Biochemical and Biophysical Research Communications 434 (2013) 210–216

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Voltage-dependent N-type Ca^{2+} channels in endothelial cells contribute to oxidative stress-related endothelial dysfunction induced by angiotensin II in mice

Motohiro Nishida [a,1], Tatsuya Ishikawa [b,1], Shota Saiki [a], Caroline Sunggip [a], Shizuka Aritomi [b], Eri Harada [b], Koichiro Kuwahara [c], Katsuya Hirano [d], Yasuo Mori [e], Shokei Kim-Mitsuyama [f,*]

[a] Department of Drug Discovery and Evolution, Graduate School of Pharmaceutical Sciences, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812-8582, Japan
[b] Ajinomoto Pharmaceuticals Co., Ltd., 1-1 Irifune 2-chome, Chuo-ku, Tokyo 104-0042, Japan
[c] Department of Medicine and Clinical Science, Kyoto University Graduate School of Medicine, Kyoto, Japan
[d] Division of Molecular Cardiology, Research Institute of Angiocardiology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan
[e] Laboratory of Environmental Systems Biology, Graduate School of Environmental Studies and Laboratory of Molecular Biology, Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University, Kyoto, Japan
[f] Department of Pharmacology and Molecular Therapeutics, Kumamoto University Graduate School of Medical Sciences, 1-1-1 Honjo, Kumamoto 860-8556, Japan

ARTICLE INFO

Article history:
Received 2 March 2013
Available online 26 March 2013

Keywords:
N-type Ca^{2+} channel
Cilnidipine
Reactive oxygen species
Hypertension
Vascular relaxation

ABSTRACT

N-type voltage-dependent Ca^{2+}channels (VDCCs), expressed predominantly in the nervous system, play pivotal roles in sympathetic regulation of the circulatory system. Although N-type VDCCs are also reportedly expressed in the vasculature, their pathophysiological role is obscure. We demonstrated that oxidative stress-related endothelial dysfunction induced by angiotensin (Ang) II is suppressed in mice lacking the N-type VDCC α_{1B} subunit (Ca_v 2.2). Impairment of endothelium-dependent relaxation of the thoracic aorta observed following Ang II treatment in wild-type (WT) mice was significantly attenuated in the Ang II-treated Ca_v 2.2-deficient mice, despite the comparable increase of the blood pressure in the two groups of mice. The thoracic aorta of the Ca_v 2.2-deficient mice showed a smaller positive area of oxidative stress markers as compared to the WT mice. The Ang II-induced endothelial dysfunction was also suppressed by cilnidipine, an L/N-type VDCC blocker, but not by amlodipine, an L-type VDCC blocker; however, this unique effect of cilnidipine was completely abolished in the Ca_v 2.2-deficient mice. Furthermore, selective inhibition of N-type VDCCs by ω-conotoxin GVIA dramatically suppressed the production of reactive oxygen species (ROS) as well as agonist-induced Ca^{2+} influx in the vascular endothelial cells. These results suggest that N-type VDCCs expressed in the vascular endothelial cells contribute to ROS production and endothelial dysfunction observed in Ang II-treated hypertensive mice.

1. Introduction

Vascular endothelium is a major modulator of the vascular tone, releasing vasoactive substances such as endothelium-derived relaxing factor (EDRF), endothelium-derived hyperpolarizing factors (EDHFs), cycloxygenase metabolites, and endothelium-derived constricting factors (EDCFs) [1,2]. The balanced release of these factors is altered in several cardiovascular pathologies, such as hypertension and heart failure. The resultant apparent deterioration of endothelium-dependent vasodilatation is termed 'endothelial dysfunction'. Endothelial dysfunction presents decreased bioavailability of nitric oxide, a potent EDRF, in patients with essential hypertension, which may also be a key determinant of the premature development of atherosclerosis and thrombosis [2,3]. Thus, therapeutic strategies directed at improving endothelial dysfunction may result in decreased morbidity and mortality in hypertensive patients.

A growing body of evidence suggests that oxidative stress in the vasculature plays a pivotal role in endothelial dysfunction, and that the physical and chemical stresses induced by high pressure lead to augmented oxidative stress [4]. Vascular tone is largely dependent on the Ca^{2+} influx across the plasma membrane through volt-

Abbreviations: ACh, acetylcholine; Ang, angiotensin; AT_1R, Ang type1 receptor; BP, blood pressure; BW, body weight; DHE, dihydroethidium; DHP, dihydropyridine; EDCF, endothlium-derived constricting factor; EDHF, endothelium-derived hyperpolarizing factor; EDRF, endothelium-derived relaxing factor; 4-HNE, 4-hydroxy-2-nonenal; HW, heart weight; KW, kidney weight; LTCCs, L-type Ca^{2+} channels; Nox, NADPH oxidase; 8-OH-dG, 8-hydroxy-2′-deoxyguanosine; ROS, reactive oxygen species; VDCCs, voltage-dependent Ca^{2+} channels; WT, wild-type.

* Corresponding author. Fax: +81 96 373 5082.
E-mail addresses: nishida@phar.kyushu-u.ac.jp (M. Nishida), tatsuya_ishikawa@ajinomoto.com (T. Ishikawa), kimmitsu@gpo.kumamoto-u.ac.jp (S. Kim-Mitsuyama).

[1] These authors contributed equally to this work.

0006-291X © 2013 Elsevier Inc. Open access under CC BY-NC-ND license.
http://dx.doi.org/10.1016/j.bbrc.2013.03.040

age-dependent Ca^{2+} channels (VDCCs) in the vascular smooth muscle cells. Subtypes of VDCC, namely, the L-, N-, T- and P/Q-type channels are present in the vasculature, and blockers of L-type VDCCs are widely used in the treatment of hypertension [5,6]. Thus, lowering of the blood pressure (BP) by the blockade of L-type VDCCs is expected to reduce oxidative stress in the vasculature. However, blockade of L-type VDCCs also results in activation of the sympathetic tone because of its rapid vasodilatory effect [6,7]. Development of new-generation L-type VDCC blockers to reduce the undesired reflex sympathetic activation has been attempted, however, to date, success has not been achieved in avoiding such sympathetic reflex activation by L-type VDCC blockers [7,8]. In contrast, cilnidipine, a dihydropyridine (DHP) derivative that inhibits both N-type and L-type VDCCs [9,10], has been shown to be more effective than blockers of L-type VDCCs alone in patients with essential hypertension [11,12]. As N-type VDCCs are predominantly distributed in the sympathetic nervous system and regulate the noradrenaline release from the sympathetic nerve endings [13,14], suppression of hemodynamic and autonomic reflexes by blockade of N-type VDCCs may underlie the potent antihypertensive effect of cilnidipine [11,15]. However, it is unclear whether cilinidipine suppresses oxidative stress-related endothelial dysfunction by inhibiting sympathetic tone. In addition, it has been reported recently that N-type VDCCs are also expressed in the vasculature and that their gene expression levels are increased in hypertensive rats [16].

Activation of the renin-angiotensin system (RAS) is one of the major pathophysiological mechanisms underlying the development of essential hypertension [17]. Stimulation of the angiotensin (Ang) II, type1 receptor (AT_1R) induced by Ang II causes hypertension by enhancing oxidative stress in the vasculature partly through mechanical stress-dependent production of reactive oxygen species (ROS) [18–21]. Therefore, we investigated whether the N-type VDCCs participate in the endothelial dysfunction associated with the augmented oxidative stress induced by Ang II, using mice lacking the α_{1B} subunit (Ca_v 2.2) of N-type VDCCs.

2. Materials and methods

2.1. Materials

Val^5-Ang II was purchased from Peptide Institute. Paraform aldehyde and 10% formalin neutral buffer solution were purchased from Wako. Horseradish peroxidase-conjugated anti-rabbit IgG antibody was obtained from Santa Cruz Biotech. Heparin solution was from Mochida Pharmaceutical Co. Ltd. Mayer's Hematoxylin and eosin were from Muto Chemical Co. Ltd. Alexa Fluor 488 anti-rat IgG, Alexa Fluor 546 anti-mouse IgG and dihydroethidium (DHE) were from Molecular Probes. Amlodipine was from Sigma. Mouse anti-4-hydroxy-2-nonenal (4-HNE) and anti-8-hydroxy-2′-deoxyguanosine (8-OH-dG) antibodies were from the Japan Institute for the Control of Aging (JaICA). Anti-CD68 antibody was from AbD Serotec, and anti-Ca_v 2.2 antibody was from Alomone. Cilnidipine was synthesized in Ajinomoto Pharmaceutical Co. Ltd.

2.2. Animals and drug treatment

The Ca_v 2.2-deficient mice were developed by Dr. Yasuo Mori [14,22]. Their wild-type littermates (WT) served as the controls for all the studies. A mini osmotic pump (Alzet) filled with cilnidipine (30 mg/kg/day), amlodipine (10 mg/kg/day), or 100% polyethylene glycol (vehicle) was implanted intraperitoneally into 8-week-old mice, and after 3 days, another mini osmotic pump filled with Val^5-Ang II (1 mg/kg/day) was implanted for continuous administration of Ang II for 4 weeks. Increase in BP induced by Ang II was confirmed using the tail-cuff measurement method (Softron, BP-98A). Genotyping of the Ca_v 2.2-deficient mice was performed using the polymerase chain reaction [14]. All animals were maintained in a conventional air-conditioned room with a time-controlled lighting system. The handling and sacrificing of the animals were carried out in accordance with nationally prescribed guidelines, and ethical approval for the studies was obtained from the Animal Care and Use Committee of Kyushu University.

2.3. Isometric force measurements of mouse aortic rings

Mice were sacrificed with sodium pentobarbital (50 mg/kg, i.p.); the descending thoracic aorta was carefully isolated with any adherent connective tissue dissected out, and cut into 2-mm-long rings [23]. Each ring was mounted between a pair of triangular stainless steel hooks, one of which was stationary and the other connected to a strain-gauge transducer (UL10GR, Minebea), to record the isometric tension (MC6625, Graphtec, Tokyo, Japan). The rings, placed in 20 ml organ baths containing Krebs Henseleit (KH) solution maintained at 37 °C and aerated continuously with 95% O_2 and 5% CO_2, were stretched to a resting tension of 1.5 g and allowed to equilibrate for at least 30 min, with occasional changes of the KH solution. After equilibration, the rings were set at 1.5 g tension and incubated for 10 min. The endothelial function was determined by examining the degree of relaxation in response to acetylcholine (ACh) of the aortic rings precontracted by KCl (60 mM).

2.4. Analysis of the oxidative stress level and degree of macrophage infiltration in mouse aorta

The aortic vessels were embedded in OCT compound (Leica) and frozen sections were prepared using a microtome (Cryostat; Leica, CM1100). The OCT-embedded sections (5 μm in thickness) were stained with anti-4-HNE (1/500), anti-8-OH-dG (1/500), and anti-CD68 (1/200) antibodies. The 4-HNE adducts and 8-OH-dG were visualized with Alexa Fluor 546 anti-mouse IgG antibody (1/1,000, Molecular Probes). Digital photographs were taken at ×60 magnification using confocal microscopy (FV10i, Olympus), and 5 regions were selected at random for each specimen of the vessel. The average intensity was analyzed using the MetaMorph Software (Molecular Devices).

2.5. Measurement of ROS production and the intracellular Ca^{2+} concentration

Measurement of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was performed using fura-2 [24]. Production of ROS in bovine aortic endothelial cells (BAECs) was measured using DHE [25]. Cells plated on 35-mm glass-based dishes were loaded with DHE (10 μM) at 37 °C for 10 min prior to ATP treatment. The DHE fluorescence was observed using confocal microscopy at an emission wavelength of 560 nm and excitation wavelength of 543 nm.

2.6. Statistical analysis

The results are shown as means ± SEM. All experiments were repeated at least three times. Statistical comparisons were performed using two-tailed Student's *t*-test or one-way analysis of variance followed by the Student–Newman-Keuls procedure, with the level of significance set at $P < 0.05$.

3. Results

3.1. Involvement of N-type VDCCs in oxidative stress-related endothelial dysfunction induced by Ang II

As shown in a previous report [22], the basal heart rate and BP in Ca_v 2.2-deficient mice were almost equivalent to those in WT mice (Table 1). Chronic Ang II treatment produced equivalent increases of BP in both WT and Ca_v 2.2-deficient mice, indicating that Ang II-induced signaling was not affected by the deletion of N-type VDCCs. Following 4-weeks' treatment with Ang II, the WT mice showed impairment of ACh-induced endothelium-dependent vascular relaxation of the thoracic aorta (Fig. 1A). In contrast, this Ang II-induced endothelial dysfunction was significantly attenuated in Ca_v 2.2-deficient mice (Fig. 1A and B). Oxidative stress due to loss of reduction/oxidation (redox) homeostasis has been implicated in the occurrence of Ang II-induced endothelial dysfunction [4,19]. Chronic treatment with Ang II was shown to increase the expression of oxidized nucleotide (8-OH-dG) and fatty acid (4-HNE) in WT mouse thoracic aorta, while accumulation of these oxidative stress markers was significantly reduced in Ca_v 2.2-deficient mouse thoracic aorta (Fig. 1C–F). These results indicate that N-type VDCCs participate in the augmentation of the oxidative stress and induction of endothelial dysfunction by Ang II in the mouse thoracic aorta.

Table 1
Hemodynamic parameters and organ weights in the WT (Ca_v 2.2 (+/+)) and Ca_v 2.2-deficient (Ca_v 2.2 (−/−)) mice.

Ca_v 2.2 (+/+)	Vehicle (n = 6)	Ang II (n = 4)	Ang II + CIL (n = 5)	Ang II + AML (n = 4)
HR (bpm)	581 ± 15	531 ± 22	579 ± 53	740 ± 18*,#
SBP (mmHg)	105 ± 3	143 ± 7*	106 ± 3#	104 ± 6#
HW/BW (mg/g)	4.3 ± 0.0	5.2 ± 0.1*	4.2 ± 0.1#	5.3 ± 0.2*
KW/BW (mg/g)	5.8 ± 0.1	7.4 ± 0.1*	6.2 ± 0.3#	7.2 ± 0.2*
Ca_v 2.2 (−/−)	(n = 4)	(n = 4)	(n = 4)	(n = 4)
HR (bpm)	623 ± 41	609 ± 50	655 ± 27	669 ± 24
SBP (mmHg)	103 ± 2	137 ± 5*	110 ± 6#	95 ± 10#
HW/BW (mg/g)	3.8 ± 0.1	4.4 ± 0.1*	4.0 ± 0.1	4.1 ± 0.2
KW/BW (mg/g)	5.2 ± 0.1	6.4 ± 0.2*	5.4 ± 0.2	5.7 ± 0.2

Abbreviations: CIL, cilnidipine; AML, amlodipine; HR, heart rate; SBP, systolic blood pressure; HW, heart weight; BW, body weight; KW, kidney weight.
* $P < 0.05$ *versus* vehicle group.
# $P < 0.05$ *versus* Ang II group.

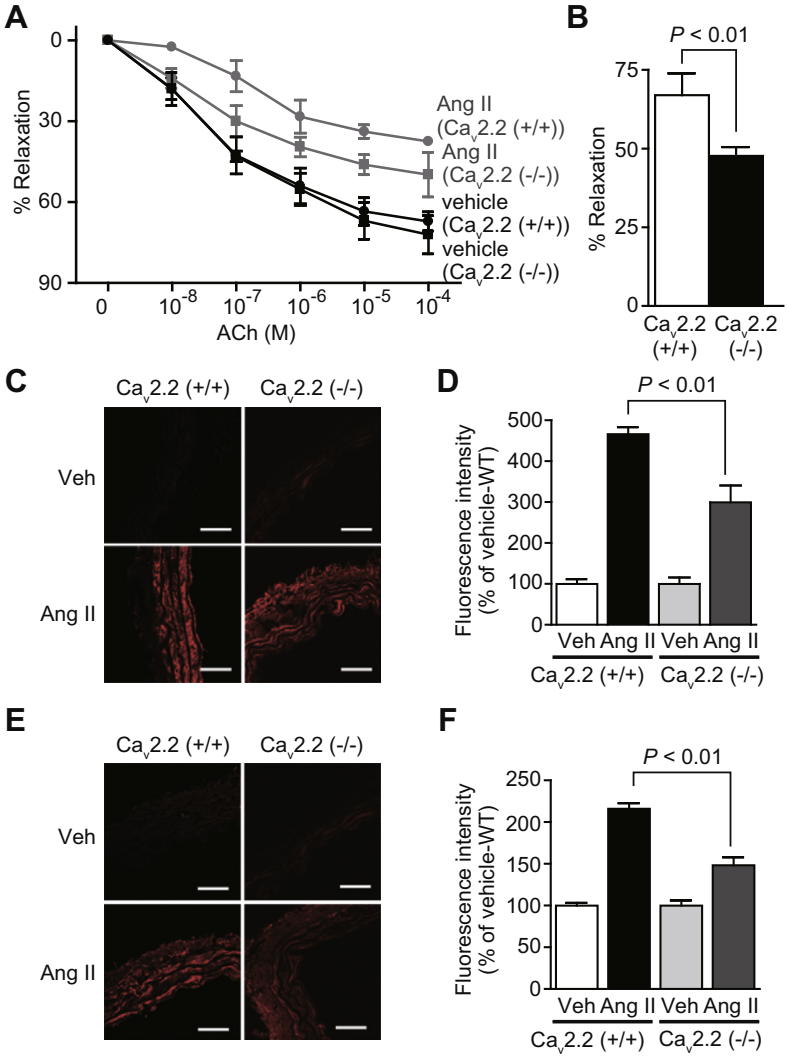

Fig. 1. Inhibition of N-type VDCCs attenuates Ang II-induced impairment of endothelium-dependent vascular relaxation produced by ACh in the mouse thoracic aorta. (A) Dose-dependent relaxation induced by ACh in WT (Ca_v 2.2 (+/+)) and Ca_v 2.2-deficient (Ca_v 2.2 (−/−)) mice with or without Ang II. n = 6. (B) Peak relaxation induced by ACh (10 μM) in the thoracic aorta of Ang II-infused Ca_v 2.2 (+/+) and Ca_v 2.2 (−/−) mice. (C) Fluorescence images of 8-OH-dG formation in the thoracic aorta. Scale bar = 50 μm. (D) Average fluorescence intensities of the 8-OH-dG images. (E) Fluorescence images of 4-HNE formation. Scale bar = 50 μm. (F) Average fluorescence intensities of the 4-HNE images. n = 3–5.

3.2. Potent inhibition of Ang II-induced endothelial dysfunction by cilnidipine

We next examined whether inhibition of the L/N-type VDCCs by cilnidipine, in comparison to that of the L-type VDCCs by amlodipine (a DHP derivative), exerts a beneficial effect against Ang II-induced endothelial dysfunction in mice [26]. Treatment with amlodipine completely suppressed Ang II-induced increase of BP, but caused significant reflex tachycardia (Table 1). Treatment with cilnidipine also completely suppressed the increase of BP induced by Ang II, but without producing reflex tachycardia. While both cilnidipine and amlodipine suppressed the Ang II-induced impairment of endothelium-dependent vascular relaxation caused by ACh in WT mice, the magnitude of the protective effect was greater for cilnidipine (Fig. 2A). However, this potent protective effect of cilnidipine as compared to that of amlodipine was completely abolished in Ca_v 2.2-deficient mice (Fig. 2B). While both cilnidipine and amlodipine significantly suppressed the accumulations of 4-HNE/8-OH-dG and macrophage infiltration in the thoracic aorta of WT mice with Ang II, the magnitude of suppression was greater for cilnidipine (Fig. 2C–F). This potent inhibitory effect of cilnidipine of Ang II-induced augmentation of oxidative stress and macrophage infiltration was also abrogated in Ca_v 2.2-deficient mice. These results indicate that cilnidipine exerts its potent vascular

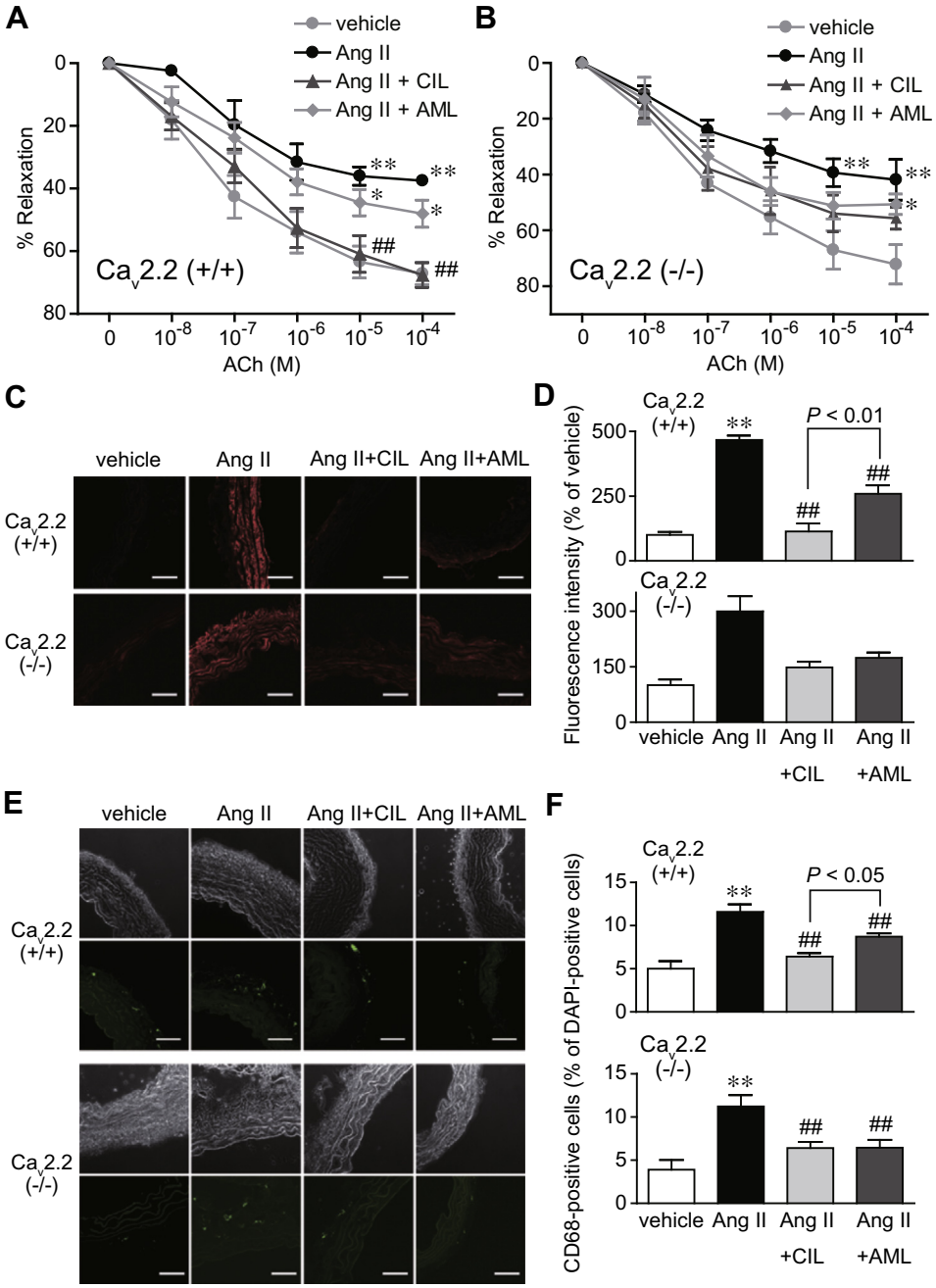

Fig. 2. Protection against Ang II-induced impairment of endothelial function by cilnidipine via inhibition of N-type VDCCs. (A, B) Relaxation of the thoracic aorta induced by ACh in Ca_v 2.2 (+/+) mice (A) and Ca_v 2.2 (−/−) mice (B) treated or not treated with Ang II. An osmotic pump containing cilnidipine (CIL) or amlodipine (AML) was implanted 3 days before Ang II infusion. $n = 4–6$. (C) Fluorescence images of 8-OH-dG formation. Scale bar = 50 μm. (D) Average fluorescence intensities of 8-OH-dG images. (E) DIC and Fluorescence images of macrophage infiltration. Scale bar = 50 μm. (F) Average proportions of CD68-positive macrophages. $^{*}P < 0.05$ and $^{**}P < 0.01$ *versus* vehicle group, $^{\#\#}P < 0.01$ *versus* Ang II group.

protective effects against Ang II-induced endothelial dysfunction in part via inhibition of the N-type VDCCs.

3.3. Requirement of N-type VDCCs for agonist-induced ROS production in endothelial cells

Although stimulation of the AT_1R by Ang II has been reported to induce ROS production in the vasculature via NADPH oxidase activation, it also induces ROS production via a mechanical stress-mediated mechanism [20,21]. We further examined whether N-type VDCCs are expressed and contribute to Ang II-induced ROS production in the endothelial cells, using human vascular endothelial cells (HUVECs). Western blotting using anti-Ca_v 2.2 antibody showed that the N-type VDCCs were expressed in HUVECs, but not in rat vascular smooth muscle cells (Fig. 3A). We also found membrane expression of N-type VDCCs in bovine aortic endothelial cells (BAECs), however, neither the HUVECs nor the BAECs were responsive to Ang II or noradrenaline (Fig. 3B), suggesting the scarce expression of AT_1Rs and adrenergic receptors in these endothelial cell lines. We have previously reported that extracellular nucleotides mediate mechanical stress-induced cardiac fibrosis in mice [27], and that extracellular nucleotides are well known as potent endogenous ligands activating intracellular Ca^{2+} signaling in a variety of cells [24]. Treatment with ATP, a specific agonist for purinergic receptors, led to a marked increase in ROS production as well as intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) in BAECs (Fig. 3B–E). N-type VDCCs are high voltage-activated Ca^{2+} channels that can be selectively blocked by ω-conotoxin (ωCTx)-GVIA, a 27 amino-acid peptide isolated from the venom of the fish-hunting cone snail, *Conus geographus* [28]. The ATP-induced ROS production and sustained elevation of the $[Ca^{2+}]_i$ were suppressed by ωCTx-GVIA. We also found that treatment of BAECs with KCl (10 mM) induced moderate increase of the DHE fluorescence intensity, which was suppressed by ωCTx (data not shown). These results strongly suggest that N-type VDCCs contribute to ROS production associated with a sustained increase in $[Ca^{2+}]_i$ in vascular endothelial cells.

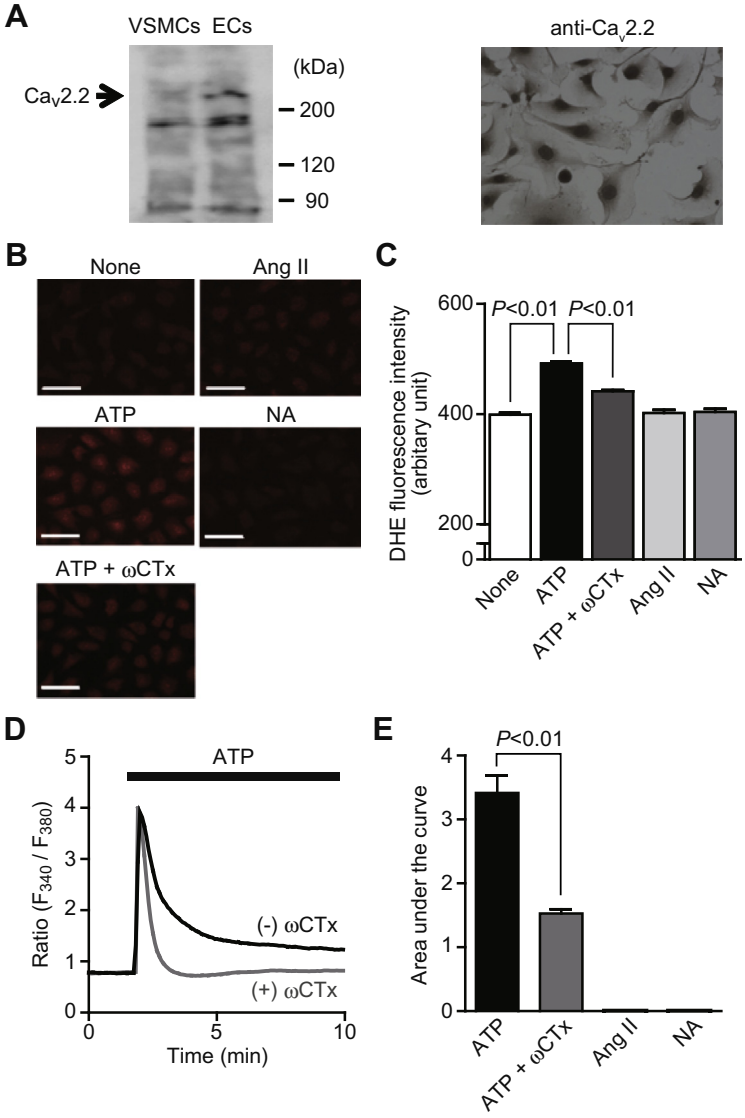

Fig. 3. N-type VDCCs participate in ATP-induced ROS production in endothelial cells (ECs). (A) Left, expression of Ca_v 2.2 proteins in human ECs and rat vascular smooth muscle cells (VSMCs). Right, localization of Ca_v 2.2 proteins in bovine aortic ECs (BAECs). (B) Effects of ω-conotoxin (ωCTx) on the increase in DHE fluorescence intensity by ATP (100 μM), Ang II (1 μM) and noradrenaline (NA, 100 μM) treatment for 1 h in BAECs. Cells were treated with ωCTx (100 ng/ml) 30 min prior to ATP stimulation. (C) Average increases in DHE fluorescence intensities. (D) Time-courses of ATP-induced Ca^{2+} responses in BAECs treated with or without ωCTx. (E) Cumulative increases in $[Ca^{2+}]_i$ induced by ATP, Ang II and NA.

4. Discussion

The involvement of N-type VDCCs in oxidative stress has been implicated previously in the kidney [29,30]. Cilnidipine has a potent renoprotective effect that is mediated by the blockade of N-type VDCCs, including suppression of the RAS, renal fibrosis and inflammation [29,31]. In fact, N-type VDCCs expressed in the nerve terminals have been revealed to mediate neurotransmitter release, leading to constriction of afferent and efferent arterioles, and increase of the glomerular capillary pressure [32]. Slight reduction of heart rate by Ang II treatment was observed in WT mice, which was diminished in Ca_v 2.2-deficient mice (Table 1). Chronic Ang II infusion has been reported to decrease renal noradrenaline overflow in conscious dogs [33]. Thus, our data support the renoprotective effect by inhibition of N-type VDCCs against the adverse effects of high BP. As Ang II-induced increase in BP remained unaltered by inhibition of N-type VDCCs, N-type VDCCs may not participate in the Ang II-induced BP elevation.

We also demonstrated that the N-type VDCCs in endothelial cells contribute to the extracellular nucleotide (ATP)-induced ROS production as well as sustained increase of the $[Ca^{2+}]_i$ in aortic endothelial cell lines. Stimulation of purinergic P2X receptors is known to induce membrane depolarization via cation influx, leading to sustained Ca^{2+} influx through the VDCCs [34]. Our previous reports have shown that sustained increase in $[Ca^{2+}]_i$ is required for agonist-induced ROS production in cardiac cells [25]. As ωCTx-GVIA partially suppressed ATP-induced ROS production (Fig. 3), N-type VDCCs may be one of the key players in agonist-induced ROS production in endothelial cells. We suggest for the first time that N-type VDCCs in endothelial cells, but not in nerve terminals, directly contribute to ROS production, and that this participates in oxidative stress-related endothelial dysfunction in hypertensive mice. DHP derivatives work as lipophilic chain-breaking antioxidants [35], and potent antioxidant activity of cilnidipine has been reported [30]. The cilnidipine *versus* amlodipine randomized trial for evaluation in renal disease (CARTER) suggests that cilnidipine is superior to amlodipine for preventing the progression of proteinuria in hypertensive patients under treatment with a RAS inhibitor [26]. The same group has also reported that cilnidipine showed a significantly higher antioxidant activity than amlodipine against ionomycin-stimulated superoxide production in cultured human mesangial cells [30]. Another report has shown that cilnidipine suppresses cardiac remodeling and diastolic dysfunction through inhibiting NADPH oxidase activity in Dahl salt-sensitive rats [36]. We did not investigate the mechanism underlying the N-type VDCC-mediated ROS production, but these reports support the idea that Ca^{2+} influx through N-type VDCCs contribute to NADPH oxidase-mediated ROS production in endothelial cells.

In conclusion, we demonstrated that the N-type VDCCs expressed in endothelial cells contribute to agonist-induced ROS production and oxidative stress-related endothelial dysfunction in Ang II-treated hypertensive mice. Our findings provide a novel insight into the mechanism underlying the progression of cardiovascular diseases caused by hypertension, and is thereby expected to pave the way for the development of a new therapeutic strategy to decrease the morbidity and mortality of hypertensive patients.

Acknowledgments

M. N. and T. I. designed and analyzed the experiments and wrote the paper. S. S., C. S., S. A., and E. H. performed animal study, and analyze the study. K. K., H. K., and Y. M. helped design and edited the paper. K-M. S. decided to submit the article. This work was supported by a Grant-in-Aid for Ajinomoto Pharmaceuticals Co., Inc. (to M. N.), by the Uehara Memorial Foundation (to M. N., K. K., and Y. M.), and the Platform for Drug Discovery, Informatics, and Structural Life Science from the Ministry of Education, Culture, Sports, Science and Technology, Japan (to M. N.). The authors declare no conflict of interest.

References

[1] P.M. Vanhoutte, Endothelium and control of vascular function. State of the art lecture, Hypertension 13 (1989) 658–667.
[2] S. Taddei, L. Ghiadoni, A. Virdis, et al., Mechanisms of endothelial dysfunction: clinical significance and preventive non-pharmacological therapeutic strategies, Curr. Pharm. Des. 9 (2003) 2385–2402.
[3] H. Brunner, J.R. Cockcroft, J. Deanfield, et al., Endothelial function and dysfunction. Part II: association with cardiovascular risk factors and diseases. A statement by the working group on endothelins and endothelial factors of the european society of hypertension, J. Hypertens. 23 (2005) 233–246.
[4] C. Sunggip, N. Kitajima, M. Nishida, Redox control of cardiovascular homeostasis by angiotensin II, Curr. Pharm. Des., in press.
[5] E.A. Ertel, K.P. Campbell, M.M. Harpold, et al., Nomenclature of voltage-gated calcium channels, Neuron 25 (2000) 533–535.
[6] F.H. Leenen, Clinical relevance of 24 h blood pressure control by 1,4-dihydropyridines, Am. J. Hypertens. 9 (1996) 97S–104S.
[7] A. Takahara, Clinidipine: a new generation Ca^{2+} channel blocker with inhibitory action on sympathetic neurotransmitter release, Cardiovasc. Ther. 27 (2009) 124–139.
[8] M. Lindqvist, T. Kahan, A. Melcher, et al., Long-term calcium antagonist treatment of human hypertension with mibefradil or amlodipine increases sympathetic nerve activity, J. Hypertens. 25 (2007) 169–175.
[9] H. Uneyama, A. Takahara, H. Dohmoto, et al., Blockade of N-type Ca^{2+} current by cilnidipine (FRC-8653) in acutely dissociated rat sympathetic neuron, Br. J. Pharmacol. 122 (1997) 37–42.
[10] S. Fujii, K. Kameyama, M. Hosono, et al., Effect of cilnidipine, a novel dihydropyridine Ca^{2+} channel antagonist, on N-type Ca^{2+} channel in rat dorsal root ganglion neurons, J. Pharmacol. Exp. Ther. 280 (1997) 1184–1191.
[11] S. Morimoto, Y. Yano, K. Maki, et al., Renal and vascular protective effects of cilnidipine in patients with essential hypertension, J. Hypertens. 25 (2007) 2178–2183.
[12] H. Toba, M. Yoshida, C. Tojo, et al., L/N-type calcium channel blocker cilnidipine ameliorates proteinuria and inhibits the renin–angiotensin–aldosterone system in deoxycorticosterone acetate-salt hypertensive rats, Hypertens. Res. 34 (2011) 521–529.
[13] L.D. Hirning, A.P. Fox, E.W. McCleskey, et al., Dominant role of N-type Ca^{2+} channels in evoked release of noradrenaline from sympathetic neurons, Science 239 (1988) 57–61.
[14] M. Ino, T. Yoshinaga, M. Wakamori, et al., Functional disorders of the sympathetic nervous system in mice lacking the α_{1B} subunit (Cav 2.2) of N-type calcium channels, Proc. Natl. Acad. Sci. USA 98 (2001) 5323–5328.
[15] T. Takami, M. Shigemasa, Efficacy of various antihypertensive agents as evaluated by indices of vascular stiffness in elderly hypertensive patients, Hypertens. Res. 26 (2003) 609–614.
[16] S. Takai, D. Jin, S. Aritomi, et al., Powerful vascular protection by combining cilnidipine with valsartan in stroke-prone, spontaneously hypertensive rats, Hypertens. Res. (2012), http://dx.doi.org/10.1038/hr.2012.187.
[17] M.J. Ryan, S.P. Didion, S. Mathur, et al., Angiotensin II-induced vascular dysfunction is mediated by the AT1A receptor in mice, Hypertension 43 (2004) 1074–1079.
[18] K.K. Griendling, C.A. Minieri, J.D. Ollerenshaw, et al., Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells, Circ. Res. 74 (1994) 1141–1148.
[19] S. Rajagopalan, S. Kurz, T. Munzel, et al., Angiotensin II-mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NAD(P)H oxidase activation. Contribution to alterations of vasomoter tone, J. Clin. Invest. 97 (1996) 1916–1923.
[20] Z. Unqvari, M.S. Wolin, A. Csiszar, Mechanosensitive production of reactive oxygen species in endothelial and smooth muscle cells: role in microvascular remodeling?, Antioxid Redox Signal. 8 (2006) 1121–1129.
[21] K. Grote, I. Flach, M. Luchtefeld, et al., Mechanical stretch enhances mRNA expression and proenzyme release of matrix metalloproteinase-2 (MMP-2) via NADPH oxidase-derived reactive oxygen species, Circ. Res. 92 (2003) e80–e86.
[22] Y. Mori, M. Nishida, S. Shimizu, et al., Ca^{2+} channel α1B subunit (Cav 2.2) knockout mouse reveals a predominant role of N-type channels in the sympathetic regulation of the circulatory system, Trends Cardiovasc. Med. 12 (2002) 270–275.
[23] K. Nishioka, M. Nishida, M. Ariyoshi, et al., Protein kinase A-mediated phosphorylation of TRPC6 channels underlies suppression of angiotensin II-induced vasoconstriction, Arterioscler. Thromb. Vasc. Biol. 31 (2011) 2278–2286.
[24] M. Nishida, M. Ogushi, R. Suda, et al., Heterologous down-regulation of angiotensin type1 receptors by purinergic $P2Y_2$ receptor stimulation through S-nitrosylation of NF-κB, Proc. Natl. Acad. Sci. USA 108 (2011). 6662-6627.
[25] N. Kitajima, K. Watanabe, S. Morimoto, et al., TRPC3-mediated Ca^{2+} influx contributes to Rac1-mediated production of reactive oxygen species in MLP-deficient mouse hearts, Biochem. Biophys. Res. Commun. 409 (2011) 108–113.

[26] T. Fujita, K. Ando, H. Nishimura, et al., Antiproteinuric effect of the calcium channel blocker cilnidipine added to renin-angiotensin inhibition in hypertensive patients with chronic renal disease, Kidney Int. 72 (2007) 1543–1549.
[27] M. Nishida, Y. Sato, A. Uemura, et al., $P2Y_6$ receptor-$G\alpha 12_{/13}$ signaling in cardiomyocytes triggers pressure overload-induced cardiac fibrosis, EMBO J. 27 (2008) 3104–3115.
[28] B.M. Olivera, W.R. Gray, R. Zeikus, et al., Peptide neurotoxins from fish-hunting cone snails, Science 230 (1985) 1338–1343.
[29] T. Soeki, M. Kitani, K. Kusunose, et al., Renoprotective and antioxidant effects of cilnidipine in hypertensive patients, Hypertens. Res. 35 (2012) 1058–1062.
[30] K. Hishikawa, O. Takase, M. Idei, et al., Comparison of antioxidant activity of cilnidipine and amlodipine, Kidney Int. 76 (2009) 230–231.
[31] S. Aritomi, K. Niinuma, T. Ogawa, et al., Additive effects of cilnidipine and angiotensin II receptor blocker in preventing the progression of diabetic nephropathy in diabetic spontaneously hypertensive rats, Clin. Exp. Nephrol. 17 (2013) 41–50.
[32] K. Hayashi, S. Wakino, N. Sugano, et al., Ca^{2+} channel subtypes and pharmacology in the kidney, Circ. Res. 100 (2007) 342–353.
[33] R.G. Carroll, T.E. Lohmeier, A.J. Brown, Chronic angiotensin II infusion decreases renal norepinephrine overflow in conscious dogs, Hypertension 6 (1984) 675–681.
[34] M. Bräter, S.N. Li, I.J. Gorodezkaya, et al., Voltage-sensitive Ca^{2+} channels, intracellular Ca^{2+} stores and Ca^{2+}-release-activated Ca^{2+} channels contribute to the ATP-induced $[Ca^{2+}]_i$ increase in differentiated neuroblastoma $\times$ glioma NG 108-15 cells, Neurosci. Lett. 264 (1999) 97–100.
[35] I.T. Mak, W.B. Weglicki, Comparative antioxidant activities of propranolol, nifedipine, verapamil, and diltiazem against sarcolemmal membrane lipid peroxidation, Circ. Res. 66 (1990) 1449–1452.
[36] M. Takatsu, T. Hattori, T. Murase, et al., Comparison of the effects of cilnidipine and amlodipine on cardiac remodeling and diastolic dysfunction in Dahl salt-sensitive rats, J. Hypertens. 30 (2012) 1845–1855.