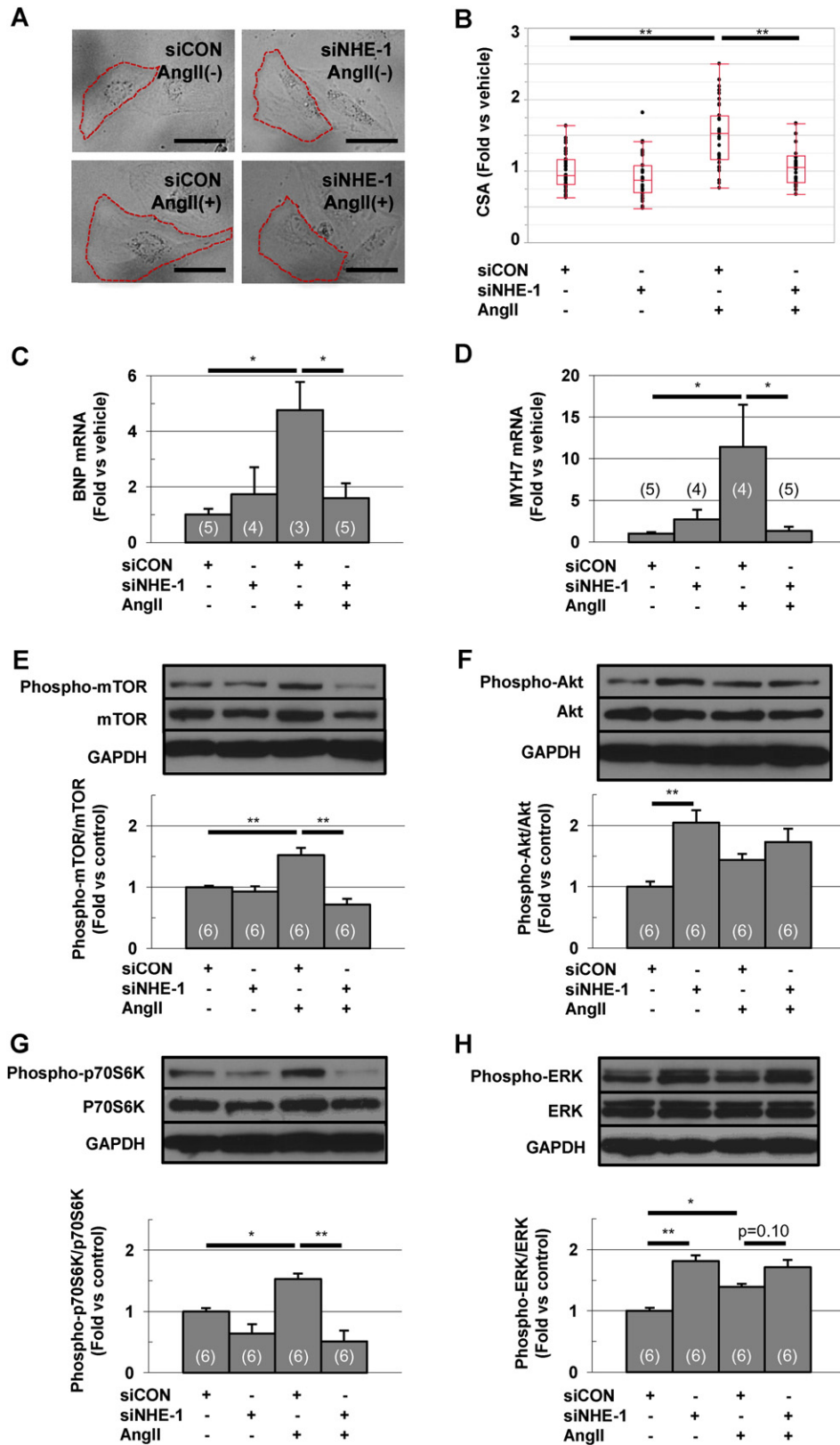


Fig. 6. Impact of RNA silencing of NHE-1 on cardiomyocyte hypertrophy in vitro. Effects of NHE-1 inhibition induced by RNA silencing on cardiomyocyte hypertrophy were assessed in terms of morphological changes (CSA; A and B. Scale bar: 100 μ m), prohypertrophic markers (BNP and MYH7 mRNA; C and D), and hypertrophic cell signaling (changes in mTOR, Akt, p70S6K, and ERK; E-H). Representative images (A) and box plot summary (B) of cardiomyocytes treated with AngII (100 nM) with NHE-1 siRNA (siNHE-1) or negative control siRNA (siCON). * $P < 0.05$, ** $P < 0.01$.

the ACE-independent AngII production [47], however, we could not specify the responsible factors for the TEN-sensitive and ACE-independent decline in AngII level in the present study.

The present study has 2 limitations. First, this study was designed to observe the impact of TEN on hypertension independently of the unwanted influence(s) of comorbid diabetes; however, if one considers the clinical indication of TEN as antidiabetic remedy, to test the impact of TEN on diabetic models is supposed to be addressed. Second, we have yet to find the reason why there are differences between TEN and SITA in the BP-lowering effect (Fig. 1E). Indeed, previous evidences consistently reported the context-dependent action of DPP4i on hypertension [9,48] and such drug-specific effects might be reasoned by the structural difference observed among DPP-4 inhibitors [12].

The present study is beyond the drug-specific effect(s) of TEN that provides the more comprehensive evidences regarding the cardiac hypertrophy via NHE-1. NHE-1 modulates intracellular acidity and its activity is, at least in part, AngII-dependent [14,49]. In heart, the role of NHE-1 has been evaluated in the limited models, namely, myocardial ischemia [14] and pressure-overload [15]. We therefore evaluated NHE-1 expression (Figs. 4, 5, and 6) and activity (Fig. 4D and E) in hypertensive model (Figs. 5 and 6). We found that AngII enhanced NHE-1 expression and increased pHi, which was restored by its specific inhibitor EIPA (Fig. 4D and Fig. 5) and RNA silencing (Fig. 4E and Fig. 6). TEN suppressed NHE-1 expression (Fig. 4B and C) and its activity (Fig. 4D), leading to the amelioration of hypertrophic response of cardiomyocytes (Fig. 5). However, we could not elucidate the further mechanism underlying the AngII/TEN axis that may regulate NHE-1 activity and expression. NHE-1 is an integral membrane transport protein that localizes in cellular plasma membrane (Fig. 7), implicating the possibility that small molecule may interact directly with the NHE-1 independently of any specific receptors. Interestingly, NHE-1 is up-regulated in various malignancies and NHE-1 becomes activated during malignant cell transformation [50]. NHE-1 activation

is known to be important in particular at the beginning of cell division and during cell proliferation, at least in part, via extracellular signal-regulated protein kinases 1/2 (ERK1/2) mechanism. AngII plays a primary role in tumor growth via activation of ERK1/2 [51], implicating the presumable link between AngII and NHE-1. Notably, some statins inhibit tumor NHE-1 presumably via isoprenylation thereby augment tumor apoptosis [52]. Inhibition of isoprenylation by lovastatin is responsible for the occurrence of apoptosis in leukemic HL-60 cells associated with dose-dependent intracellular acidification that augments DNA degradation. In cardiomyocytes, several statins have been demonstrated to exert anti-hypertrophic actions against AngII-induced cardiac remodeling [53,54], suggesting the action of statins on cardiac remodeling might be mediated by NHE-1 presumably via isoprenylation, if the case in tumor cells might be applicable for cardiomyocyte growth.

In conclusion, we provide the evidences regarding not only the drug action of TEN but also the novel pathophysiological role(s) of the AngII/NHE-1 axis in hypertensive cardiac remodeling. The present study reinforces the primary role of AT-II pathway in cardiac remodeling particularly in hypertensive population.

Conflicts of interest/disclosure

TM has received lecture fees and research grants from Astellas, AstraZeneca, Bayer, Boehringer Ingelheim, Daiichi Sankyo, Denso, Kowa, MSD, Pfizer, Takeda, and Tanabe-Mitsubishi. YKB received lecture fees and research grants from Astera, AstraZeneca, Boehringer Ingelheim, MSD, Takeda, and Tanabe-Mitsubishi. All authors and his/her family members hold stock directly or indirectly in any of these companies.

Acknowledgments

The authors extend their appreciation to the staff and volunteers who supported this study and, in particular, Dr. Mikito Takefuji for his technical advice and Ms. Yoko Inoue (Nagoya University) for her laboratory assistance.

This work was supported in part by Grant-in-Aid for Scientific Research No. 20249045 (to Dr. Murohara) and No. 23591080 and 16K09496 (to Dr. Bando) from the Ministry of Education, Culture, Sports, Science, and Technology Japan.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jmcc.2016.06.066>.

References

- [1] C. Hampp, V. Borders-Hemphill, D.G. Moeny, D.K. Wysowski, Use of antidiabetic drugs in the U.S., 2003–2012. *Diabetes Care* 37 (2014) 1367–1374.
- [2] J.B. Green, M.A. Bethel, P.W. Armstrong, J.B. Buse, S.S. Engel, J. Garg, et al., Effect of Sitagliptin on cardiovascular outcomes in type 2 diabetes. *N. Engl. J. Med.* 373 (2015) 232–242.
- [3] W.B. White, C.P. Cannon, S.R. Heller, S.E. Nissen, R.M. Bergenstal, G.L. Bakris, et al., Alogliptin after acute coronary syndrome in patients with type 2 diabetes. *N. Engl. J. Med.* 369 (2013) 1327–1335.
- [4] B.M. Scirica, D.L. Bhatt, E. Braunwald, P.G. Steg, J. Davidson, B. Hirshberg, et al., Saxagliptin and cardiovascular outcomes in patients with type 2 diabetes mellitus. *N. Engl. J. Med.* 369 (2013) 1317–1326.
- [5] K.B. Filion, L. Azoulay, R.W. Platt, M. Dahl, C.R. Dormuth, K.K. Clemens, et al., A multicenter observational study of incretin-based drugs and heart failure. *N. Engl. J. Med.* 374 (2016) 1145–1154.
- [6] D.K. McGuire, F. Van de Werf, P.W. Armstrong, E. Standl, J. Koglin, J.B. Green, et al., Association between sitagliptin use and heart failure hospitalization and related outcomes in type 2 diabetes mellitus: secondary analysis of a randomized clinical trial. *JAMA Cardiol.* 1 (2016) 126.
- [7] L. Liu, J. Liu, W.T. Wong, X.Y. Tian, C.W. Lau, Y.X. Wang, et al., Dipeptidyl peptidase 4 inhibitor sitagliptin protects endothelial function in hypertension through a glucagon-like peptide 1-dependent mechanism. *Hypertension* 60 (2012) 833–841.

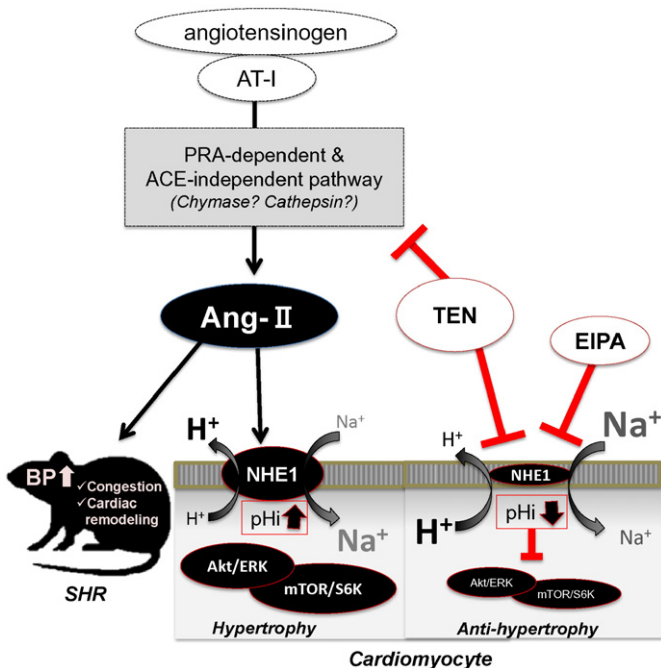


Fig. 7. Summary of the present study and putative model of AngII/NHE-1 axis in hypertensive cardiac remodeling. TEN lowers hypertension of rat model and comorbid cardiac remodeling presumably by attenuating the ACE-independent AngII generation, leading to normalization of mild congestion, BP elevation, and cardiomyocyte remodeling in hypertension. Another impact of AngII is direct action on NHE-1 expression and activity of cardiomyocytes that promotes hypertensive cardiac hypertrophy. TEN attenuated NHE-1 independently of AngII and attenuated the cardiomyocyte hypertrophy.

- [8] J.K. Devin, M. Pretorius, H. Nian, C. Yu, F.T. Billings, N.J. Brown, Substance P increases sympathetic activity during combined angiotensin-converting enzyme and dipeptidyl peptidase-4 inhibition, *Hypertension* 63 (2014) 951–957.
- [9] E.K. Jackson, Z. Mi, S.P. Tofovic, D.G. Gillespie, Effect of dipeptidyl peptidase 4 inhibition on arterial blood pressure is context dependent, *Hypertension* 65 (2015) 238–249.
- [10] B.A. Maron, Hemodynamics should be the primary approach to diagnosing, following, and managing pulmonary arterial hypertension, *Can. J. Cardiol.* 31 (2015) 515–520.
- [11] A. Kollias, S. Lagou, M.E. Zeniodi, N. Boubouchairopoulou, G.S. Stergiou, Association of central versus brachial blood pressure with target-organ damage: systematic review and meta-analysis, *Hypertension* 67 (2016) 183–190.
- [12] M. Nabeno, F. Akahoshi, H. Kishida, I. Miyaguchi, Y. Tanaka, S. Ishii, et al., A comparative study of the binding modes of recently launched dipeptidyl peptidase IV inhibitors in the active site, *Biochem. Biophys. Res. Commun.* 434 (2013) 191–196.
- [13] R. Morishita, H. Nakagami, Tenueligiptin: expectations for its pleiotropic action, *Expert. Opin. Pharmacother.* 16 (2015) 417–426.
- [14] A.C. Reid, C.J. Mackins, N. Seyedi, R. Levi, R.B. Silver, Coupling of angiotensin II AT1 receptors to neuronal NHE activity and carrier-mediated norepinephrine release in myocardial ischemia, *Am. J. Physiol. Heart Circ. Physiol.* 286 (2004) H1448–H1454.
- [15] J. Voelkl, Y. Lin, I. Alesutan, M.S. Ahmed, V. Pasham, S. Mia, et al., Sgk1 sensitivity of Na⁽⁺⁾/H⁽⁺⁾ exchanger activity and cardiac remodeling following pressure overload, *Basic Res. Cardiol.* 107 (2012) 236.
- [16] T. Shigeta, M. Aoyama, Y.K. Bando, A. Monji, T. Mitsui, M. Takatsu, et al., Dipeptidyl peptidase-4 modulates left ventricular dysfunction in chronic heart failure via angiogenesis-dependent and -independent actions, *Circulation* 126 (2012) 1838–1851.
- [17] M.M. Zaruba, H.D. Theiss, M. Vallaster, U. Mehl, S. Brunner, R. David, et al., Synergy between CD26/DPP-IV inhibition and G-CSF improves cardiac function after acute myocardial infarction, *Cell Stem Cell* 4 (2009) 313–323.
- [18] A.M. Lambeir, C. Durinx, S. Scharpe, I. De Meester, Dipeptidyl-peptidase IV from bench to bedside: an update on structural properties, functions, and clinical aspects of the enzyme DPP IV, *Crit. Rev. Clin. Lab. Sci.* 40 (2003) 209–294.
- [19] U. Forstermann, W.C. Sessa, Nitric oxide synthases: regulation and function, *Eur. Heart J.* 33 (2012) 829–837 (37a–37d).
- [20] M. Kim, M.J. Platt, T. Shibasaki, S.E. Quaggin, P.H. Backx, S. Seino, et al., GLP-1 receptor activation and Epac2 link atrial natriuretic peptide secretion to control of blood pressure, *Nat. Med.* 19 (2013) 567–575.
- [21] M.A. Zaman, S. Oparil, D.A. Calhoun, Drugs targeting the renin-angiotensin-aldosterone system, *Nat. Rev. Drug Discov.* 1 (2002) 621–636.
- [22] M. Emdin, C. Fatini, G. Mirizzi, R. Poletti, C. Borrelli, C. Prontera, et al., Biomarkers of activation of renin-angiotensin-aldosterone system in heart failure: how useful, how feasible? *Clin. Chim. Acta* 443 (2015) 85–93.
- [23] M. Volpe, T. Unger, Plasma renin and cardiovascular risk: what is the evidence for an association? *Cardiology* 125 (2013) 50–59.
- [24] J.E. Sealey, M.H. Alderman, C.D. Furberg, J.H. Laragh, Renin-angiotensin system blockers may create more risk than reward for sodium-depleted cardiovascular patients with high plasma renin levels, *Am. J. Hypertens.* 26 (2013) 727–738.
- [25] N.J. Brown, S. Byiers, D. Carr, M. Maldonado, B.A. Warner, Dipeptidyl peptidase-IV inhibitor use associated with increased risk of ACE inhibitor-associated angioedema, *Hypertension* 54 (2009) 516–523.
- [26] R.F. Gillum, L.E. Teichholz, M.V. Herman, R. Gorlin, The idiopathic hyperkinetic heart syndrome: clinical course and long-term prognosis, *Am. Heart J.* 102 (1981) 728–734.
- [27] J.T. Davis, F. Rao, D. Naqshbandi, M.M. Fung, K. Zhang, A.J. Schork, et al., Autonomic and hemodynamic origins of pre-hypertension: central role of heredity, *J. Am. Coll. Cardiol.* 59 (2012) 2206–2216.
- [28] I.H. Zucker, L. Xiao, K.K. Haack, The central renin-angiotensin system and sympathetic nerve activity in chronic heart failure, *Clin. Sci. (Lond.)* 126 (2014) 695–706.
- [29] H. Matsui, W.H. Barry, C. Livsey, K.W. Spitzer, Angiotensin II stimulates sodium-hydrogen exchange in adult rabbit ventricular myocytes, *Cardiovasc. Res.* 29 (1995) 215–221.
- [30] M. Karmazyn, A. Kilic, S. Javadov, The role of NHE-1 in myocardial hypertrophy and remodelling, *J. Mol. Cell. Cardiol.* 44 (2008) 647–653.
- [31] L.M. Ruilope, R.E. Schmieder, Left ventricular hypertrophy and clinical outcomes in hypertensive patients, *Am. J. Hypertens.* 21 (2008) 500–508.
- [32] G.B. Mancini, B. Dahlöf, J. Diez, Surrogate markers for cardiovascular disease: structural markers, *Circulation* 109 (2004) IV22–IV30.
- [33] P. Verdecchia, G. Schillaci, C. Borgioni, A. Ciucci, M. Battistelli, C. Bartocchini, et al., Adverse prognostic significance of concentric remodeling of the left ventricle in hypertensive patients with normal left ventricular mass, *J. Am. Coll. Cardiol.* 25 (1995) 871–878.
- [34] G. de Simone, J.R. Kizer, M. Chinali, M.J. Roman, J.N. Bella, L.G. Best, et al., Normalization for body size and population-attributable risk of left ventricular hypertrophy: the strong heart study, *Am. J. Hypertens.* 18 (2005) 191–196.
- [35] T. Kahan, L. Bergfeldt, Left ventricular hypertrophy in hypertension: its arrhythmogenic potential, *Heart* 91 (2005) 250–256.
- [36] P.M. Okin, R.B. Devereux, S. Jern, S.E. Kjeldsen, S. Julius, M.S. Nieminen, et al., Regression of electrocardiographic left ventricular hypertrophy by losartan versus atenolol: the losartan intervention for endpoint reduction in hypertension (LIFE) study, *Circulation* 108 (2003) 684–690.
- [37] N.K. Hollenberg, N.D. Fisher, D.A. Price, Pathways for angiotensin II generation in intact human tissue: evidence from comparative pharmacological interruption of the renin system, *Hypertension* 32 (1998) 387–392.
- [38] R. Kumar, V.P. Singh, K.M. Baker, The intracellular renin-angiotensin system: implications in cardiovascular remodeling, *Curr. Opin. Nephrol. Hypertens.* 17 (2008) 168–173.
- [39] R.E. Schmieder, M.R. Langenfeld, A. Friedrich, H.P. Schobel, C.D. Gatzka, H. Wehprecht, Angiotensin II related to sodium excretion modulates left ventricular structure in human essential hypertension, *Circulation* 94 (1996) 1304–1309.
- [40] J.J. Sim, S.K. Bhandari, J. Shi, K. Kalantar-Zadeh, S.A. Rasgon, J.E. Sealey, et al., Plasma renin activity (PRA) levels and antihypertensive drug use in a large healthcare system, *Am. J. Hypertens.* 25 (2012) 379–388.
- [41] A. Mamey, S. Kunchakarra, L. Byrne, N.J. Brown, Interactive hemodynamic effects of dipeptidyl peptidase-IV inhibition and angiotensin-converting enzyme inhibition in humans, *Hypertension* 56 (2010) 728–733.
- [42] J. Shanks, S. Manou-Stathopoulou, C.J. Lu, D. Li, D.J. Paterson, N. Herring, Cardiac sympathetic dysfunction in the prehypertensive spontaneously hypertensive rat, *Am. J. Physiol. Heart Circ. Physiol.* 305 (2013) H980–H986.
- [43] B.D. Hoit, Y. Shao, A. Kinoshita, M. Gabel, A. Husain, R.A. Walsh, Effects of angiotensin II generated by an angiotensin converting enzyme-independent pathway on left ventricular performance in the conscious baboon, *J. Clin. Invest.* 95 (1995) 1519–1527.
- [44] R. Kumar, M.A. Boim, Diversity of pathways for intracellular angiotensin II synthesis, *Curr. Opin. Nephrol. Hypertens.* 18 (2009) 33–39.
- [45] E. Yahiro, S. Miura, S. Imaizumi, Y. Uehara, K. Saku, Chymase inhibitors, *Curr. Pharm. Des.* 19 (2013) 3065–3071.
- [46] X.W. Cheng, Z. Huang, M. Kuzuya, K. Okumura, T. Murohara, Cysteine protease cathepsins in atherosclerosis-based vascular disease and its complications, *Hypertension* 58 (2011) 978–986.
- [47] Y. Uehara, S. Miura, E. Yahiro, K. Saku, Non-ACE pathway-induced angiotensin II production, *Curr. Pharm. Des.* 19 (2013) 3054–3059.
- [48] A. Sufiun, K. Rafiq, Y. Fujisawa, A. Rahman, H. Mori, D. Nakano, et al., Effect of dipeptidyl peptidase-4 inhibition on circadian blood pressure during the development of salt-dependent hypertension in rats, *Hypertens. Res.* 38 (2015) 237–243.
- [49] J.M. Costa-Pessoa, C.F. Figueiredo, K. Thieme, M. Oliveira-Souza, The regulation of NHE(1) and NHE(3) activity by angiotensin II is mediated by the activation of the angiotensin II type I receptor/phospholipase C/calcium/calmodulin pathway in distal nephron cells, *Eur. J. Pharmacol.* 721 (2013) 322–331.
- [50] S.J. Reshkin, A. Bellizzi, S. Caldeira, V. Albarani, I. Malanchi, M. Poignee, et al., Na⁺/H⁺ exchanger-dependent intracellular alkalization is an early event in malignant transformation and plays an essential role in the development of subsequent transformation-associated phenotypes, *FASEB J.* 14 (2000) 2185–2197.
- [51] I. Sipahi, S.M. Debanne, D.Y. Rowland, D.I. Simon, J.C. Fang, Angiotensin-receptor blockade and risk of cancer: meta-analysis of randomised controlled trials, *Lancet Oncol.* 11 (2010) 627–636.
- [52] R.G. Mihaila, A minireview on NHE1 inhibitors. A rediscovered hope in oncohematology, *Biomed. Pap. Med. Fac. Univ. Palacky Olomouc Czech Repub.* 159 (2015) 519–526.
- [53] S. Yagi, K. Aihara, Y. Ikeda, Y. Sumitomo, S. Yoshida, T. Ise, et al., Pitavastatin, an HMG-CoA reductase inhibitor, exerts eNOS-independent protective actions against angiotensin II induced cardiovascular remodeling and renal insufficiency, *Circ. Res.* 102 (2008) 68–76.
- [54] S. Kudo, K. Satoh, M. Nogi, K. Suzuki, S. Sunamura, J. Omura, et al., SmgGDS as a crucial mediator of the inhibitory effects of statins on cardiac hypertrophy and fibrosis: novel mechanism of the pleiotropic effects of statins, *Hypertension* 67 (2016) 878–889.