Simultaneous Treatment with Azelnidipine and Olmesartan Inhibits Apoptosis of Hl-1 Cardiac Myocytes Expressing E334k cMyBPC

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Abstract

Background: Apoptosis appears to play an important role in the pathogenesis of hypertrophic cardiomyopathy (HCM). We have previously reported 3 HCM patients carrying the E334K *MYBPC3*, and that heterologous expression of E334K cMyBPC in cultured cells induced apoptosis. The purpose of this study was to identify pharmacological agents that would inhibit apoptosis in HL-1 cardiomyocytes expressing E334K cMyBPC.

Methods and Results: E334K cMyBPC expression in cells increased levels of pro-apoptosis (p53, Bax and cytochrome c) and decreased levels of anti-apoptosis (Bcl-2 and Bcl-XL). While the beta blocker carvedilol (1 µM) normalized the level of p53 and Bcl-2 and the calcium channel blocker (CCB) bepridil (0.5 µM) normalized that of Bcl-2, both the CCB azelnidipine (1 µM) and the angiotensin receptor blocker (ARB) olmesartan (10 µM) normalized those of p53, Bax, cytochrome *c*, and Bcl-XL. Among those proteins, cytochrome *c* was the one which showed the highest degree of change. Both azelnidipine (0.1 µM) and olmesartan (1 µM) reduced the level of cytochrome *c* by 40.2 ± 4.3 % and 31.3 ± 5.1 %, respectively. The CCB amlodipine and the ARB valsartan reduced it only by 19.1 ± 2.1 % and 20.1 ± 5.2 %, respectively. Flow cytometric analysis and annexin V staining showed that treatment of cells with azelnidipine (0.1 µM) plus olmesartan (0.3 µM) or that with amlodipine (0.1 µM) plus valsartan (0.3 µM) reduced the number of apoptotic cells by 35.8 ± 10.5 % and 18.4 ± 3.2 %, respectively.

Conclusion: Azelnidipine plus olmesartan or amlodipine plus valsartan inhibited apoptosis of HL-1 cells expressing E334K cMyBPC, and the former combination was more effective than the latter.

Abbreviations

HCM	hypertrophic cardiomyopathy
ARB	angiotensin receptor blocker
CCB	calcium channel blocker
ER	endoplasmic reticulum

Introduction

Hypertrophic cardiomyopathy (HCM) is caused by mutations in the genes encoding proteins of the cardiac sarcomere and non-cardiac sarcomeric proteins [1]. The mortality of HCM is determined by heart failure as well as arrhythmia. Several lines of evidence obtained in animal and clinical studies have suggested the involvement of cellular apoptosis in the pathogenesis of cardiomyopathy [2]. Two major mechanisms for cellular apoptosis have been proposed; apoptotic cell death via mitochondrial Ca^{2+} overload, and the induction of transcriptional factors such as CHOP/GAPD153, caspase 12, and JNK-dependent signaling trigerred by endoplasmic reticulum (ER) stress. Thus, the protecting the heart from apoptosis is essensial for patients with hypertrophic cardiomyopathy.

Several pharmacological agents including beta blockers, calcium channel blockers (CCBs), and angiotensin receptor blockers (ARBs) are recommended for patients with HCM [3]. These agents have been reported to attenuate cellular apoptosis. While chronic beta-adrenergic receptor hyperactivation is known to cause ER Ca^{2+}-depletion inducing thereby a perpetual ER stress response in the failing heart, beta blockers have been shown to suppress ER stress and protect myocytes from apoptosis in a rat model of cardiac

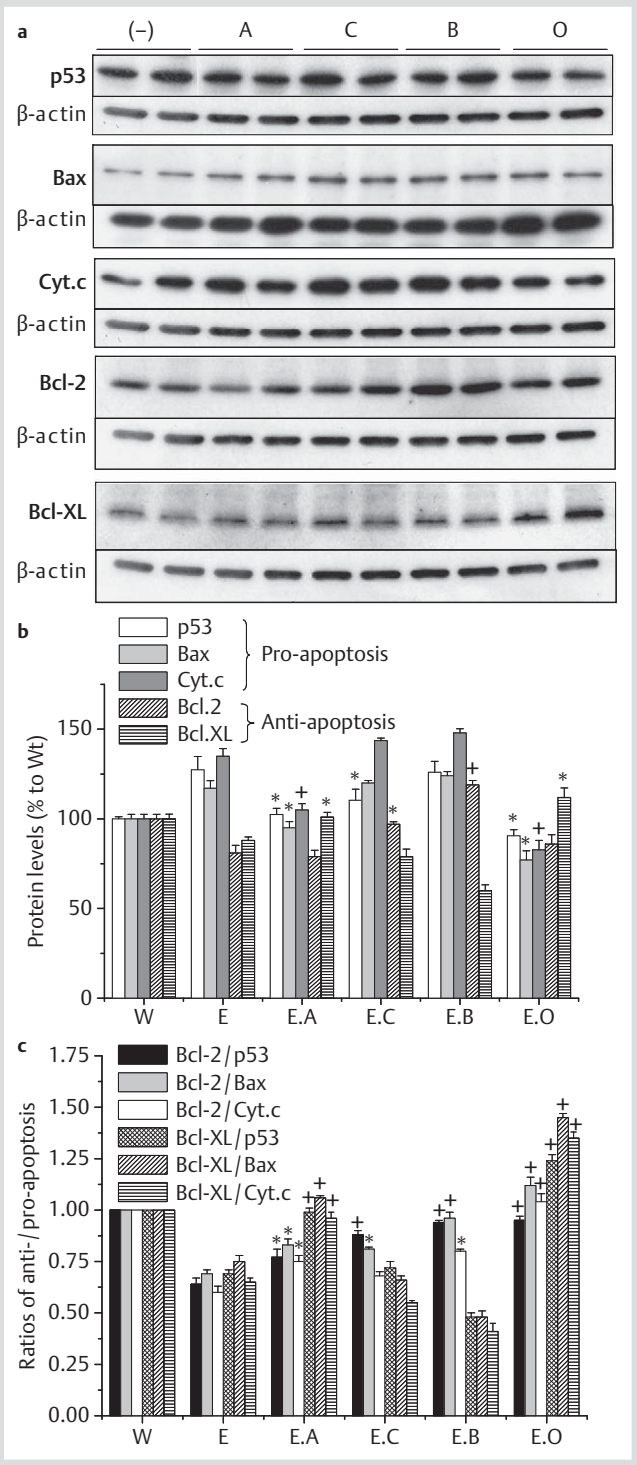

Fig. 1 Effect of the single dose of azelnidipine, carvedilol, bepridil, or olmesartan on the level of proteins regulating apoptosis in HL-1 cells expressing either Wt or E334K cMyBPC. **a** Representative Western blot of pro-apoptotic proteins p53, Bax, and cytochrome *c* as well as anti-apoptotic proteins Bcl-2 and Bcl-XL in HL-1 cells transfected with either E334K *MYBPC3* plasmid or the Wt in the presence or absence of drugs as indicated. β-actin used as a control for protein loading. **b** Summary of quantitative densitometric scan of the single dose of drugs on the level of indicated proteins taken from **a**; * and + were compared to the corresponding proteins in E. **c** The ratios of anti-apoptotic/pro-apoptotic proteins. W and E indicated HL-1 cells expressing Wt and E334K cMyBPC, respectively. A, C, B, and O indicated treatment with azelnidipine (1 μM), carvedilol (1 μM), bepridil (0.5 μM), and olmesartan (10 μM), respectively. (–) indicated no treatment. $n=4$, $^{*}p<0.05$, $^{+}p<0.001$. Cyt. *c*, cytochrome *c*.

hypertrophy induced by aortic constriction or isoproterenol injection [4]. On the other hand, while angiotensin II can further increase ER stress-induced apotosis, the ARB olmesartan attenuates ER stress-induced renal apoptosis in mice rendered diabetic by streptozotocin injection [5]. Furthermore, mitochondrial Ca^{2+} overload seems to be a consequence of the rise in the cytosolic Ca^{2+} concentration promoted by Ca^{2+} entry through plasma membrane receptor-operated and voltage-dependent Ca^{2+} channels, because the CCB azelnidipine prevented cardiac dysfunction in experimentally-induced diabetic rats by reducing intracellular calcium accumulation, oxidative stress, and apoptosis [6].

We previously reported HCM patients carrying the E334K *MYBPC3*, who exhibited cardiac dysfunction and arrhythmia [7,8]. Heterologous expression of E334K cMyBPC inhibited cellular proteasome activity, induced accumulation of pro-apoptotic proteins, increased the levels of cardiac ion channels and Ca^{2+} handling proteins, and caused apoptosis. In this study, we tested whether pharmacological agents -beta blocker, CCB, and ARB- inhibited apoptosis of cardiomyocytes expressing E334K cMyBPC. We found that azelnidipine and olmesartan normalized the levels of proteins that regulate apoptosis, and that azelnidipine plus olmesartan suppressed apoptosis of HL-1 cells expressing E334K cMyBPC. This combination was more efficient than the CCB amlodipine plus the ARB valsartan.

Material and Methods

Cells culture and heterologous expression

HL-1 cardiac myocytes were provided by Dr. Claycomb (Louisiana State University) and cultured according to instructions [9]. cDNA encoding either Wt or E334K cMyBPC with a 6-myc tag at the N-terminus [7] was ligated to pCS^{2+} at *BamHI* and *XhoI* sites to generate plasmid expression vectors (pCS-6myc-*MYBPC3*). Transfection into HL-1 cells was performed using lipofectamin 2000 (Invitrogen) following the manufacturer's instructions.

Used drugs and justification of their concentration

Azelnidipine, carvedilol, bepridil, olmesartan, amlodipine, and valsaratan were used in this study. Azelnidipine, carvedilol, and olmesartan were kindly donated by Daiichi Sankyo, Japan, and the others were purchased from Sigma-Aldrich Japan.

Justification of the concentration of each agent used in this study was as follow. The single dose experiment (**Fig. 1**) was justified from the comparable dose to the therapeutic concentration as well as by looking the available previous studies (azelnidipine [8,10–12], carvedilol [13,14], bepridil [15], olmesartan [16], amlodipine [17], and valsartan [18]). The dose-dependent effect (**Fig. 2**) was done in lower concentration than the single dose experiment (**Fig. 1**) to know the lower concentration which remains effective. The doses of combination of agents (**Fig. 3**) were determined from the dose-dependent experiment (**Fig. 2**) as well as from the previous experiment. The combination of lower doses of azelnidipine and olmesartan was then selected [10,11].

Western blotting

Wt or E334K *MYBPC3* was transfected into HL-1 cells in the absence or presence of azelnidipine, amlodipine, olmesartan, valsaratan, carvedilol and/or bepridil. Protein extracts of cells were prepared 48 h post-transfection, as described elsewhere [7]. Proteins (15 μg)

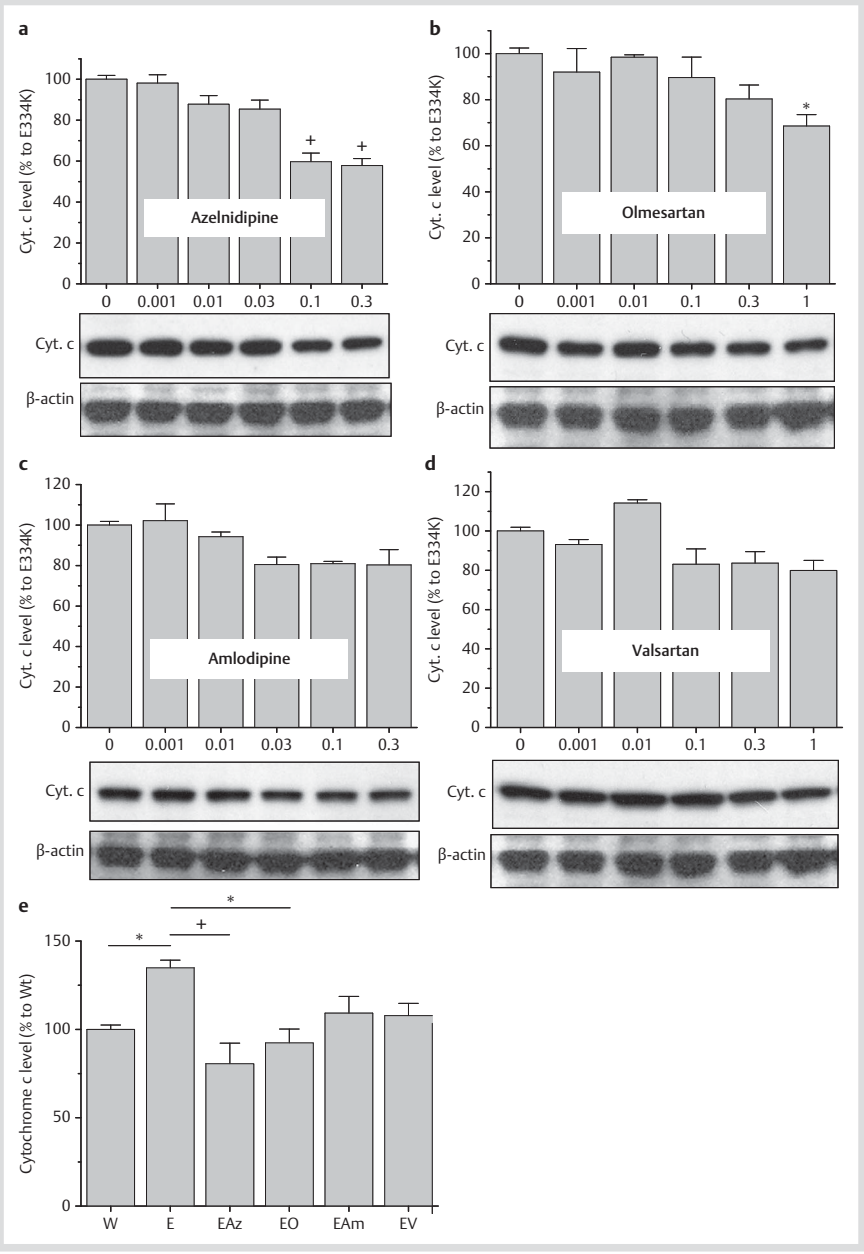

Fig. 2 The dose-dependent effect of azelnidipine, olmesartan, amlodipine, or valsartan on the level of pro-apoptotic protein cytochome *c* in HL-1 cells expressing E334K cMyBPC. Western blotting of cytochrome *c* in HL-1 cells transfected with E334K *MYBPC3* plasmid in the presence of 0, 0.001, 0.01, 0.03, 0.1, and 0.3 µM of azelnidipine **a** or amlodipine **c**, or 0, 0.001, 0.01, 0.1, 0.3 and 1 µM olmesartan **b** or valsartan **d**. For each panel **a**–**d**; **bottom**, representative Western blot, with β-actin used as a control for protein loading; **top**, quantitative densitometric scan of indicated proteins. **e** Summary of cytochrome *c* levels in cells expressing either Wt (W) or E334K (E) cMyBPC in the absence of presence of drugs, taken from **a** to **d**; the comparison level of W and E was taken from **Fig. 1a, b**. EAz, EO, EAm, EV indicated the cells expressing E334K cMyBPC in the presence of azelnidipine (0.1 µM), olmesartan (1 µM), amlodipine (0.1 µM), and valsartan (1 µM), respectively. $n = 4$, $^{*}p < 0.05$, $^{+}p < 0.001$. Cyt. *c*, cytochrome *c*.

were separated by SDS-PAGE and electrotransferred to PVDF membrane. Membranes were probed with antibodies to actin (Calbiochem, La Jolla, CA), p53 (Santa Cruz Biotechnology, Santa Cruz, CA), Bax (Santa Cruz), cytochrome *c* (BD Biosciences, Franklin Lakes, NJ), Bcl-2 (Santa Cruz), Bcl-XL (Santa Cruz) and were developed using an ECL system (Amersham Bioscience, Piscataway, NJ). The intensities of the bands were quantified using NIH image Software.

Annexin V staining and flow cytometry

Annexin V staining and flow cytometry of Wt or E334K *MYBPC3*-transfected cells and data analysis were performed as reported elsewhere [7].

Statistical analysis

OriginR for Windows software version 7.0 (OriginLab Corporation, Northampton, MA, USA) was used for statistical analysis. Differences between 2 groups were assessed using the 2-sample *t*-test. One-way ANOVA with the Bonferroni test for post-hoc analysis was used for multiple comparisons. All experimental data are expressed as the mean ± SEM. Differences with *p*-values < 0.05 were considered significant.

Results

Fig. 1 shows the effects of a single dose of azelnidipine, carvedilol, bepridil, and olmesartan on the level of proteins that regulate apoptosis in HL-1 cells expressing either Wt or E334K cMyBPC. Compared with the Wt, E334K cMyBPC increased levels of the pro-apoptotic proteins p53, Bax, and cytochrome *c* and decreased levels of the anti-apoptotic proteins Bcl-2 and Bcl-XL. Carvedilol (1 µM) normalized the level of both p53 and Bcl-2 while bepridil (0.5 µM) normalized only the level of Bcl-2. Interestingly, both azelnidipine (1 µM) and olmesartan (10 µM) normalized the levels of p53, Bax, cytochrome *c* and Bcl-XL in cells expressing E334K cMyBPC (**Fig. 1b**).

It has been known that both the ratio of Bcl-2/pro-apoptotic proteins and that of Bcl-XL/pro-apoptotic proteins are indexes of

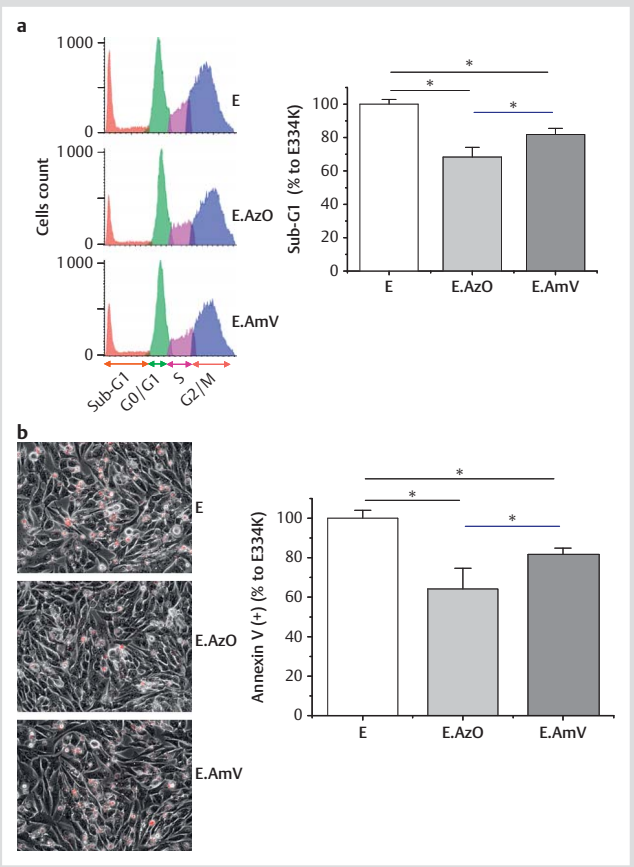

Fig. 3 Effect of combination of azelnidipine plus olmesartan vs. amlodipine plus valsartan on apoptosis of HL-1 cells expressing E334K cMyBPC. Flow cytometric analysis to count the sub-G1 cell population corresponding to apoptosis **a** and annexin V staining detected apoptosis **b** in HL-1 cells transfected with E334K *MYBPC3* plasmid, in the presence or absence of combination drugs as indicated. AzO and AmV indicated combination of azelnidipine (0.1 µM) plus olmesartan (0.3 µM) and amlodipine (0.1 µM) plus valsartan (0.3 µM), respectively. The bar graph in **a** shows relative numbers of cells in the sub-G1 population to the cells without treatment, $n = 4$ for each group; and in **b** shows relative numbers of annexin V(+) cells to the cells without treatment, $n = 3$ for each group. $^*p < 0.05$.

apoptosis through the mitochondrial pathway [18]. Compared with the Wt, E334K cMyBPC expression resulted in decreased ratios of anti-apoptotic/pro-apoptotic proteins (**Fig. 1c**). The ratio of Bcl-2/pro-apoptotic proteins was normalized by olmesartan and partially restored by azelnidipine, carvedilol, and bepridil; however, the ratio of Bcl-XL/pro-apoptotic proteins was only normalized by both azelnidipine and olmesartan, which was reflected by normalization of the level of cytochrome *c*, the ratio of Bcl-2/cytochrome *c* as well as the ratio of Bcl-XL/cytochrome *c*. To compare the normalization of the level of cytochrome *c* with CCBs and ARBs, we investigated the effects of azelnidipine and olmesartan at different dose levels. In this experiment, we used concentrations lower than those used in the single dose experiment. Both azelnidipine at 0.1 and 0.3 µM and olmesartan at 1 µM reduced the level of cytochrome *c* in HL-1 cells expressing E334K cMyBPC (**Fig. 2**). The level of cytochrome *c* was significantly reduced by azelnidipine (40.2 ± 4.3%; $p < 0.001$) and to a lower level compared with the rate of reduction obtained with olmesartan (31.3 ± 5.1%; $p < 0.05$) (**Fig. 2a, b**). We examined the effects of amlodipine and valsartan on the level of cytochrome *c*. Both amlodipine at 0.1 and 0.3 µM and valsartan at 1 µM reduced the level of cytochrome *c* by 19.1 ± 2.1% and 20.1 ± 5.2%, respectively (**Fig. 2c, d**), but none of them reached a statistically significant level.

It has been recently reported that a CCB combined with an ARB has protective effects on the cardiovascular system [19]. Thus, we examined the effect of a combination of those drugs on apoptosis. **Fig. 3a** shows the effects of azelnidipine (0.1 µM) plus olmesartan (0.3 µM) on the number of cells in the sub-G1 population, in comparison with the effects of amlodipine (0.1 µM) plus valsartan (0.3 µM). Both combinations significantly reduced the number of sub-G1 cells. The reduction was more marked when cells were treated with azelnidipine plus olmesartan (31.6 ± 5.7%, $p < 0.05$) than treated with amlodipine plus valsartan (18.9 ± 3.6%, $p < 0.05$). Both combinations reduced the number of annexin V(+) cells among cells expressing E334K cMyBPC (**Fig. 3b**). Again, this effect was more prominent when cells were treated with azelnidipine plus olmesartan (35.8 ± 10.5%, $p < 0.05$) than when treated with amlodipine plus valsartan (18.4 ± 3.2%, $p < 0.05$).

Discussion

In this study, we demonstrated that azelnidipine and olmesartan normalized the level of proteins that regulate apoptosis such as p53, Bax, cytochrome *c*, and Bcl-XL in HL-1 cells expressing E334K cMyBPC (**Fig. 1**). On the other hand, carvedilol normalized the level of p53 and Bcl-2, while bepridil normalized the level of Bcl-2 alone. The normalization of cytochrome *c* level after treatment with azelnidipine and olmesartan suggested that apoptosis was partly mediated by the mitochondrial pathway. This is supported by a report demonstrating that mitochondrial dysfunction contributed to cardiac cellular apoptosis in a mouse model of hypertrophic and failing heart [20]. Moreover, we found that besides the normalization of the level of cytochrome *c* in cells expressing E334K cMyBPC (**Fig. 2**) azelnidipine plus olmesartan reduced the number of apoptotic cells (**Fig. 3**). Although the reduction of the level of cytochrome c by either amlodipine or valsartan did not reach a statistically significant level (**Fig. 2**), a combination of these drugs significantly reduced the number of apoptotic cells (**Fig. 3**).

Pharmacological suppression of apoptosis has been reported in several studies. Azelnidipine suppressed apoptosis of cardiac cells in streptozotocin-diabetic rats and of hypoxic renal tubular cells through suppression of calcium accumulation in the mitochondria [6,21]. In the present study, azelnidipine also normalized the ratio of Bcl-2 or Bcl-XL/pro-apoptotic proteins (**Fig. 1**). The attenuation of cardiac apoptosis by carvedilol has been demonstrated in several studies [22–25]. Zeng et al. (2003) reported that inhibition of cardiomyocyte apoptosis by carvedilol was related to an increase of the Bcl-2/Bax ratio [23]. Although we found that carvedilol or bepridil did not increase the level of cytochrome *c* in HL-1 cells expressing E334K cMyBPC (**Fig. 1b**) and failed to normalize the ratio of Bcl-XL/pro-apoptotic proteins, these drugs normalized the Bcl-2/Bax ratios and Bcl-2/p53 ratios (**Fig. 1c**). Olmesartan attenuated cardiomyocyte apoptosis of obese and diabetic mice [26], in rats with experimentally induced autoimmune myocarditis [27], in the kidney of streptozotocin diabetic mice [5], and in subtotally nephrectomized rats [28]. ER stress by reactive oxygen species and angiotensin II also has been known to trigger apoptosis by induction of transcrip-

tional factors such as CHOP/GAPD153, caspase 12, and the JNK-dependent pathway. Attenuation of apoptosis by olmesartan is also related to suppression of ER stress [5,27,28]. In the present study, olmesartan normalized the level of proteins that regulate apoptosis (**Fig. 1**), indicating the involvement of the mitochondrial pathway.

The normalization of Bcl-2 or Bcl-XL/pro-apoptotic proteins and the level of cytochrome *c*, suggested protective effects of CCBs and ARBs from apoptosis mediated mitochondrial pathway. Amlodipine inhibited cardiomyocyte apoptosis in rats with cardiac hypertrophy due to hypertension [29], in mice with cardiac infarction induced by ligation of the left coronary artery [30], and in neonatal rat cardiac myocytes treated with doxorubicin [31]. Yamanaka et al. (2003) showed that inhibition of apoptosis by amlodipine occured through suppression of the mitochondrial apoptotic pathway [31]. Valsartan inhibited apoptosis in a model of cardiac hypertrophy induced by catecholamines [32], in rat hearts with chronic pressure overload induced by spontaneous hypertension [33], in the canine model of reperfused myocardial infarction [34], and of cardiomyocytes in mice with diabetic cardiomyopathy [35]. Wu et al. (2011) reported that valsartan suppressed ER stress-induced myocardial apoptosis via activation of the CHOP/Puma signaling pathway [35]. In the present study, normalization of the level of cytochrome *c* was significant in cells treated with azelnidipine plus olmesartan, but not in cells treated with amlodipine plus valsartan, indicating that the action of azelnidipine and olmesartan in HL-1 cells expressing E334K cMyBPC was independent from their class effect as CCB and ARB.

Several studies have reported the effects of CCBs combined with ARBs on non-cardiac tissues or cells. Tanifuji et al. (2009) showed that combination therapy using olmesartan plus azelnidipine suppressed extracellular signal-regulated kinase activation, interstitial fibrosis, and oxidative stress in a mouse model of polycystic kidney disease [36]. This drug combination also ameliorated the impairment of endothelium-derived, hyperpolarizing factor-mediated responses in apolipoprotein E-deficient mice with streptozocin-induced diabetes [37]. This is the first study demonstrating the effect of olmesartan plus azelnidipine as well as amlodipine plus valsartan on apoptosis in a cellular model of cardiomyopathy induced by mutant cMyBPC. The addition of the CCB azelnidipine to the ARB olmesartan has been reported to be more effective in reducing oxidative stress in hypertensive diabetic patients with chronic kidney disease than the addition of amlodipine [38]. In this study, we found that azelnidipine plus olmesartan supressed apoptosis more effeciently than amlodipine plus valsartan (**Fig. 3**).

In conclusion, we demonstrated that azelnidipine plus olmesartan as well as amlodipine plus valsartan inhibited apoptosis in HL-1 cells expressing E334K cMyBPC, and that the former combination appeared to have better protective effects than the latter. These findings provide a new insight into the pharmacological treatment of patients with hypertrophic cardiomyopathy.

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Conflict of Interest

The authors declare no conflict of interest.

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