Efonidipine, a Ca\(^{2+}\)-Channel Blocker, Enhances the Production of Dehydroepiandrosterone Sulfate in NCI-H295R Human Adrenocortical Carcinoma Cells

Keiichi Ikeda, Takatoshi Saito and Katsuyoshi Tojo

\(^{1}\)Department of Molecular and Cellular Biology, Institute of DNA Medicine, Research Center for Medical Sciences, The Jikei University School of Medicine, Tokyo, Japan

\(^{2}\)Division of Diabetes and Endocrinology, Department of Internal Medicine, The Jikei University School of Medicine, Tokyo, Japan

Steroid biosynthesis is initiated with transportation of cholesterol along with steroidogenic acute regulatory protein (StAR) into the mitochondria and is achieved with several steroidogenic enzymes. It has been reported that Ca\(^{2+}\) channel blockers (CCBs), such as azelnidipine, efonidipine and nifedipine, suppress the biosynthesis of aldosterone and cortisol, but the overall effects of CCBs on steroid biosynthesis remain to be clarified. The present study was designed to evaluate the effects of CCBs on the expression of steroidogenic enzymes and the production of adrenal androgen, dehydroepiandrosterone sulfate (DHEA-S) that has anti-atherosclerotic actions. NCI-H295R human adrenocortical carcinoma cells and HepG2 human hepatoma cells were cultured for 24 hours with or without a CCB (amlodipine, efonidipine, nifedipine, azelnidipine \(R(-)\)-efonidipine, verapamil or diltiazem). HepG2 hepatoma cells were used to confirm the effects of CCBs on the expression of StAR. In fact, efonidipine and nifedipine increased the expression of StAR in HepG2 cells. Efonidipine and nifedipine, but not other examined CCBs, also increased the \(N^6,2\'-O\)-dibutyryladenosine \(3',5'\)-cyclic monophosphate (dbcAMP)-induced StAR mRNA, which reflects the action of adrenocorticotropic hormone, and efonidipine and \(R(-)\)-efonidipine enhanced the dbcAMP-induced DHEA-S production in NCI-H295R adrenocortical carcinoma cells. Therefore, efonidipine and nifedipine might increase the expression of StAR and, in turn, efonidipine enhanced the dbcAMP-induced DHEA-S production, independent of Ca\(^{2+}\) channel blockade. These results indicate that such effects are not associated with Ca\(^{2+}\) influx. Moreover, only efonidipine enhanced the angiotensin II-induced expression of StAR mRNA (\(P < 0.01\) vs. angiotensin II alone). In conclusion, efonidipine might exert an additional action beyond anti-hypertensive actions.

Keywords: Ca\(^{2+}\) antagonists; dehydroepiandrosterone-sulfate; HepG2 human hepatoma cell; NCI-H295R human adrenocortical carcinoma cell; steroid biosynthesis


The adrenal cortex synthesizes various bioactive steroids, such as mineralocorticoids (e.g., aldosterone), gluocorticoids (e.g., cortisol and corticosterone), and adrenal androgens (e.g., dehydroepiandrosterone [DHEA], testosterone, and estrone). Some adrenal steroids, such as aldosterone, cortisol, and corticosterone, are involved in cardiovascular diseases such as hypertension. Recent studies showed that some Ca\(^{2+}\) channel blockers (CCBs) have inhibitory actions on the expression of 11\(\beta\)-hydroxylase and steroid 18-hydroxylase, leading to the suppression of secretion of cortisol and aldosterone in NCI-H295R human adrenocortical carcinoma cells (Imagawa et al. 2006; Isaka et al. 2009), in addition to their primary role involving their anti-hypertensive action. However, adrenal steroid biosynthesis is initiated with transport of cholesterol by steroidogenic acute regulatory protein (StAR), which is a rate-limiting enzyme of steroid biosynthesis (Christenson and Strauss 2000), and detailed evidence for the effect of CCBs on the expression of steroidogenic enzymes has not been elucidated. In addition, another group recently reported that nifedipine, which is a widely used dihydropyridine CCB, enhance the expression of StAR in MA-10 mouse Leydig cells (Pandey et al. 2010). The present study was, therefore, designed to evaluate the effects of L-type and/or T-type CCBs, such as amlodipine, efonidipine, nifedipine, azelnidipine, and \(R(-)\)-efonidipine, on the expression of StAR using NCI-H295R human adrenocortical carcinoma cells, in which the L-, T-, and N-type Ca\(^{2+}\) channels are all...
expressed (Isaka et al. 2009; Aritomi et al. 2011). We also used HepG2 human hepatoma cells to confirm the effects of CCBs on the expression of StAR, because the HepG2 human hepatoma cell imports cholesterol into its mitochondria by the StAR protein, like NCI-H295R human adrenocortical carcinoma cells, and in turn, synthesizes bile acid (Hall et al. 2005). In addition, we have attempted to evaluate the involvement of the effects of CCBs on the expression of other steroidogenic enzymes and on the production of DHEA sulfate (DHEA-S), which has been reported to have several actions against atherosclerosis and vascular remodeling (Alexandersen et al. 1996; Li et al. 2009).

Methods

Cells and Chemicals

NCI-H295R human adrenocortical adenoma cells were purchased from American Type Culture Collection and HepG2 human hepatoma cells from Dainippon Sumitomo Pharma Co., Ltd. (Osaka, Japan). Dulbecco’s Modified Eagle’s Medium (DMEM, Ca2+-free [Catalog No. 21068-028] and low glucose), DMEM/Ham’s F12 (DMEM/F12), fetal bovine serum (FBS), GlutaMAX Supplement I, and Invitrool PVDF were purchased from Life Technologies Co. (Carlsbad, CA, USA). Bovine serum albumin (BSA), N-defue, 2’-O-Dibutyryladenosine 3’,5’-cyclic monophosphate sodium salt (dbcAMP), amiodipine (2-(2-Aminoethoxy)-methy1)-4-(2-chlorophenyl)-1,4-dihydro-6-methyl-3,5-pyridinedicarboxylic acid 3-ethyl-5-methyl ester benzene sulfonate) besylate, nifedipine (1,4-Dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester), diltiazem ((2S,3S)-(＋)-cis-3-Acetoxy-5-(2-dimethylaminoethyl)-2,3-dihydro-2-(4-methoxyphenyl)-1,5-benzothiazepin-4(5H)-one) hydrochloride, and verapamil (5-[3,4-Dimethoxyphenylamino]-2-(3,4-dimethoxyphenyl)-2-isopropylvaleronitrile) hydrochloride were from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Efonidipine (5-(5,5-Dimethyl-1,3,2dioxaphosphorinan-2-yl)-1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3-pyridinedicarboxylic acid 2-[phenyl[phenethyl]amino] ethyl ester) hydrochloride was generously provided by Shionogi & Co., Ltd. (Osaka, Japan). Azelnidipine (2-Amino-1,4-dihydro-6-methyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylic acid 3-[1(diphenylmethyl)-3-azetidinyl] 5-(1-methylhe1yl) ester) was generously provided by Daichi-Sankyo Co., Ltd. (Tokyo, Japan). R(＋)-efonidipine, which is an R-(＋)-enantiomer of efonidipine and a selective blocker for T-type, but not L-type, Ca2+ channels (Tanaka et al. 2004), was generously provided by Nissan Chemical Industries, Ltd. (Tokyo, Japan). Angiotensin II was purchased from Peptide Institute, Inc., (Minoh, Japan), Prime Script RT Reagent Kit and SYBER PremixEX Taq were from Takara Bio, Inc. (Osu, Japan), ISOGEN was from Nippon gene Co. (Tokyo, Japan), CaCl2, and ethylene glycol bis (β-aminoethyl ether)-N,N',N'-tetraacetic acid (EGTA) were from Nacalai Tesque, Inc. (Kyoto, Japan), and M-PER Mammalian Protein Extraction Reagent and the BCA protein assay kit were from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Goat anti-StAR antibody, donkey anti-goat IgG-HRP, and goat anti-rabbit IgG were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), rabbit anti-CYP11A1 and anti-CYP21A2 polyclonal antibodies from Abgent Inc. (San Diego, CA, USA), rabbit anti-HSD3B2 was from Abcam plc (Cambridge, UK), mouse monoclonal antibody for human β-actin, Anti-Human ACTB was from Cosmo Bio Co., Ltd. (Tokyo, Japan), and Amersham ECL Anti-Mouse IgG Horseradish Peroxidase-Linked Species-Specific Whole Antibody and the ECL Plus Western Blotting Detection System were from GE Healthcare UK Ltd. (Buckinghamshire, UK). NCI-H295R human adrenocortical carcinoma cells were purchased from the American Type Culture Collection (ATCC, Manasas, VA, USA) and HepG2 human hepatoma cells from Dainippon Sumitomo Pharma Co., Ltd. (Osaka, Japan). The DHEA-S-specific EIA kit was purchased from Immunospec Co. (Chatsworth, CA, USA) and the cortisol-specific enzyme EIA kit from Oxford Biomedical Research Inc. (Oxford, MI, USA).

Cell culture of NCI-H295R human adrenocortical carcinoma cells and HepG2 human hepatoma cells

NCI-H295R human adrenocortical carcinoma cells were cultured as previously described (Isaka et al. 2009). Briefly, cells were initially cultured in 10 cm culture dishes with DMEM/F12 containing 5% FBS (v/v), 6.25 mg/l transferrin, 6.25 mg/l insulin, 1.25 g/l BSA and antibiotics (100 U/ml penicillin G and 10 μg/ml streptomycin) (maintenance medium) and passed into 6-well plates at a density of 2.0 × 106 cells/well, incubated with maintenance medium for 48 hours prior to each experiment. HepG2 human hepatoma cells were also cultured in 6-well plates after seeding at a density of 4.0 × 105 cells/well for 48 hours with DMEM containing 10% FBS (v/v).

Isolation of mRNA from NCI-H295R human adrenocortical cells and HepG2 human hepatoma cells and preparation of cDNA

Isolation was carried out after culture of NCI-H295R human adrenocortical carcinoma cells in stimulation medium (DMEM/F12 containing 0.1% BSA and a stimulation agent, such as dbcAMP (10−3 mol/l), which stimulates adrenocorticotropin (ACTH)-dependent steroidogenic gene expression (Ozbay et al. 2004), or angiotensin II (10−7 mol/l) with or without CCBs (amiodipine besylate, efonidipine hydrochloride, nifedipine, azelnidipine, (+)-efonidipine, diltiazem hydrochloride, and verapamil hydrochloride, 10−6 mol/l) for 6 h and 24 h. The vehicle medium contained a maximum concentration of 0.1% dimethyl-sulfoxide (DMSO), in which all examined CCBs are dissolved. The concentration of each CBB was unified as 10−6 mol/l, because azelnidipine, efonidipine, or nifedipine at this concentration exerted maximal effects on steroid biosynthesis in the previous study (Isaka et al. 2009). NCI-H295R human adrenocortical carcinoma cells were also cultivated in Ca2+-free DMEM, which was supplemented with 0.1% BSA (w/v) and 1% GlutaMAX Supplement I (v/v), containing 10−3 mol/l CaCl2, 0.5 × 10−3 mol/l EGTA, or 10−3 mol/l EGTA with or without 10−3 mol/l dbcAMP for 24 hours. HepG2 human hepatoma cells were also cultured in FBS-free DMEM with or without a CBB (amiodipine, nifedipine, efonidipine, or (+)-efonidipine, 10−6 mol/l) for 24 h. The cells were then washed with phosphate-buffered saline and lysed with ISOGEN. Total RNA was extracted using the isothiocyanate-acid-phenol-chloroform method. cDNA was then synthesized by Prime Script RT reagent Kit with the oligo-dT primer.

Quantitative real-time PCR for StAR mRNA

Synthesized cDNA was used for real-time PCR using SYBR PremixEX Taq and Thermal Cycler Dice (Takara Bio, Inc.). The specific primers for StAR and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as follows: StAR (GenBank accession No. U17280), forward: 5′-TGGCATGGACACAGACTT-3′, reverse: 5′-GTGAAGCACCATGCAAGTGG-3′; GAPDH (Isaka et al. 2009),
Western blot analysis of the effects of CCBs on the expression of steroidogenic enzymes in NCI-H295R human adrenocortical carcinoma cells

NCI-H295R human adrenocortical carcinoma cells stimulated by dbcAMP with or without CCBs for 24 hours in 10cm culture dishes were lysed by M-PER Mammalian Protein Extraction Reagent and proteins were electrophoresed by sodium dodecyl sulfate polyacrylamide gel. The proteins were subsequently transferred onto a polyvinylidene difluoride membrane (Invitrolon PVDF) and probed by goat anti-StAR antibody (1:200), rabbit anti-CYP11A1 polyclonal antibody (1:80), rabbit anti-HSD3B2 polyclonal antibody (1:1,000), or rabbit anti-CYP21A2 polyclonal antibody (1:80) and, in turn, reprobed with mouse monoclonal antibody for human β-actin, Anti-Human ACTB (1:500). Probed proteins were then treated with donkey anti-goat IgG-HRP (1:5,000) and Amersham ECL Anti-Mouse IgG Horseradish Peroxidase-Linked Species-Specific Whole Antibody, respectively, and visualized using the ECL Plus Western Blotting Detection System. Chemiluminescence was detected by LAS-4000miniEPUV luminescent image analyzer and analyzed using Multi Gauge Ver.3.0 software (Fuji Photo Film, Tokyo, Japan). Each value was normalized to the amount of β-actin.

Measurement of DHEA-S and cortisol in culture medium of NCI-H295R human adrenocortical carcinoma cells

NCI-H295R human adrenocortical carcinoma cells plated in 6-well plates were cultured in DMEM/F12 containing 0.1% BSA, antibiotics and a stimulator, such as dbcAMP (10\(^{-3}\) mol/l) or angiotensin II (10\(^{-7}\) mol/l) with or without CCBs for 24 h. Culture medium was collected, and DHEA-S and cortisol in the same culture medium were measured using the DHEA-S- and cortisol-specific enzyme immunoassay (EIA) kits according to the manufacturer’s protocol. The data were normalized with protein content, which was measured by the BCA protein assay kit following lysis of the cells using albumin standard.

Statistical Analysis

Statistical analysis was performed using analysis of variance followed by a post hoc test for between-group comparison (StatView 5.0, SAS Institute, Inc., Cary, NC). P values less than 0.05 were considered to indicate statistical significance, and all data were expressed as the mean ± standard deviation.

Results

Effects of CCBs on the expression of StAR mRNA and protein in H295R human adrenocortical carcinoma cells and HepG2 human hepatoma cells

Each CCB (10\(^{-5}\) mol/l) alone, except for nifedipine, did not exert significant effects on the expression StAR mRNA in NCI-H295R human adrenocortical cells at 24 hours (data not shown). Nifedipine alone significantly increased the expression of StAR mRNA small (about 1.5-fold increase of DMSO). dbcAMP, which increases intracellular cAMP like ACTH (Ozbay et al. 2004), significantly increased the expression of StAR mRNA (about 2.7 folds of DMSO, P < 0.01 vs. vehicle, Fig. 1A-E) and its protein (about 16.4 folds of DMSO, Fig. 1F). Efonidipine or nifedipine dose-dependently and significantly enhanced the expression of the dbcAMP-induced StAR mRNA in NCI-H295R human adrenocortical carcinoma cells (P < 0.01 vs. dbcAMP alone), whereas amlodipine, azelnidipine, or R(-)-efonidipine significantly decreased dbcAMP-induced StAR mRNA (P < 0.01 vs. dbcAMP alone, Fig. 1A-E). The results of Western blot analysis on the expression of StAR protein are consistent with the results of real-time PCR of StAR mRNA. Angiotensin II, which is another stimulator of steroid biosynthesis in adrenocortical cells, also increased the expression of StAR mRNA (P < 0.01 vs. DMSO, about 1.4 fold of DMSO, Fig. 2). Efonidipine also enhanced the angiotensin II-induced expression of StAR mRNA (P < 0.01 vs. angiotensin II alone), whereas azelnidipine dose-dependently decreased angiotensin II-induced expression of StAR mRNA (P < 0.05 vs. angiotensin II alone) (Fig. 2). In contrast, amlodipine and nifedipine did not exert significant action on angiotensin II-induced expression of StAR mRNA (Fig. 2). Efonidipine, nifedipine, or R(-)-efonidipine also significantly increased the expression of StAR mRNA in HepG2 human hepatoma cells, whereas amlodipine and azelnidipine significantly decreased its expression (P < 0.01 vs. DMSO, Fig. 3A). The results of Western blot analysis were also consistent with those of the real-time PCR (Fig. 3B, C). The expression of StAR mRNA was decreased by the L-type Ca\(^{2+}\) channel blockade with non-dihydropyridine CCBs, such as diltiazem or verapamil, and by chelation of extracellular Ca\(^{2+}\) with EDTA, indicating that the efonidipine, nifedipine, or R(-)-efonidipine-induced increase in StAR mRNA may be independent to extracellular Ca\(^{2+}\) (Fig. 4).

Regulation of other steroidogenic enzymes by dihydropyridine CCBs in NCI-H295R human adrenocortical carcinoma cells

The CCBs did not exert significant effects on the expression of both db-cAMP- and angiotensin II-induced changes of 17α-hydroxylase/17,20-lyase (CYP17), sulfotransferase (SULT2A1), and steroid sulfatase (STS) mRNAs analyzed by real time RT-PCR (data not shown). Therefore, we analyzed the protein expression of CYP11A1, HSD3B2, and CYP21A2 with Western blot analysis, because the expression levels of their mRNAs were marginally increased by the CCBs (about 1 to 2-fold increases in both dbcAMP- and angiotensin II-induced levels of each mRNA expression). As shown in Fig. 5, the expression levels of these enzymes were not noticeably increased by dbcAMP with or without the CCBs, whereas angiotensin II increased the expression of these enzymes.
NCI-H295R human adrenocortical carcinoma cells were cultured for 24 hours with or without a CCB (azelnidipine, amlodipine, nifedipine, efonidipine, or $R^-(−)$-efonidipine). Efonidipine and nifedipine dose-dependently enhanced the dbcAMP-induced expression of StAR at mRNA and protein levels, while amlodipine and $R^-(−)$-efonidipine suppressed the dbcAMP-induced expression of StAR both at mRNA and protein levels at $10^{-6}\text{mol/l}$. A)-E) Dose-dependent effects of CCBs on the expression of StAR mRNA quantified with real-time PCR ((A) azelnidipine, (B) amlodipine, (C) nifedipine, (D) efonidipine, and (E) $R^-(−)$-efonidipine). The qualification and gel image of the expression of StAR protein in Western Blot analysis at $10^{-6}\text{mol/l}$ of dihydropyridine CCBs were shown in F) and G), respectively. The data were reproduced in at least two independent experiments. *$P<0.01$ vs. DMSO, **$P<0.05$ vs. DMSO, #$P<0.01$ vs. dbcAMP alone. D: DMSO (vehicle, 0.1%, [●]), dbcAMP: dbcAMP $10^{-3}\text{mol/l}$, dbcAMP $10^{-3}\text{mol/l}$ alone (●), Aze: azelnidipine $10^{-6}\text{mol/l}$ (△), Aml: amlodipine $10^{-6}\text{mol/l}$ (▽), Nif: nifedipine $10^{-6}\text{mol/l}$ (▲), Efo: efonidipine $10^{-6}\text{mol/l}$ (▼), $R^-(−)$-Efo: $R^-(−)$-efonidipine (□).
Importantly, co-treatment with each CCB further enhanced the angiotensin II-induced increase of such enzymes.

The effects of dihydropyridine CCBs on dbcAMP- or angiotensin II-induced DHEA-S and cortisol production

As shown in Fig. 6A, CCBs except efonidipine and R(−)-efonidipine did not exert significant actions on the production of DHEA-S in NCI-H295R human adrenocortical carcinoma cells, whereas efonidipine and R(−)-efonidipine significantly increased the production of DHEA-S. On the contrary, angiotensin II alone decrease in the production DHEA-S, and treatment with amlodipine, efonidipine, or R(−)-efonidipine further decreased the production of DHEA-S, but azelnidipine and nifedipine did not exert significant action on angiotensin II-induced decrease in DHEA-S (Fig. 6B). The CCBs, except for R(−)-efonidipine, decreased the production of cortisol, consistent with our previous results (Isaka et al. 2009).

Discussion

The adrenal steroids were synthesized with many steroidogenic enzymes. Steroid biosynthesis was initiated by StAR, which is a rate-limiting enzyme of steroid biosynthesis and consisted with many steroidogenic enzymes, such as CYP11A1, HSD3B2, CYP21A2, CYP17, SULT2A1, and STS. Although the dihydropyridine CCB is one of major compounds to treat hypertension, recent studies revealed that the dihydropyridine CCBs have various action on steroid biosynthesis and steroid actions beyond anti-hypertensive actions (Imagawa et al. 2006; Dietz et al. 2008; Isaka et al. 2009). We previously described the suppressive action of azelnidipine on steroid biosynthesis, such as cortisol and aldosterone by modulating the expression of CYP11B1 and CYP11B2 (Isaka et al. 2009). But the change of the expression pattern of steroidogenic enzymes by CCBs was not evaluated in our previous study. Therefore, the present study was designed to evaluate the
some CCBs enhance dbcAMP-induced expression of StAR at mRNA and protein levels in NCI-H295R adrenocortical carcinoma cells and HepG2 human hepatoma cells in addition to MA-10 mouse Leydig cells (Pandey et al. 2010), which may be dihydropyridine-specific and independent to Ca\(^{2+}\) blockade, because blockade of influx of extracellular Ca\(^{2+}\) by BAYK8644 (Clark et al. 1995) or the non-dihydropyridine Ca\(^{2+}\) channel antagonist and chelating the extracellular Ca\(^{2+}\) cause the decrease in the expression of StAR. The effects of CCBs on the expression of StAR in HepG2 human hepatoma cells is consistent with those in NCI-H295R human adrenocortical carcinoma cells, indicating that transportation of cholesterol into mitochondria may be facilitated by CCBs such as efonidipine or nifedipine. But such CCBs suppressed the production of aldosterone and cortisol (Isaka et al. 2009), which are final products in adrenal cells in steroidogenic system (Miller 2008). Then, a question is arisen; “How the steroidogenic system treat the transported cholesterol?” The results of the present study also revealed that the CCBs, especially, efonidipine and \(R(-)\)-efonidipine, may enhanced dbcAMP-induced production of DHEA-S, which has potentially beneficial actions against atherosclerosis and vascular remodeling (Alexandersen et al. 1996; Li et al. 2009), whereas the CCBs enhanced angiotensin II-induced decrease of the production of DHEA-S. Age-associated declines in adrenal androgen production may contribute to decreased immune function, osteoporosis, and atherosclerosis (Dharia and Parker 2004) and the prevalence of hypertension raised in aged population. Therefore, efonidipine, which exerts cardio- and renal protective actions via the blockade of the T-type Ca\(^{2+}\) channel resulting in suppression of release of aldosterone from adrenal cells and the activation of nuclear factor-\(\kappa\)B (Imagawa et al. 2006; Isaka et al. 2009; Hayashi et al. 2010), may exert additional cardiovascular actions, such as an anti-atherosclerotic action, through holding up the age-associated declines in adrenal androgen production in the aged patients with hypertension. Our present results showed that, at least, efonidipine and \(R(-)\)-efonidipine enhanced the dbcAMP-induced production of DHEA-S which reflects increased cholesterol transport to mitochondria due to increased expression of StAR because other steroidogenic enzymes are not significantly changed by dbcAMP with efonidipine or \(R(-)\)-efonidipine. On the contrary, angiotensin II decreased the production of DHEA-S, and CCBs enhanced the decrease of DHEA-S induced by angiotensin II, possibly due to enhancement of the expression of the steroidogenic enzymes, such as HSD3B2 and CYP21A2. The detailed mechanism of enhancement by CCBs on angiotensin II is not clarified and the further studies should be required.

In conclusion, the present results indicate that efonidipine may enhance the production of adrenal DHEA-S via the modulation of StAR independent blockade of Ca\(^{2+}\) influx through Ca\(^{2+}\) channels. Therefore, although the detailed mechanism still remains to be clarified, efonidipine
Fig. 5. The effects of dihydropyridine CCBs on the expression of dbcAMP- and angiotensin II-induced steroidogenic enzymes in NCI-H295R adrenocortical carcinoma cells. CCBs enhanced angiotensin II-induced expression of these enzymes, whereas CCBs did not exert the significant action on the protein expression of these enzymes. A) Image of western blot analysis of CYP11A1, HSD3B2, and CYP21A2. B)-D) Quantification of the protein expression of CYP11A1, HSD3B2, and CYP21A2. D: DMSO (vehicle, 0.1%), Aml: amlodipine $10^{-6}$ mol/l, Nif: nifedipine $10^{-6}$ mol/l, Efo: efonidipine $10^{-6}$ mol/l, Aze: azelnidipine $10^{-6}$ mol/l, R(−)-Efo: R(−)-efonidipine $10^{-6}$ mol/l.
may exert an additional action beyond anti-hypertensive actions via slowing the age-associated decline of adrenal androgen production in aged patients with hypertension.

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Conflict of Interest
All authors have no conflict of interests.

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