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Study on the anti-hypertensive mechanism of hesperidin in spontaneously hypertensive rats and vascular endothelial cells

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Study on the anti-hypertensive mechanism of hesperidin in spontaneously hypertensive rats and vascular endothelial cells

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Abbreviations

- ACE, angiotensin-converting enzyme
- ACh, acetylcholine
- AGT, angiotensinogen
- AKT, serine/threonine-protein kinases
- Ang I, angiotensin I
- Ang II, angiotensin II
- ARBs, angiotensin receptor blockers
- AT_1R, Ang II type 1 receptor
- AT_2R, Ang II type 2 receptor
- BK_2R, bradykinin B2 receptor
- BW, body weight
- CaMKII, Ca^{2+}/calmodulin-dependent kinase II
- cAMP, cyclic adenosine monophosphate
- CAP, capsaicin
- cGMP, cyclic guanosine monophosphate
- CPZ, capsazepine
- CVDs, cardiovascular diseases
- DBP, diastolic blood pressure
- EGCG, epigallocatechin-3-*O*-gallate
- eNOS, endothelial NO synthase
- ER, estrogen receptor
- HBSS, Hanks' Balanced Salt solution
- HD, hesperidin
- HO-1, heme oxygenase 1
- HR, heart rate
- HUVECs, human umbilical vein endothelial cells
- L-NMMA, N^G-methyl-L-arginine acetate salt
- MasR, Mas receptor
- NO, nitric oxide
- Nrf2, nuclear factor erythroid-2-related factor 2
- p38 MAPK, p38 mitogen-activated protein kinase
- PE, phenylephrine
- PGI2, prostacyclin
- PKA, protein kinase A
- RAS, renin-angiotensin system
- RIPA, radio immunoprecipitation assay
- ROS, reactive oxygen species
- SBP, systolic blood pressure
- SEM, standard error of the mean
- SHRs, spontaneously hypertensive rats
- siRNA, silencing RNA
- TRPV1, transient receptor potential vanilloid 1
- 2K-1C, two-kidney, one-clipped

Chapter I

Introduction

Hypertension, also called high blood pressure, is a chronic medical disorder that the blood pressure in the arteries is persistently higher than normal [1], which is diagnosed as the systolic blood pressure (SBP) ≥140 millimeters mercury (mmHg) and/or the diastolic blood pressure (DBP) ≥90 mmHg [2]. Hypertension has been the most prevalent in both developed and developing countries in the past decades. Estimates suggested that the global number of people with hypertension was 0.97 billion (26.4% of adults) in 2000, increased to 1.39 billion (31.1% of adults) in 2010, and likely raised to 1.56 billion (29.0% of adults) by 2025 (Fig. 1-1) [3,4]. Hypertension is one of the most critical risk factors for cardiovascular diseases (CVDs) that are the first cause of death in the world [5], including stroke, coronary artery disease, heart failure, atrial fibrillation, and peripheral arterial disease [6]. Consequently, hypertension is a major worldwide public-health challenge and has been identified as the leading contributor to mortality and disability-adjusted life years lost [3]. Such high prevalence of hypertension exacts a tremendous global public health burden.

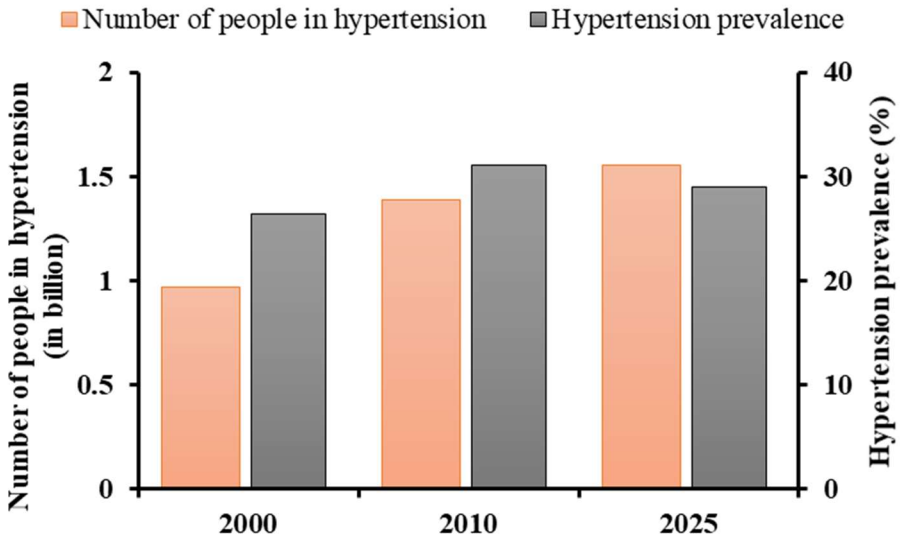

Fig. 1-1 Global estimates of the prevalence of hypertension in adults aged ≥20 years since 2000 [3,4]

The development of hypertension is usually closely associated with several risk factors, including unhealthy diets (excessive dietary sodium, diet high in saturated fats and trans fats, low intake of fruits and vegetables), physical inactivity, excess alcohol consumption, and obesity [7]. Diet and lifestyle modification are approaches to preventing the onset of this pathology. Several epidemiological studies suggested that diet plays an essential role in preventing and managing hypertension [8–10]. Some dietary patterns such as the Mediterranean diet, the dietary approach to stop hypertension diet, the Nordic diet, and the Lacto-ovo vegetarian diet have been reported to be beneficial for lowering blood pressure in patients with hypertension [11,12]. A common characteristic of these dietary patterns is a high intake of plant-derived foods containing high naturally occurring polyphenols, which have been investigated extensively over the last decades to explain the potential anti-hypertensive effects of the above-mentioned diets.

Naturally occurring polyphenols are a group of compounds characterized by their structures having at least two phenyl rings found in natural products, including fruits, vegetables, cereals, and beverages [13]. Naturally occurring polyphenols are secondary metabolites that protect plants against diseases, infections, and damages [14]. The general chemical structure of polyphenols is characterized by phenolic rings and the interconnection of these rings. Based on their structural characteristics, polyphenols can be classified into flavonoids (further divided into flavanones, flavones, isoflavones, flavonols, flavanonols,

flavan-3-ols, anthocyanidins, and chalcones), and nonflavonoids, comprising phenolic acids, stilbenes, lignans (Fig. 1-2) [15]. Over 10,000 polyphenol compounds have been identified from plant-based foods consumed daily in human diets [16]. Many animal, human and epidemiologic studies have revealed that consumption of polyphenols has been proven to exert numerous human health benefits, *e.g.*, preventing the development of cardiovascular diseases and hypertension by antioxidant and free radical scavenging potentials as well as anti-inflammatory action [17]. For instance, it has been reported that consumption of epigallocatechin-3-*O*-gallate (EGCG), a major polyphenol in green tea, attenuated hypertension *via* exerting anti-oxidant, anti-inflammatory action in Dahl salt-sensitive rats [18]. A double-blind, randomized clinical trial demonstrated that quercetin, widely distributed in foods such as onions, apples, and tea, could significantly reduce systolic blood pressure [19] and provide protection against CVDs [20]. Consumption of naringin, a flavanone, could exert an anti-hypertensive effect [21] in human studies. These naturally occurring polyphenols have attracted emerging interest due to their health beneficial physiological potentials in controlling blood pressure.

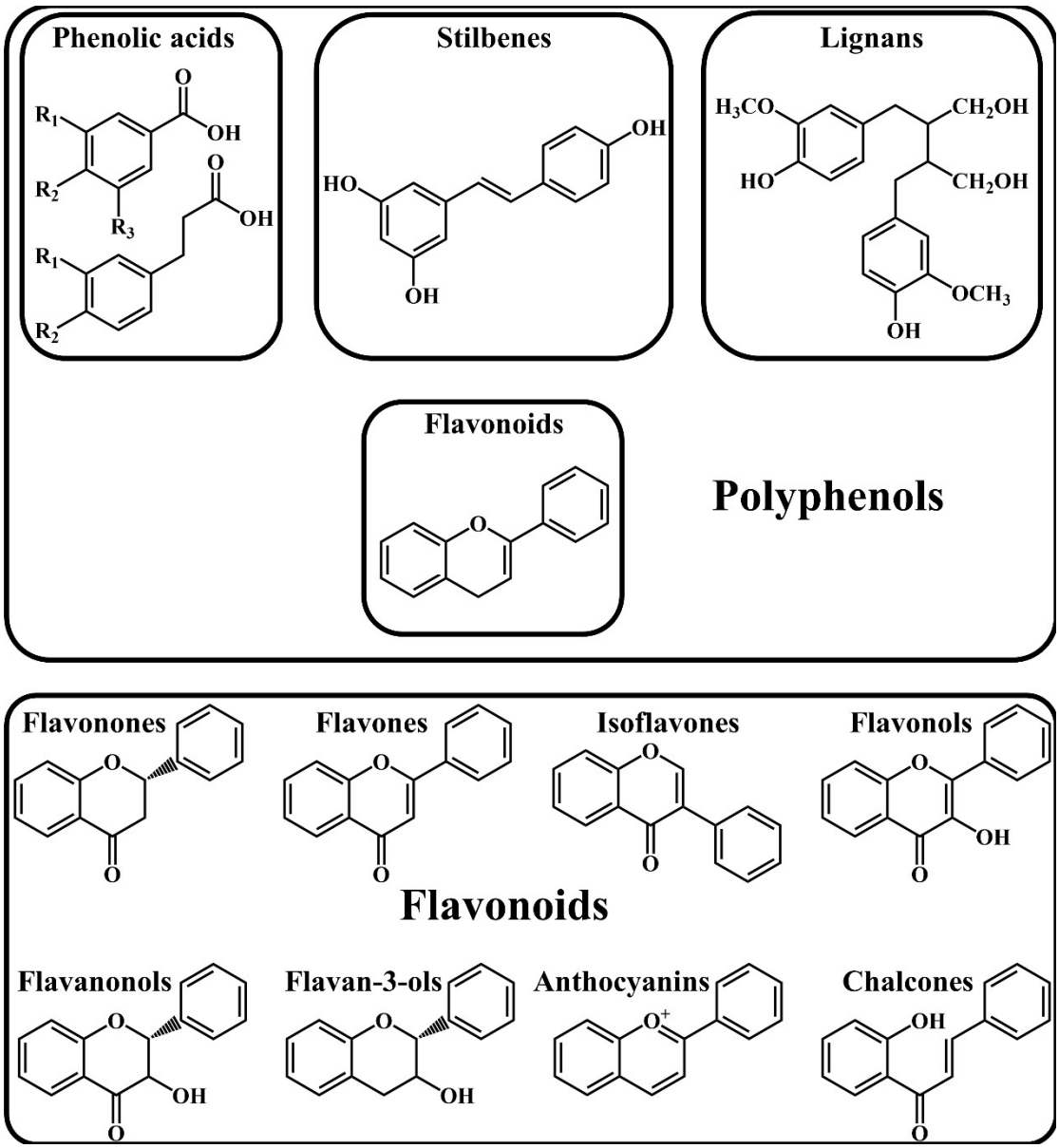

Fig. 1-2 Schematic classification of the main naturally-occurring polyphenols and their chemical structures.

Polyphenols are classified into four principal classes: phenolic acids, stilbenes, lignans, and flavonoids. Flavonoids are further divided into flavanones, flavones, isoflavones, flavonols, flavanonols, flavan-3-ols, anthocyanidins, and chalcones.

Among these naturally occurring polyphenols, hesperidin (4′-methoxy-7-*O*-rutinosyl-3′,5-dihydroxyflavanone; hesperetin-7-*O*-rutinoside), the glycoside form of hesperetin (Fig. 1-3), is a naturally occurring flavanone glycoside abundantly found in citrus fruits [22]. It was first isolated from the peel of orange by the French chemist Lebreton in 1828 [23], and is the predominant polyphenol consumed from citrus fruits and juices [24]. In a research on polyphenol consumption in Finland, hesperidin was shown to be the most highly consumed flavonoid, with a consumption of 28.3 mg/day, approximately 30% of the total flavonoid consumption [25].

Diverse physiological functions of hesperidin have been reported so far, including anti-hypertensive, cardioprotective, anti-diabetic, anti-tumor, neuroprotective activity, antioxidant potentials, and anti-inflammatory action [26]. The health benefits of hesperidin reported in the literature are listed in Table 1-1.

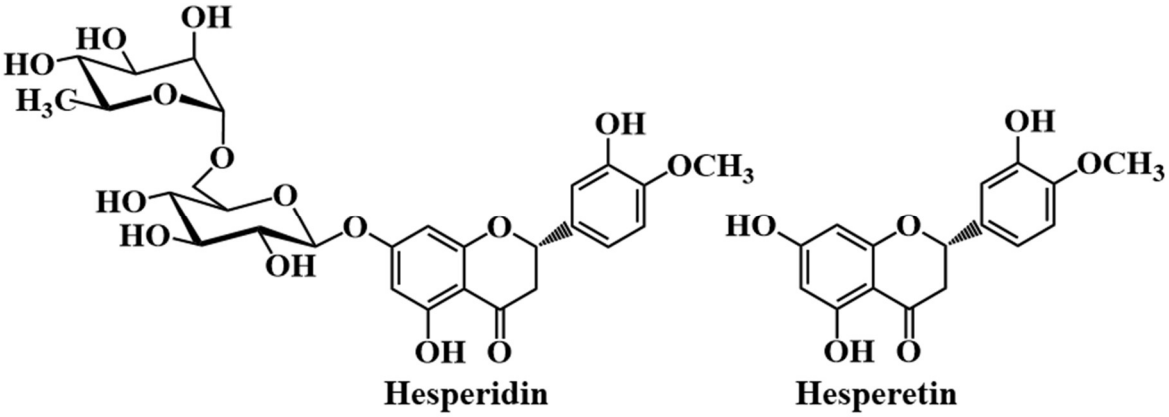

Fig. 1-3 Chemical structures of hesperidin and hesperetin.

Table 1-1 Health benefits of hesperidin *in vivo* and *in vitro*.

health claim	compound	cell /animal	mechanism	reference
anti-hypertensive	hesperidin	bovine aortic endothelial cells (BAEC)	Activated nitric oxide (NO) production	[27]
	hesperidin/glucosyl hesperidin	spontaneously hypertensive rats (SHRs)/ Wistar-Kyoto rats	Increased NO bioavailability	[28,29]
	hesperidin	two-kidney, one-clipped (2K-1C) hypertensive rats	Suppressed oxidative stress markers and NADPH oxidase over-expression	[30]
	hesperidin	healthy volunteers	Increased endothelium-dependent microvascular reactivity	[31]
	hesperidin	mildly hypertensive volunteers/ healthy volunteers	Reduced the systolic blood pressures (SBP)/decreased the arterial velocity pulse index	[32,33]
anti-diabetic	hesperidin/ hesperetin	L6 myoblasts (rat skeletal muscle cell)	Stimulated glucose uptake *via* upregulation of glucose transporter 4	[34]
	hesperidin	alloxan and high fat diet-induced SD rat	Prevented insulin resistance	[35]
	hesperidin	streptozotocin-induced SD rats	Ameliorated pancreatic β-cell dysfunction and apoptosis by *via* inhibition of oxidative stress and endoplasmic reticulum stress	[36]
anti-tumor	hesperidin	HepG2 cells	Induced apoptosis *via* both mitochondrial and death receptor pathways	[37]
	hesperidin	A549 and NCI-H358 cells	Inhibited caspase-3 and decreased mitochondrial membrane potential	[38]
	hesperidin	Diethylnitrosamine/CCl_4-induced rats	Inhibited hepatocarcinogenesis by activating nuclear factor erythroid-2-related factor 2 (Nrf2)/heme oxygenase 1 (HO-1) and peroxisome proliferator activated receptor gamma pathways	[39]
	hesperidin	Thioacetamide-induced hepatocarcinoma in Rats	Involvement of Wnt signaling pathways	[40]

Table 1-1 Health benefits of hesperidin *in vivo* and *in vitro* (continued).

health claim	compound	cell /animal	mechanism	reference
neuroprotective	hesperidin	SH-SY5Y cells (Human neuroblastoma cell)	Activated the Nrf2/HO-1 pathways	[41,42]
	hesperidin	Parkinson's disease mice induced by 6-hydroxydopamine	Inhibited reactive oxygen species (ROS) formation and glutathione reductase activity	[43]
	hesperidin	APP/PS1–21 mouse model of Alzheimer's Disease	Suppressed β-amyloid deposition, microglial activity, and transforming growth factor beta immunoreactivity	[44]
anti-oxidative	hesperidin	*in vitro*/yeast cells	Free radical scavenging activity and cellular antioxidative activity	[45]
	hesperidin	Human red blood cells	Protected cellular membrane from oxidative damage	[46]
	hesperidin	Wistar rats	Upregulated the activity of antioxidants and Nrf2	[47]
anti-inflammatory	hesperidin	murine macrophage RAW264.7 cell	Inhibited cyclooxygenase and nitric oxide synthase	[48,49]
	hesperidin	HMC-1 cell (human mast cell)	Inhibited hypoxia-inducible factor-1a	[50]
	hesperidin	Ovalbumin-induced asthma (mice)	Suppressed Th2 cytokines and GATA-binding protein 3	[51,52]

Regarding the anti-hypertensive effect, hesperidin was initially named "Vitamin P" due to its preventive effect against CVDs by enhancing capillary resistance [53]. In the past decade, numerous *in vivo* and *in vitro* studies have demonstrated the anti-hypertensive effect of hesperidin. Ohtsuki *et al.*, Yamamoto *et al.*, and Ikemura *et al.* have found that oral administration of hesperidin induced a significant reduction of SBP in spontaneously hypertensive rats (SHRs) [28,29,54]. Wunpathe *et al.* revealed that hesperidin reduced blood pressure in a dose-dependent manner in two-kidney, one-clipped (2K-1C) hypertensive rats [30]. Moreover, a randomized, controlled, cross-over clinical trial demonstrated that the consumption of orange juice, or its major flavonoid hesperidin, led to a significantly lower DBP in healthy overweight individuals [31]. Additionally, it has been demonstrated that hesperidin can cross the intestinal membrane as an intact form and enter the portal blood system with a significant absorption (C_{max}, 49 nM mg/dose) after a single oral administration of hesperidin in Sprague-Dawley rats [55].

However, like other polyphenols, hesperidin presents a poor *in vivo* bioavailability due to its low aqueous solubility [56]. Taking all consideration, we developed a new fermented Mikan tea, made with third crop green tea (*Camellia sinensis*) leaves and unripe Satsuma Mandarin (*Citrus unshiu*) fruits. The fermented Mikan tea enhanced about 6-fold more hesperidin solubility and improved the absorption of hesperidin *in vivo* than that of hesperidin dissolved in water [57,58]. It was reported that natural products derived from tea, like EGCG,

theaflavin-3,3-digallate, and theasinensin A, could lead to a ca. 3-fold increase in the solubility of hesperidin *via* the formation of a complex through π–π and/or hydrophobic interactions between hesperidin and catechins (e.g., theaflavins and theasinensins) in the mikan tea [59,60]. It has been demonstrated that intake of the fermented Mikan tea, containing hesperidin, caused a significant 8 mmHg SBP reduction in mildly hypertensive volunteers (36.7 mg hesperidin/day, 12 weeks) [32] and improved the impaired vascular function with a significant reduction in arterial velocity pulse index in healthy volunteers (53.8 mg hesperidin/day, 8 weeks) [33]. These implied that hesperidin in the Mikan tea might exert anti-hypertensive effects *in vivo*.

Blood pressure is the force of circulating blood pushing against the walls of blood vessels. It is primarily determined by the circulating blood volume and systemic vascular resistance. Changes in circulating blood volume would change the quantity of blood within the system, affecting blood pressure. For instance, severe dehydration or excessive bleeding (decreasing circulating blood volume) would reduce blood pressure, and sodium and water reabsorption would increase the circulating blood volume and then increase blood pressure. Systemic vascular resistance refers to the resistance of the systemic vasculature that is used to create blood pressure [61]. Vascular resistance plays a central role in the pathophysiology of hypertension [62]. It is well known that the renin-angiotensin system (RAS) controls vascular resistance and plays a fundamental role in regulating blood pressure and cardiovascular function [63]. The components of

the RAS are shown in Fig. 1-4. When the blood pressure in the arteriole falls, juxtaglomerular cells in the kidneys cleave the precursor prorenin into renin and secrete it into circulation. Plasma renin converts angiotensinogen (AGT) produced in the liver to angiotensin I (Ang I, [Ang-(1–10)]). Then, Ang I is cleaved to generate angiotensin II (Ang II, [Ang-(1–8)]) by angiotensin-converting enzyme (ACE) localized in the vascular endothelium of different organs. Ang II, the most critical effector peptide hormone of the RAS, acts on specific receptors to regulate the blood pressure. When Ang II binds to Ang II type 1 receptor (AT_1R), it causes blood vessels to narrow (vasoconstriction), aldosterone secretion, reabsorption of Na^+ and water (increasing circulating blood volume), oxidative stress, and endothelial dysfunction, resulting in increased blood pressure. The ACE/Ang II/AT_1R axis is the classical RAS axis. Ang II can also activate Ang II type 2 receptor (AT_2R), and can be hydrolyzed by ACE2 to generate the Ang (1-7) that acts *via* Mas receptor (MasR) to counteract these effects, lowering blood pressure. These two axes (the AngII/ AT_2R axis and the ACE2/Ang (1-7)/MasR axis) are the alternative RAS axes [64].

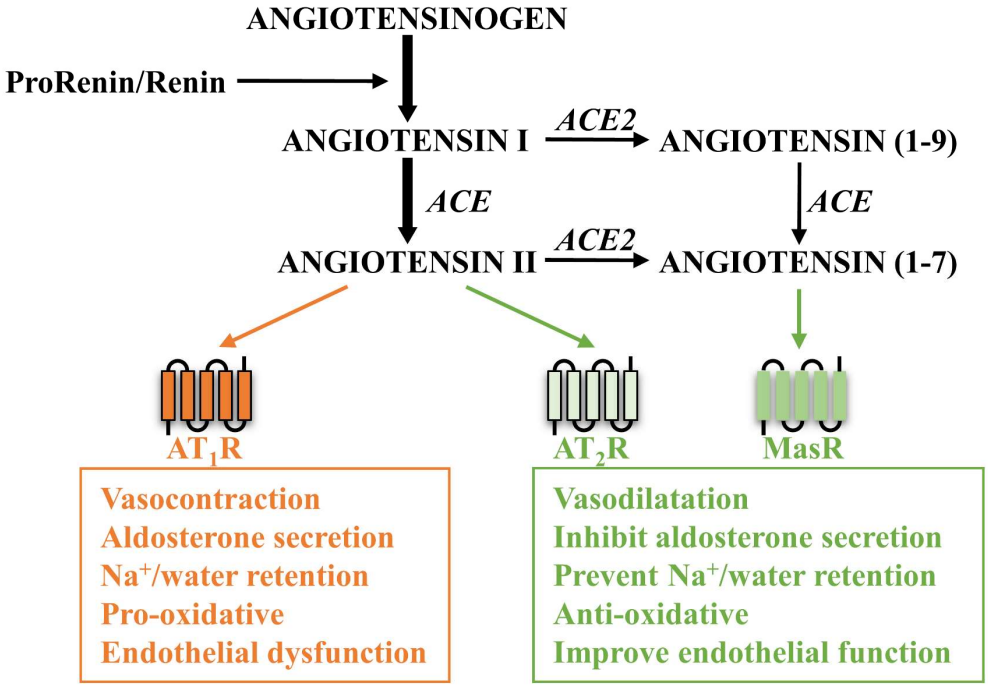

Fig. 1-4 The renin–angiotensin system (RAS).

Abbreviations: ACE, angiotensin converting enzyme; ACE2, angiotensin converting enzyme 2; AT_1R, angiotensin type 1 receptor; AT_2R, angiotensin type 2 receptor; MasR, Mas receptor.

Nowadays, a strategy for hypertension treatment is to target RAS, such as ACE inhibitors (captopril and enalapril) and angiotensin receptor blockers (valsartan and losartan). However, it should be noted that the long-term application of these drugs may cause side effects such as headaches, dizziness, constipation, coughing, and angioneurotic edema [65]. There is, thus, a growing interest in the preventive potential of alternative medicinal foods or naturally occurring polyphenols with anti-hypertensive effects by, *e.g.*, inhibiting renin activity, inhibiting ACE activity, blockade of angiotensin receptor, or activation of ACE2/Ang (1-7)/MasR. Four possible strategies for the prevention of hypertension are introduced as shown below.

Strategies for the prevention of hypertension:

Inhibition of renin

Renin, a 335-amino acid protease polypeptide, specifically converts angiotensinogen to generate Ang I, which is the first and rate-limiting step in RAS [66]. It has been identified as a vasoconstrictor, a major factor in the pathogenesis of hypertension. Thus, food-derived inhibitors or natural accruing flavonoids would be expected to achieve the optimal inhibition of renin, like therapeutic drugs (*e.g.*, Aliskiren) [67,68]. So far, it has been reported that various food-derived peptides have exhibited *in vitro* renin inhibitory activity and induced anti-hypertensive effects in SHRs and even in hypertensive humans

[69,70]. Moreover, some natural products, including soyasaponin I, oleic acid, linoleic acid, (-)-kaur16-en-19-oic acid [71], catechins [72], and baicalin [73], also exerted renin inhibitory activity and improved hypertension *in vivo* [73,74]. These indicated the promising prospect of discovering naturally occurring polyphenols as candidates for renin inhibitors in controlling blood pressure.

Inhibition of ACE

ACE is a carboxypeptidase that plays a vital role in regulating blood pressure by catalyzing the conversion of inactive Ang I into the potent vasoconstrictor Ang II. Too much Ang II can lead to excessive fluid retention and ultimately raise blood pressure [75]. ACE has become an important potential pharmacological target for preventing and treating hypertension. Thus, the ACE inhibitor drugs, such as captopril, enalapril, and lisinopril, have been prescribed for hypertension in clinical practice. However, the long-term application of these ACE inhibitor drugs could initiate adverse side effects like dizziness, coughing, and angioneurotic edema [65]. As their high efficacy and low risk of side effects, natural products have recently gained growing attention as potential ACE inhibitors. Natural food protein-derived peptides, such as peptides from soybean [76], eggs, milk [77], meat, and fish [78], have been reported to exhibit ACE inhibitory activity *in vitro* and *in vivo* studies.

Moreover, the natural accruing flavonoids, *e.g.*, isoquercitrin [79], quercetin

[80], and kaempferol [81] have also exerted ACE inhibitory activity and their potential as drug candidates *in vitro* and *in vivo* studies. Their inhibition of ACE activity depends on double bond C2=C3, 4′-*O*-methoxylation, 4-carbonyl group, and 3′-hydroxylation [82].

Angiotensin II receptor blockers

Similar to ACE inhibitors that prevent the formation of Ang II, angiotensin receptor blockers (ARBs) block the binding of Ang II to AT_1R on blood vessels, thereby inhibiting the vasoconstriction actions of Ang II [83]. ARBs are one of two medications commonly used to control high blood pressure, treat heart failure, and prevent kidney failure in people with diabetes. Valsartan, losartan, and irbesartan, a class of this drug family, have been used to treat high blood pressure and heart failure. ARBs can be a therapeutic approach to preventing hypertension [84]. The components with AT_1R inhibitory activity have also been found in natural products over the past decades. Fernández-Musoles *et al*. revealed that lactoferrin-derived peptides selectively inhibit angiotensin II-induced vasoconstriction by blocking AT_1R [85]. Laskar *et al*. demonstrated *in silico* that the phytochemicals (morin and orotic acid) might be ARBs against cardiovascular diseases [86]. Lukiati *et al*. also predicted the potency of the phenolic compounds (10-gingerol, 8-gingerol, 6-gingerol) from red ginger as AT_1R antagonists through molecular docking [87]. Although the research on

natural products as ARBs has just started, they have already indicated that natural products as ARBs would be a promising approach for preventing hypertension.

Activation of ACE2/Ang (1-7)/MasR

Except for ACE inhibitors and AT_1R blockers, activation of the ACE2/Ang (1-7)/MasR axis has been identified as a novel strategy for the treatment of hypertension [88]. Ang (1-7) cleaved from Ang II by ACE2, binds to the MasR, then activates endothelial NO synthase (eNOS) *via* serine/threonine-protein kinases (AKT) to produce nitric oxide (NO) and enhances the phospholipase A2 activity to produce prostacyclin (PGI2). NO stimulates soluble guanylate cyclase to increase intracellular cyclic guanosine monophosphate (cGMP), and PGI2 stimulates adenylate cyclase to increase intracellular cyclic adenosine monophosphate (cAMP) in vascular smooth muscle cells. Both cGMP and cAMP indirectly reduce intracellular calcium levels, resulting in smooth muscle relaxation [89]. Activation of ACE2 causes a decrease in Ang II level and increases the formation of Ang (1-7), thus eliciting the reduction of blood pressure [88]. ACE2, Ang (1-7), and MasR play vital roles in regulating blood pressure that counteracts the ACE/Ang II/AT_1R axis [90]. Some drugs that act as ACE2 activators (diminazene aceturate, xanthone, and NCP-2454) [91] or MasR agonists (AVE0991 and CGEN-856S) [88] have been developed for the prevention of hypertension. It has been reported that some natural compounds could activate

the ACE2/Ang (1-7)/MasR axis *via* increasing ACE2 expression, Ang (1-7) levels, and MasR expression *in vivo*, such as egg-derived tripeptide Ile-Arg-Trp [92], rapeseed protein-derived peptides, LY, RALP, and GHS [93], and resveratrol [94]. These suggested that the activation of ACE2/Ang (1-7)/ MasR must also be a promising strategy for prevention of hypertension.

According to the aforementioned research background, naturally occurring polyphenols or food components may elicit anti-hypertensive physiological potential *via* inhibiting renin activity, inhibiting ACE activity, blockade of angiotensin receptor, or activation of ACE2/Ang (1-7)/MasR. Additionally, recent studies have revealed that transient receptor potential vanilloid type 1 (TRPV1), a ligand-gated cationic channel, plays a vital role in regulating cardiovascular functions and the pathogenesis of cardiovascular diseases *via* releasing NO, neuropeptide substance P, and calcitonin gene-related peptide [95,96]. Some dietary phytochemicals, such as EGCG [97] and sesamin [98], have been reported to activate TRPV1, trigger Ca^{2+} influx, and enhance the phosphorylation of Ca^{2+}/calmodulin-dependent kinase II (CaMKII)/eNOS and NO generation, leading to preventing the endothelial dysfunction in endothelial cells. These indicated that naturally occurring polyphenols or food components might exert an anti-hypertensive effect *via* the TRPV1- Ca^{2+} signaling pathway.

Thus far, it has been established that hesperidin suppressed RAS cascades, such as the reduction of ACE activity, Ang II levels, and AT_1R expression, then inhibited the RAS cascades-mediated NADPH oxidase overexpression and the reactive oxygen species (ROS) generation, finally restored the decreased NO bioavailability induced by excessive ROS and reduced the blood pressure in hypertensive rats [30]. Other studies also found that the hypotensive effect of hesperidin involved increasing NO production and reducing NADPH oxidase expression in rat blood vessels [29,54,99]. Although a lot of studies on hesperidin anti-hypertensive effects have been performed, the underlying mechanisms of hesperidin action in vessels are not fully elucidated yet.

Thus, this study aimed to investigate the anti-hypertensive effects of hesperidin and the underlying mechanisms giving rise to the anti-hypertensive effect by focusing on the RAS in animal and cell experiments using SHRs and human umbilical vein endothelial cells (HUVECs), respectively. The objectives for each Chapter in this study are described as follows:

1) In **Chapter II**, in order to clarify the anti-hypertensive effects of hesperidin and hesperidin-containing Mikan tea *in vivo*, hesperidin and hesperidin-containing Mikan tea were administrated for 20 weeks in SHRs. The underlying mechanisms of the anti-hypertensive effect were investigated by focusing on the circulating and local RAS. It was found that hesperidin can cause a significant ($P < 0.05$) reduction in the systolic blood pressure (SBP) and ameliorate the impaired vascular function in 28-week-old SHRs. The intake of

hesperidin induced a significant upregulation of MasR expression in the aorta, but not influenced any other factors related to the circulating and local RAS, suggesting that the anti-hypertensive effect of hesperidin was involved in vessel regulation *via* the activation of the aortic MasR axis.

2) In **Chapter III**, since the daily intake of hesperidin resulted in an elevated aortic MasR expression in SHRs (**Chapter II**), the mechanism underlying hesperidin-stimulated aortic MasR expression was investigated using HUVECs. We also examined the structure-activity relationship of flavonoids to determine the structural factors required to enhance MasR expression using hesperidin analogs. It showed that hesperidin activated TRPV1, leading to activating two signaling axes, CaMKII/p38 mitogen-activated protein kinase (p38 MAPK) /MasR expression and CaMKII/eNOS/NO production in HUVECs. TRPV1-initiated MasR and NO augmentation in the HUVECs by hesperidin indicated a novel vasorelaxant potential of hesperidin. Furthermore, the structure-activity relationship analysis demonstrated that the twisted B ring in the flavonoid skeleton with an OH group at the 3' position was crucial for the agonistic TRPV1 binding.

Taken together, the present study has demonstrated for the first time that hesperidin exerted an anti-hypertensive effect by the activation of the aortic MasR axis *via* binding to TRPV1. It will provide new insights into the mechanism of the anti-hypertensive effect of hesperidin.

Chapter II

Anti-hypertensive effect of hesperidin in spontaneously hypertensive rats

1. Introduction

Polyphenols are naturally occurring micronutrients in plant-based foods, which have been extensively investigated due to their numerous health benefits to humans, including preventing the development of CVDs and hypertension through their antioxidant properties, free radical scavenging potentials, and anti-inflammatory action [17]. However, some naturally occurring polyphenols, such as hesperidin, showed a low bioavailability due to poor aqueous solubility. Thus, we developed a hesperidin-containing fermented Mikan tea, consisting of unripe

satsuma mandarin (*Citrus unshiu*) fruit and third-crop green tea (*Camellia sinensis*) leaves [57], which improved the solubilization of hesperidin in this Mikan tea due to the formation of stable complexes with the condensed catechins in the fermented tea [100]. Additionally, daily intake of the Mikan tea (36.7 mg hesperidin/day, 12 weeks) significantly reduced the elevated SBP in mildly hypertensive volunteers (ΔSBP, ca. 8 mmHg) [32] and improved the impaired vascular function with a significant reduction in arterial velocity pulse index in healthy volunteers (53.8 mg hesperidin/day, 8 weeks) [33]. These findings suggested that hesperidin in the Mikan tea might exhibit an anti-hypertensive effect *via* the regulation of vessel functions.

RAS is thought to be closely associated with the onset of hypertension. The components of the RAS, such as Ang II, AT_1R, AT_2R, and MasR, play a fundamental role in the regulation of blood volume and systemic vascular resistance. Thus far, hesperidin has been reported to reduce the blood pressure in SHRs [28] and humans [31] *via* lowering the elevated vascular resistance by promoting NO production [30] or inhibiting NADPH oxidase [101]. However, the physiological mechanism of Mikan tea or hesperidin action in vessels has not been elucidated. Thus, in **this Chapter**, the physiological mechanism of Mikan tea or hesperidin action in vessels was investigated. The anti-hypertensive effects of long-term intake of hesperidin or hesperidin-containing Mikan tea were evaluated using SHRs. The underlying mechanisms of their anti-hypertensive effect by focusing on the circulating and local RAS were investigated.

2. Materials and methods

2.1. Materials

Mikan tea was prepared according to our previous report [102]. Briefly, fresh third-crop green tea (*Camellia sinensis*) leaves were dried by blowing air at 70 °C for 20 min in a primary tea-rolling dryer (60k-type; Kawasaki Co.). Then, the leaves and sliced unripe satsuma mandarin (*Citrus unshiu*) fruit were mixed in a ratio of 3:1, followed by kneading with a tea roller (60k-type, Kawasaki Co.) at 26 °C for 20 min. Finally, the mixture was heated for 30 min at 110 °C in a tea dryer (120k-2 type; Kawasaki Co.) to terminate enzymatic oxidation. For the administration experiments, a hot-water extract of Mikan tea was employed, in which 1 L of hot water was added to 20 g of Mikan tea product, and the tea was stirred for 10 min. The extract powder (5.6 g) was obtained by lyophilizing the supernatant, which contained 62.7 mg of hesperidin. Hesperidin (H1673; Lot, 2594109) was obtained from Funakoshi Co. (Tokyo, Japan). Phenylephrine (PE) and acetylcholine (ACh) were purchased from Wako Pure Chemical Ind. (Osaka, Japan). Anti-AT_1R primary antibodies (ab124505, Lot: GR3188953-21) and anti-AT_2R primary antibodies (ab92445, Lot: GR109798-31) were sourced from Abcam (Cambridge, MA, USA). Anti-MasR primary antibody (AAR-013, Lot: AAR013AN1102) was purchased from Alomone Labs (Jerusalem, Israel). Rat Ang (1-7) ELISA kit (MBS731540, Lot: 20191216C) was obtained from My Biosource (San Diego, CA, USA). cAMP ELISA kit (581002, Lot: 0580287) was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). NO Assay-

FX (fluorometric) kit (NK08, Lot: ND826) was sourced from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). Ang II-EIA kit (ADI-900-204, Lot: 06041801C) was purchased from Enzo Life Sciences, Inc. (Farmingdale, NY, USA). All other chemicals were of analytical grade and were used without further purification.

2.2. Animal experiments

Seven-week-old male SHRs (SHR/Izm, Disease Model Cooperative Research Association, Shizuoka, Japan) were fed on a laboratory diet (CRF-1 diet, Charles River Co., Kanagawa, Japan) and provided with distilled water *ad libitum*. SHR is the most commonly used animal model of essential (or primary) hypertension and cardiovascular disease. Okamoto and colleagues produced the SHR strain during the 1960s by selective inbreeding of Wistar–Kyoto (WKY) rats with high blood pressure [103]. All rats were individually housed for one week at 21 ± 1 °C and 55.5 ± 5% humidity under controlled lighting from 8:30 to 20:30. The doses of hesperidin and mikan tea were chosen based on previous studies about absorption and metabolic behavior [55,58]. 8-week-old SHRs were randomly assigned into three groups (n = 4/group): control group, saline solution; hesperidin group, 50 mg/kg of hesperidin dissolved in a saline solution containing 0.5% carboxymethyl cellulose (50 mg hesperidin/kg/day); Mikan group, 50 mg/kg of Mikan tea extract (containing 0.56 mg hesperidin/kg/day)

dissolved in saline solution. Oral gavage administration of samples was performed daily for 20 weeks. Food intake and body weight (BW) were measured every week during the experimental period. At 8, 10, 12, 14, 16, 20, 23, and 28 weeks of age, SBP/DBP and heart rate (HR) were measured successively five times by using a non-invasive tail-cuff microsensor device (model MK-2000A; Muromachi Kikai, Tokyo, Japan) without warming the rats after the oral gavage. An average of three measurements was recorded for each animal after eliminating the highest and lowest blood pressure measurements. All animal experiments were in accordance with the Guidance for Animal Experiments by the Faculty of Agriculture and the Graduate Course of Kyushu University under the Law (no. 105, 1973) and notification (no. 6, 1980, of the Prime Minister's Office and no.71, 2006, of the Ministry of Health, Labor and Welfare) of the Japanese Government. The experimental design was reviewed and approved by the Animal Care and Use Committee of Kyushu University (permit number: A30-015-2).

2.3. Measurement of vasomotor response in isolated aortic rings

After the oral gavage, all rats were fasted for 16 h and then anesthetized using sevoflurane (Maruishi Pharmaceutical Co., Ltd, Osaka, Japan) at 28 weeks of age. Blood was collected from the abdominal aorta in EDTA-2Na blood collection tubes and centrifuged at 3,500 × *g* for 15 min at 4 °C to obtain plasma

for further biochemical measurements. The thoracic aorta was obtained for vasomotor response experiments, and the aorta rings were prepared according to our previous study [104]. Briefly, the thoracic aorta from euthanized rats was carefully excised and placed in a dissecting chamber filled with physiological salt solution buffer (PSS, pH 7.4, composition in mM: NaCl 145, KCl 5, Na_2HPO_4 1, $CaCl_2$ 2.5, $MgSO_4$ 0.5, glucose 10, and HEPES 5) at 37 °C to remove connective and adipose tissue. The vessels were then sliced into 2-mm-long rings. The rings were mounted on stainless steel hooks within a 5-mL organ bath filled with PSS buffer with 95 % O_2/5% CO_2 gas at 37 °C. The rings were stretched until resting tension reached 2 g and allowed to equilibrate for 45 min. After equilibration, contractions were assayed with the addition of 1 mM PE for 20 min. Relaxation was evaluated by the addition of 100 μM ACh. The tissue response (isometric tension, in g) was assayed by using a force transducer (Micro Tissue Organ Bath, Model MTOB-1Z; Labo Support, Osaka, Japan) and a recorder (4-channel amplifier; EMKA Technologies, Paris, France). The ratio of reduced tension by ACh to increased tension by PE was used to evaluate the relative vasomotor response (contraction/relaxation potential) of a given ring [104].

2.4. Biochemical measurements

The levels of plasma Ang II, Ang (1-7), and NO were measured using their corresponding assay kits, according to the manufacturer's instructions. The

thoracic aorta was homogenized with a Polytron homogenizer (25,000 rpm, 20 s, 10 repetitions, 4 °C; Bohemia, NY, USA), and the arterial cAMP in the thoracic aorta was measured with the cAMP ELISA kit according to the manufacturer's instruction. All measurements were performed with a FlexStation 3 microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2.5. Measurement of protein expression by a Wes analysis

The thoracic aorta was used to measure protein levels of AT_1R, AT_2R, and MasR in the aorta by using a capillary electrophoretic-based immunoassay Wes instrument (ProteinSimple Co., San Jose, CA, USA). The aorta was homogenized with a microtube homogenizer (Bohemia, NY, USA) in a cold lysis buffer containing PBS with 0.1% Triton X-100, 1% NP-40, 1% protease inhibitor cocktail (Nacalai Tesque Co., Kyoto, Japan) and 1% PhosSTOP (Roche, Indianapolis, IN, USA). The homogenate was centrifuged at 16,000 × *g* for 15 min at 4 ºC, and the supernatant was subjected to the Wes measurement. The Wes measurements were performed using a 12–230 kDa separation module (8 × 25 mm capillary cartridge, ProteinSimple Co.) according to our previous paper [105]. The samples were diluted with a 0.1 × sample diluent buffer and 5 × fluorescent master mix denaturing buffer to obtain a 0.5 μg/μL (AT_1R and AT_2R, assays) or 1 μg/μL (MasR assay) loading concentration, and then heated for denaturation at 95 °C for 5 min. Primary antibodies for the targeted proteins

AT_1R, AT_2R, and MasR were diluted at 1:100. The Wes reagents (biotinylated ladder and primary antibodies) were dispensed into a microplate and subjected to the Wes automated capillary electrophoresis, followed by automated immune detection using a horseradish peroxidase-conjugated anti-rabbit secondary antibody and a chemiluminescent substrate. A pentafluorophenyl ester-biotin labeling reagent that could attach to the applied proteins was used for total protein detection. The resulting chemiluminescent signal was displayed as a virtual blot-like image, and the electropherogram was based on the molecular weight using the Compass software (ProteinSimple Co.). Protein expression of AT_1R, AT_2R, and MasR was normalized to the electropherogram peak areas of total protein applied to each lane. The data were expressed as ratios against the control.

2.6. Statistical analysis

Results were expressed as the mean ± standard error of the mean (SEM). All analyses were conducted with GraphPad Prism software (La Jolla, CA, USA). Statistical differences between the two groups were performed by using Student's *t*-test. A P value < 0.05 was considered significant.

3. Results and discussion

3.1. Effects of hesperidin or Mikan tea intake on SHRs

BW, food intake, SBP, and HR of SHRs with long-term hesperidin or Mikan tea intake for 20 weeks are shown in Fig. 2-1 and summarized in Table 2-1. During the experiment period, the intake of hesperidin and Mikan tea did not show significant differences in body weight and food intake compared to the control group, suggesting that the intake of hesperidin or Mikan tea at a dose of 50 mg/kg did not affect the growth parameters of SHRs (Fig. 2-1A&B). As shown in Fig. 2-1C, the intake of hesperidin and Mikan tea tended to suppress the elevation of SBP in SHRs from 8 to 23 weeks of age. At the end of the protocol (28 weeks of age), a significantly low value in SBP was observed in the hesperidin (ΔSBP: 62 mmHg, $P < 0.05$) and Mikan (ΔSBP: 61 mmHg, $P < 0.01$) groups, compared to the control group (Table 2-1). During the experimental period, no significant difference in HR was observed for all groups (Fig. 2-1D), suggesting that the observed SBP reduction did not involve the nervous system [106].

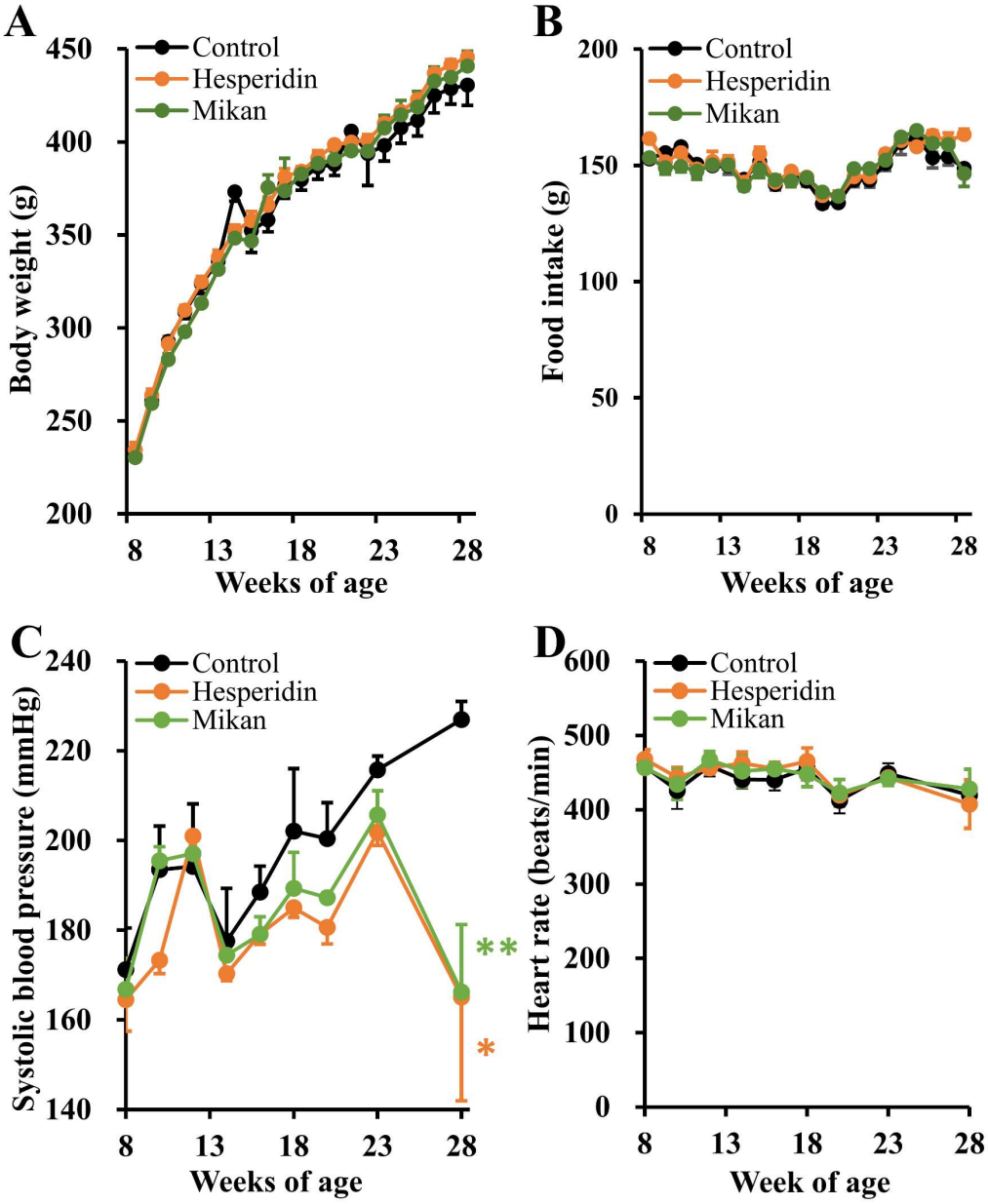

Fig. 2-1 Changes in body weight (A), food intake (B), systolic blood pressure (C), and heart rate (D) of SHRs induced by the intake of hesperidin and Mikan tea.

8-week-old male SHRs were administered hesperidin (50 mg/kg) or Mikan tea (50 mg/kg) daily for 20 weeks. Values are expressed as the mean ± SEM (n = 4). *P < 0.05, **P < 0.01 compared to the control by the Student's t-test.

Table 2-1 Changes in body weight, food intake, heart rate (HR), and blood pressure (SBP and DBP) in SHRs treated with hesperidin, Mikan tea, and saline solution (Control) during the 20-week protocol.

	0-week			20-week		
	Control	**Hesperidin**	**Mikan**	**Control**	**Hesperidin**	**Mikan**
Body weight (g)	231±3	235±3	230±3	431±12	446±0	441±9
Food intake (g/week)	153±2	162±5	153±3	149±10	163±11	147±8
HR (beats/min)	458±9	468±13	457±4	420±13	408±33	428±26
SBP (mmHg)	171±11	172±10	168±6	227±4	165±23*	166±15**
DBP (mmHg)	114±12	124±4	106±3	147±11	135±15	116±11

8-week-old male SHRs were daily administered saline solution (Control), 50 mg/kg of hesperidin (Hesperidin), or 50 mg/kg of Mikan tea (Mikan) for 20 weeks. Values are expressed as the mean ± SEM (n = 4). *P < 0.05, **P < 0.01 compared to the control group by the Student's t-test.

3.2. Effect of hesperidin or Mikan tea intake on the vasomotor response of the isolated thoracic aorta

The effect of hesperidin or mikan tea intake on maximal contraction/relaxation responses of the thoracic aorta against vaso-agonists was investigated with 1 mM PE- and subsequently 100 μM ACh-stimulation in 28-week-old SHRs (Fig. 2-2A). As shown in Fig. 2-2B, intake of hesperidin or Mikan tea did not cause a significant difference in the PE-induced contraction response compared with that of control, whereas intake of hesperidin tended to a higher response in ACh-induced relaxation of the aorta (tension: 0.050 ± 0.005 g, $P = 0.19$ *vs*. control) than that of the control group (tension: 0.034 ± 0.08 g) (Fig. 2-2C). Taking the resulting vasomotor profile (Fig. 2-2D), a higher contraction/relaxation response was observed in the 20-week daily intake of hesperidin (0.88 ± 0.05, $P < 0.05$) or Mikan tea (0.87 ± 0.03, $P < 0.05$) compared to that of the control group (0.55 ± 0.06). It has been reported that the contraction/relaxation response was ca. 0.4 in 40-week SHRs and ca. 0.7 in normal rats [104]. Higher contraction/relaxation response suggested that both hesperidin and hesperidin-containing Mikan tea could improve the impaired vasomotor response in SHRs.

Our previous human studies revealed that the intake of Mikan tea rich in hesperidin could cause a significant 8 mmHg SBP reduction (36.7 mg hesperidin/day, 12 weeks) in mildly hypertensive volunteers [32] and improved the impaired vascular function with a significant decrease in the arterial velocity

pulse index (53.8 mg hesperidin/day, 8 weeks) in healthy volunteers [33]. However, studies on the anti-hypertensive mechanism of candidate hesperidin present in Mikan tea have not been conducted. In the present study, long-term intake of hesperidin (50 mg/kg), or Mikan tea (50 mg/kg) containing 0.56 mg/kg of hesperidin, exerted a significant anti-hypertensive effect in SHRs, which significantly suppressed the elevation of SBP (Fig. 2-1C) and ameliorated the impaired vasomotor tone (i.e., contraction/relaxation response) of SHRs (Fig. 2-2D). Other natural phytochemicals, such as EGCG [107], quercetin [108], and (-)-epicatechin [109], have also exhibited the blood pressure-lowering effects in SHR experiments. However, the magnitude of hesperidin-induced SBP reduction (ca. 60 mmHg/50 mg dose) in this SHR study (Table 2-1) was higher than that those of the above-mentioned phytochemicals, taking their dosage into account (*e.g.*, ΔSBP of EGCG, ca. 50 mmHg/200 mg dose [107]; ΔSBP of quercetin, ca. 20 mmHg/20 mg dose [108]; ΔSBP of (-)-epicatechin, ca. 23 mmHg/250 mg dose [109]). These suggested that hesperidin, an active candidate molecule in Mikan tea, may exert more potent anti-hypertensive effects among the reported anti-hypertensive phytochemicals. However, due to the low content of hesperidin in Mikan tea (0.56 mg/kg in 50 mg-Mikan tea/kg), we cannot rule out the involvement of other compounds in the anti-hypertensive effect of Mikan tea (Table 2-1), and further research is warranted to clarify the candidates responsible for the effect in terms of their synergic and combinatorial action.

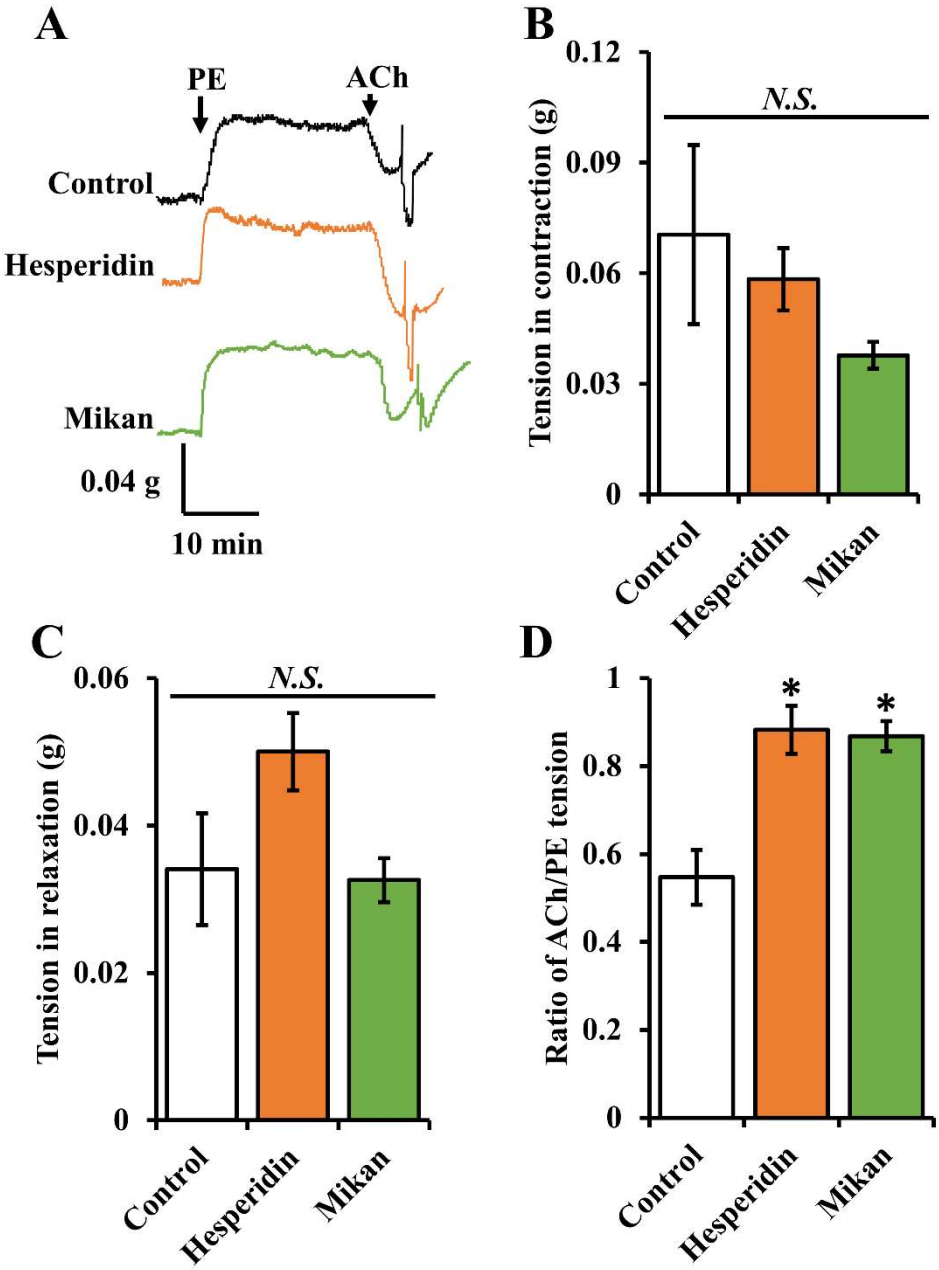

Fig. 2-2 Effects of 20-week daily intake of hesperidin and Mikan tea on the vasomotor response of thoracic aorta rings from 28-week-old SHRs.

(A) Representative traces were recorded during PE (1 mM)-induced contraction and subsequent ACh (100 μM)-induced relaxation of the aorta rings. Contraction (B) and relaxation (C) tensions were measured for aorta rings taken from each administration group. The ratio of reduced tension by ACh to increased tension

by PE was used for the vasomotor response index (D). Values are expressed as the mean ± SEM (n = 4). *P < 0.05 compared to the control by the Student's t-test. *N.S.*, no significance.

3.3. Effects of hesperidin or Mikan tea intake on plasma Ang II, Ang (1-7), NO, and arterial cAMP in SHRs

Considering the RAS plays a pivotal role in blood pressure regulation [110], and vessel resistance determines blood pressure elevation [111], the levels of circulating RAS-related metabolites, Ang II [a pressor hormone [112]], and Ang (1-7) [a depressor hormone [113]], as well as that of vasorelaxation-related NO [114], were evaluated in 28-week-old SHRs plasma; vasorelaxation factor cAMP levels were also investigated in aorta tissues [115]. There were no significant differences in plasma Ang II and Ang (1-7) levels between the hesperidin or Mikan groups and the control group (Table 2-2) after 20 weeks of administration (28 weeks of age). Hesperidin intake did not affect the levels of the plasma pressor, Ang II, as an index of ACE activity [116], nor the level of the plasma depressor, Ang (1-7), as an index of ACE2 activity [117], suggesting their anti-hypertensive effect (Fig. 2-1) was not due to suppressing circulating RAS components or the inhibitory action of ACE.

It has been reported that oxidative stress caused endothelial dysfunction and was involved in the development of cardiovascular diseases primarily due to the oxidative inactivation of NO by excessive ROS [118]. The lack of fluctuation in plasma NO levels in the hesperidin or Mikan groups indicated that the anti-hypertensive effect was not due to antioxidant action against oxidative stress in the vascular endothelium. In contrast to the present findings, previous animal investigations have suggested that RAS downregulation and/or endothelial

oxidative stress-related cascades are involved in hesperidin's anti-hypertensive mechanism in 2K-1C hypertensive Sprague-Dawley rats [30] or glycosylated hesperidin in SHRs [29]. The contradiction between the proposed anti-hypertensive mechanism and those of the aforementioned reports might be related to the differences in rat species or glycosylated hesperidin. In addition, it has been reported that the lowing blood pressure effects of glucuronized hesperetin, a possible hesperidin metabolite, differs from that of its conjugated metabolites in SHRs [101]. These allow another speculation that the anti-hypertensive action of hesperidin may be influenced by its metabolites. In our previous report [55], hesperidin can be absorbed and produce a variety of metabolic conjugates, such as homoeriodictyol and eriodictyol, in their sulfated and glucuronized forms after oral administration of hesperidin (10 mg/kg) in SD rats.

At last, the cAMP levels in thoracic aorta of 28-week-old SHRs tended to increase in the hesperidin group (P = 0.30), which allows some speculation regarding the involvement of hesperidin in the cAMP/protein kinase A (PKA) vasorelaxation axis [119]. cAMP binds to cAMP-dependent protein kinase A (PKA) [120], and the activation of PKA promotes relaxation *via* reducing the intracellular Ca^{2+} [89]. Mikan tea did not cause the increasing tendency in the cAMP might be due to its low hesperidin concentration or other compounds in the Mikan tea.

Table 2-2 Effect of hesperidin and Mikan tea on plasma levels of angiotensin II (Ang II), Ang (1-7), and NO, and arterial cAMP levels in 28-week-old SHRs.

	Control	Hesperidin	Mikan
Plasma Ang II (pmol/mL)	0.043 ± 0.003	0.034 ± 0.003	0.061 ± 0.021
Plasma Ang (1-7) (pmol/mL)	1.8 ± 0.1	1.8 ± 0.1	1.7 ± 0.1
Arterial cAMP (pmol/mg protein)	91.1 ± 18.4	178.7 ± 58.1	71.0 ± 19.4
Plasma NO (nmol/mL)	17.6 ± 0.5	15.4 ± 0.9	18.7 ± 3.9

Values are expressed as the mean ± SEM ($n = 4$). No significant differences were obtained at $P < 0.05$ by the Student's *t*-test.

3.4. Effects of hesperidin or Mikan tea intake on AT_1R, AT_2R, and MasR expression in SHRs

To further investigate the anti-hypertensive mechanism of hesperidin, Wes analyses of AT_1R, AT_2R, and MasR expression in the thoracic aorta were conducted after the 20-week daily intake of hesperidin or Mikan tea in SHRs. As shown in Fig. 2-3A&B, the hesperidin and Mikan groups with a higher vasomotor response (Fig. 2-2D) did not affect the expression of AT_1R and AT_2R (Fig 2-3A&B) and the ratio of AT_1R/AT_2R (data not shown) in the aorta compared to the control group. In contrast, levels of MasR expression, a G-protein-coupled receptor for the vasodilation factor [88], were significantly upregulated (hesperidin group: 295.6 ± 40.5 % of control, Mikan group: 242.2 ± 31.5 % of control) in the aorta of hesperidin- or Mikan tea-administered SHRs (Fig. 2-3C), suggesting that hesperidin exerts its physiological activity *via* aorta relaxation by aortic MasR activation.

As far as we know, this is the first finding that the observed hesperidin-induced anti-hypertensive action was related to the upregulation of MasR protein expression in vascular tissues. MasR, as a critical component of the Ang (1-7)/MasR axis, plays a vital role in hypertension *via* vasorelaxation cascade to counterbalancing vasoconstriction [121]. It has been reported that MasR expression is involved in the establishment and progression of cardiac diseases [122]. Recent studies revealed that Mas receptor knockout mice showed severe cardiac and renal dysfunction [123], leading to increased blood pressure [124]. As

discussed in **Chapter I**, some natural compounds, such as egg-derived tripeptide Ile-Arg-Trp [92], rapeseed protein-derived peptides, LY, RALP, and GHS [93], and resveratrol [94], could activate the ACE2/Ang (1-7)/MasR axis *via* increasing ACE2 expression, Ang (1-7) levels, and MasR expression *in vivo*. Increasing MasR expression by these compounds might be attributed to their activating effect on ACE2 and Ang (1-7), because Ang (1-7), as a MasR agonist, could increase the MasR expression [125]. Given that hesperidin intake did not increase in plasma Ang (1-7) levels (Table 2-2) and the upregulation of the MasR axis was related to p38 MAPK [125], we speculated that hesperidin might upregulate MasR expression *via* p38 MAPK rather than *via* the Ang (1-7) produced by ACE2 activation. However, the precise mechanisms, such as whether hesperidin is an agonist of MasR and how hesperidin upregulates MasR expression, will be further investigated in **Chapter III**.

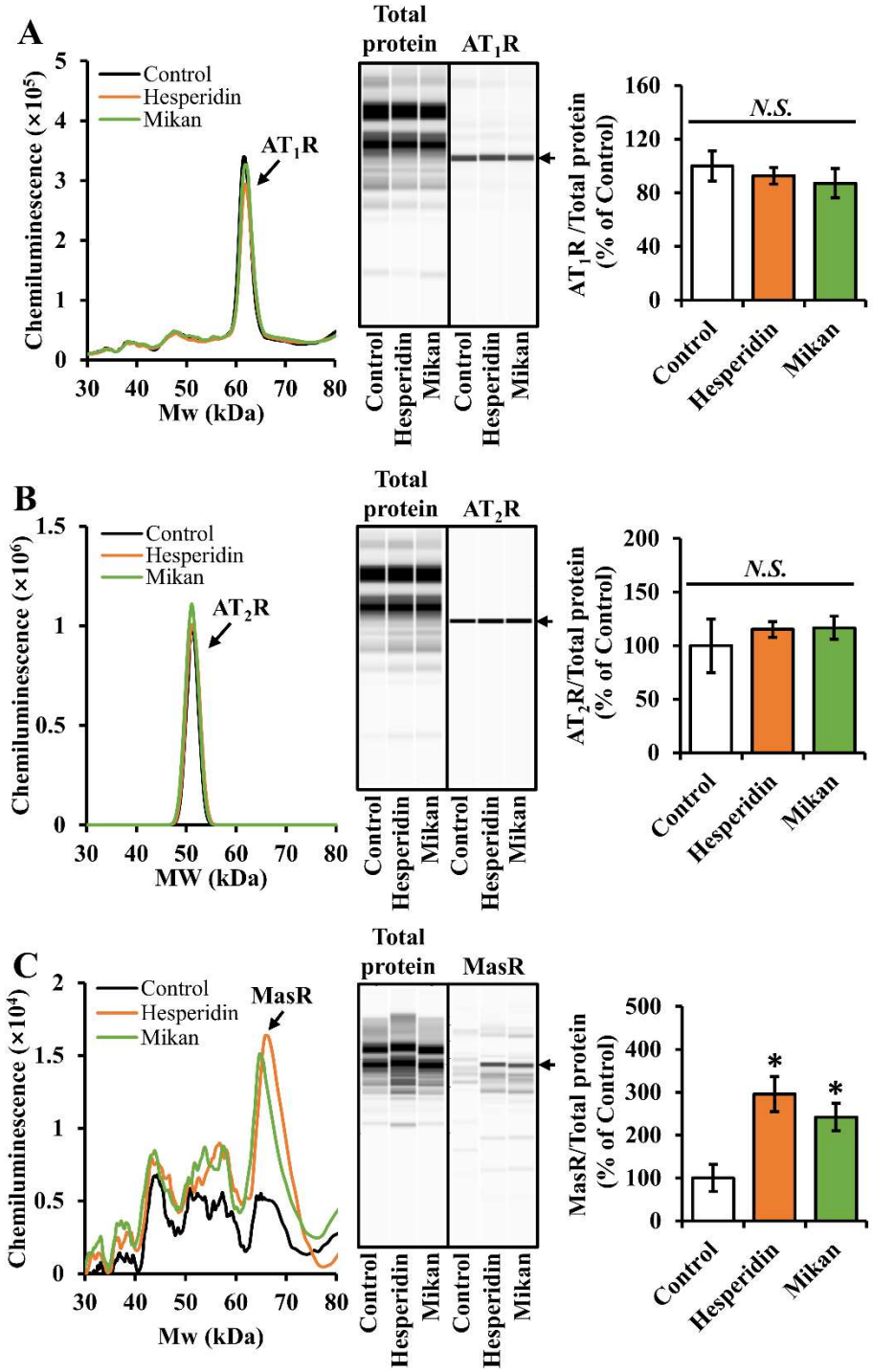

Fig. 2-3 Protein expression of AT_1R (A), AT_2R (B), and MasR (C) in the thoracic aorta of 28-week-old Mikan tea- or hesperidin-administered SHRs.

Thoracic aortas taken from Mikan tea- or hesperidin-administered SHRs were subjected to Wes analysis. The chemiluminescent signal is displayed as a virtual

blot-like image and an electropherogram was generated based on the molecular weight. Protein expressions of AT_1R (A), AT_2R (B), and MasR (C) were normalized by the electropherogram peak area of the applied total protein in each lane. Values are expressed as the mean ± SEM (n = 4). Statistical differences between the hesperidin group (or Mikan group) and the control group were evaluated by the Student's t-test. *$P < 0.05$ compared to the control. *N.S.*, no significance.

4. Summary

The present study investigated the anti-hypertensive effect of long-term intake of hesperidin and hesperidin-containing Mikan tea in 8-week-old SHRs, respectively, each at a dose of 50 mg/kg/day for 20 weeks. At the end of the protocol, the intake of either hesperidin or Mikan tea resulted in a significant ($P < 0.05$) reduction in SBP of approximately 60 mmHg, together with an amelioration of the impaired vasomotor response in the thoracic aorta. The aorta and blood taken from 28-wk SHRs also revealed that pressor Ang II and AT_1R, and depressor Ang (1-7), AT_2R, and NO levels were not influenced by hesperidin or Mikan tea intake, while MasR expression in the aorta was significantly upregulated by hesperidin or Mikan tea group. It suggested that the anti-hypertensive effect of hesperidin or Mikan tea was closely associated with the activation of the aortic MasR vasorelaxation axis (Fig. 2-4). The precise mechanisms of hesperidin on MasR activation will be further investigated in the next **Chapter III**.

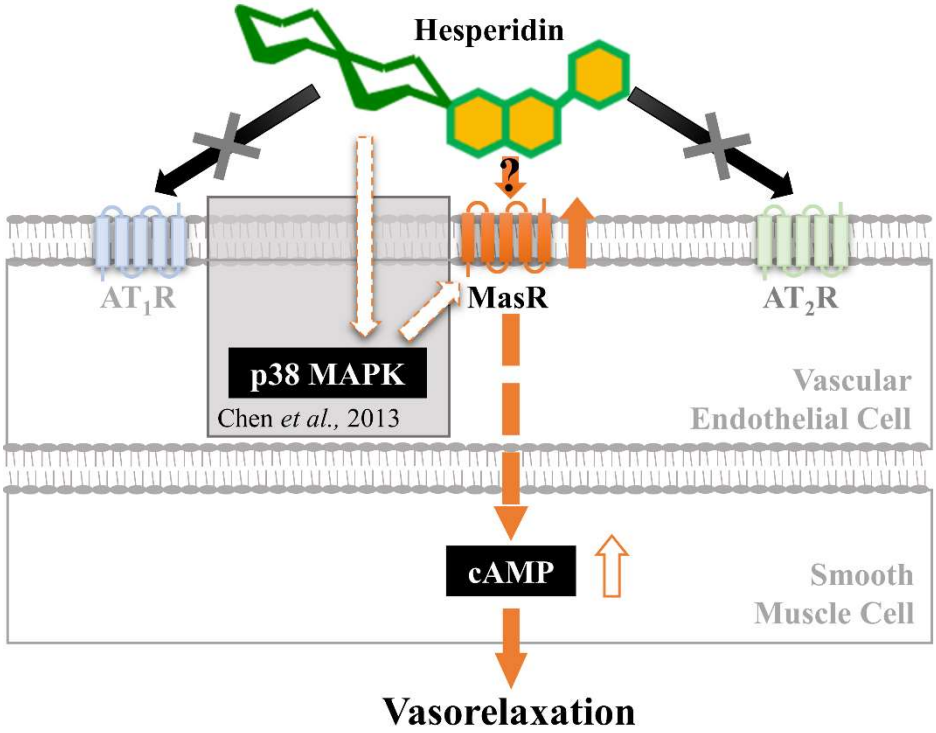

Fig. 2-4 Proposed mechanism of anti-hypertensive effect of hesperidin in SHR

Chapter III

Mechanism of hesperidin-induced MasR expression in human umbilical vein endothelial cells

1. Introduction

As shown in Table 1-1, hesperidin (hesperetin-7-*O*-rutinoside), a flavanone naturally occurring in citrus peels, has been demonstrated to exert diverse health benefits, including neuroprotective [126], antidiabetic [35], and anti-hypertensive effects [32,33,127,128]. Particularly in anti-hypertensive effects, the effect of the daily intake of hesperidin has been proved in animal [28,127] and human studies [32,33,128]. Our previous human studies also demonstrated that intake of hesperidin caused a significant 8 mmHg SBP reduction in mildly hypertensive volunteers (36.7 mg

hesperidin/day, 12 weeks) [32] and improved the impaired vascular function with a significant reduction in arterial velocity pulse index in healthy volunteers (53.8 mg/day, 8 weeks) [33]. **Chapter II** revealed that long-term hesperidin intake resulted in a significant blood pressure-lowering effect and an alleviation in the impaired vasomotor response of the abdominal aorta in SHRs. The investigation on vasomotor-related receptors revealed that hesperidin significantly upregulated the MasR rather than $AT_{1/2}R$, indicating that the hesperidin-induced anti-hypertensive effect is involved in the activation of the aortic MasR expression in SHRs. However, the mechanism(s) of elevated MasR expression in SHR aorta by hesperidin remains unclear. As shown in **Chapter I**, the physiological potency of MasR was exhibited by its stimulation of endogenous ligand Ang (1-7) in the local RAS of the vessel, kidneys, and brain [129]. MasR, as well as AT_2R, is a triggering receptor for depressor action in the system *via* activating the vasorelaxant NO/cGMP pathway to reduce vessel resistance [130,131].

Thus, in **Chapter III**, the mechanism underlying hesperidin-stimulated aortic MasR expression was investigated using HUVECs. We also investigated the structure-activity relationship of flavonoids to determine the structural factors required to induce MasR expression using hesperidin analogs.

2. Material and methods

2.1. Materials

Hesperidin (H1673; Lot, 2594109) was obtained from LKT Laboratories Inc (St. Paul, MN, USA). ICI 182,780 (ab120131; Lot, APN07104-1-1) was purchased from Abcam (Cambridge, MA, USA). D-Ala^7-Ang (1-7) or A-779 (SML-1370; Lot, 075M4712V); hesperetin (H4125; Lot, 041M1567); PD-123319 (P186; Lot, 124K4616); and SB202190 (S7067; Lot, 0000033308) were sourced from Sigma-Aldrich Co (St. Louis, MO, USA). N^G-methyl-L-arginine acetate salt (L-NMMA, N411; Lot, SN043) was purchased from Dojindo Molecular Technologies, Inc (Kumamoto, Japan). Icatibant acetate (S5695; Lot, S569501) was obtained from Selleck Chemicals (Houston, TX, USA). Capsazepine (037-23171; Lot, SAN5408), Capsaicin (034-11351; Lot, SAP2930), and KN62 (114-0063; Lot, PDK5579) were sourced from Fujifilm Wako Pure Chemicals Co (Tokyo, Japan). Ang (1-7) (17594; Lot, 0572344-4) was purchased from Cayman Chemical Co (Ann Arbor, MI, USA). Quercetin (P0042; Lot, GM01) was obtained from Tokyo Chemical Industry Co (Tokyo, Japan). Luteolin (ASB-00012461-010; Lot, 12461-215) and homoeriodictyol (ASB-00008311-010; Lot, 00008311-606) were obtained from Chromadex (Irvine, CA, USA). Eriodictyol (0056; Lot, 98081304) and taxifolin (1036; Lot, 08111206) were sourced from Extrasynthese (Geney, France). Naringenin (102430; Lot, 9784K) was purchased from MP Biomedicals (Solon, OH, USA). A NO Assay-FX (fluorometric) kit (NK08; Lot, SX600) was obtained from

Dojindo Molecular Technologies. An endpoint Calcium assay kit (36312; Lot, 2781382) was obtained from AAT Bioquest (Sunnyvale, CA, USA). An anti-MasR primary antibody (AAR-013; Lot, AAR013AN1102) was sourced from Alomone Labs (Jerusalem, Israel). An anti-AT_1R primary antibody (ab124505; Lot, GR3188953-21) and anti-AT_2R primary antibody (ab92445; Lot, GR109798-31) were purchased from Abcam (Cambridge, MA, USA). An anti-TRPV1 primary antibody (PA1-29421; Lot, WG3323715G) was sourced from Thermo Fisher Scientific Inc (Cambridge, MA, USA). An anti-eNOS primary antibody (32027S; Lot, 3), anti-p-eNOS (Ser1177) primary antibody (9571S; Lot, 14), anti-p38 MAPK primary antibody (9212S; Lot, 26), and anti-p-p38 MAPK (Thr180/Tyr182) primary antibody (9211S; Lot, 25) were purchased from Cell Signaling Technology (Danvers, MA, USA). All other chemicals were of analytical grade and were used without further purification.

2.2. Cell culture

HUVECs were purchased from Lonza Japan Ltd (C2519A, HUVEC-Umbil Vein; Lot, 18TL075837) (Tokyo, Japan), which were authenticated by short tandem repeats profiling and confirmed mycoplasma-free. HUVECs were cultured in an EGM-2 medium (CC-3162, Lonza Co, Basel, Switzerland) using a Bullet kit containing growth factors (bovine brain extract, ascorbic acid, hydrocortisone, human epidermal growth factor, fetal bovine serum, and

gentamicin/amphotericin-B) (CC-4176, Lonza Co) at 37 °C in a humidified atmosphere (5% CO2 and 95% O2). The cells at the passage of 3–6 were used for all experiments.

2.3. Detection of NO production and intracellular Ca^{2+} levels

For NO measurement, the cells at a density of 5×10^4 cells/well were seeded into a 24-well plate and incubated for 24 h. After removing the medium, the cells were rinsed twice with phosphate-buffered saline (PBS). Then the cells were treated with hesperidin (0.1 μM, 1 μM, or 10 μM), capsaicin (0.1 μM, 1 μM, or 10 μM), 1 μM hesperidin analogs, or 1 μM Ang (1-7) in a phenol red-free EBM-2 medium (CC-3129, Lonza Co) for 2 h. For time-course experiments, the HUVECs were incubated with 1 μM hesperidin for 0.5, 1, 2, 4, 8, or 12 h at 37 °C. For inhibitor experiments, the HUVECs were treated with 1 μM hesperidin in the presence or absence of either A-779 (MasR antagonist [132], 1 μM); ICI 182780 (estrogen receptor (ER) antagonist [133], 1 μM); PD-123319 (AT_2R antagonist [134], 1 μM); icatibant (bradykinin B_2 receptor (BK_2R) antagonist [135], 1 μM); capsazepine (TRPV1 antagonist [136], 10 μM); KN-62 (CaMKII inhibitor [137], 10 μM); or L-NMMA (eNOS inhibitor [138], 100 μM) for 2 h. After treatment, the medium was collected and centrifuged at 1,000 × g for 15 min at 4 °C, and the supernatant was evaluated for NO production using the NO assay-FX kit, according to the manufacturer's instruction. For intracellular

Ca^{2+} measurement, HUVECs were seeded at 1.0×10^4 cells/well in 96-well plates. After the 24-h incubation, a Fluo-8E AM dye-loading solution in Hanks' Balanced Salt solution (HBSS) was added and incubated for 1 h at 37 °C. Fluo-8E AM dye-loading medium was replaced with HBSS containing 1 μM hesperidin or 1 μM capsaicin in the presence or absence of 10 μM capsazepine. After the addition of the HBSS solution, fluorescence measurement was performed. All the fluorescence measurements were performed using a microplate reader (Perkin Elmer Life Science, Waltham, MA, USA) at excitation/emission wavelength of 360 nm/460 nm and 490 nm/525 nm for NO and Ca^{2+} measurements, respectively. The NO and Ca^{2+} levels of the sample medium relative to that of the control were used as an index of change in NO production and Ca^{2+} levels.

2.4. AT_1R, AT_2R, MasR, TRPV1, p-eNOS/eNOS, and p-p38 MAPK/p38-MAPK expression analyses in HUVECs

The HUVECs were seeded at 1.5×10^5 cells/well in a 6-well plate and incubated with 1 μM hesperidin, 1 μM capsaicin, or 1 μM Ang (1-7), in the presence or absence of 1 μM Mas receptor antagonist A-799, 10 μM TRPV1 antagonist capsazepine, 10 μM CaMKII specific inhibitor KN-62, or 10 μM p38 MAPK inhibitor SB202190 [139] in phenol red-free EBM-2 for 2 h. After treatment, the cells were rinsed twice with ice-cold PBS. After harvesting by

scraping, the cells were lysed with ice-cold radio immunoprecipitation assay (RIPA) buffer (150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1.0% NP-40, and 50 mM Tris-HCl, pH 8.0) containing 1% protease inhibitor cocktail (Nacalai Tesque Co, Kyoto, Japan) and 1% PhosSTOP (Roche, Indianapolis, IN, USA) for 15 min. The cell lysates were homogenized for 30 s (thrice) at 20,000 rpm at 4 °C with a Polytron homogenizer (Kinematica AG, Luzern, Switzerland) and centrifuged at 14,000 × *g* for 15 min at 4 °C. The supernatant was subjected to a Wes measurement for protein expression analysis. The protein concentration in the supernatant was measured using a Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Inc, Waltham, MA, USA) with bovine serum albumin as a standard.

2.5. TRPV1 siRNA knockdown

The HUVECs were transiently transfected with TRPV1 silencing RNA (siRNA) (sc-36826; Lot, F1821, Santa Cruz, CA, USA) or negative control siRNA (sc-37007; Lot, B2321) (Santa Cruz) using a HiPerFect transfection reagent (301705; Lot, 169032249) (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The HUVECs were separately seeded at 7.0×10^4 cells/well in a 24-well plate for NO production measurement or 2.1×10^5 cells/well in a 6-well plate for protein expression analysis, and the cells were transfected with siRNA at a final concentration of 10 nM for 24 h. Briefly, the

TRPV1 siRNA or negative control siRNA were diluted in EGM-2 medium without serum, then added HiPerFect Transfection reagent. The solution was mixed and incubated at room temperature for 10 min to form transfection complexes. Then the transfection complexes were added dropwise onto the cells. The cells were incubated under normal growth conditions for 3 h. After 3 h incubation, the fresh EGM-2 containing serum was added to the cells. The transfection complexes were incubated with cells for 24 h.

2.6. The Wes analysis

Protein expression levels were determined using a capillary electrophoretic-based immunoassay (Wes instrument; ProteinSimple Co, San Jose, CA, USA) according to the same protocol as described in **Chapter II**. Briefly, the supernatants were diluted with a 0.1 × sample diluent buffer and 5 × fluorescent master mix denaturing buffer (ProteinSimple Co) to obtain a 0.2 μg/μL (AT_1R, AT_2R, TRPV1, p-eNOS/eNOS, and p-p38 MAPK/p38 MAPK assays) or 0.125 μg/μL (MasR assay) loading concentration. Primary antibodies for AT_1R, AT_2R, and MasR proteins were diluted at 1:100, p-eNOS, p-p38 MAPK, p38 MAPK, and TRPV1 were diluted at 1:50, and eNOS was diluted at 1:10. A pentafluorophenyl ester-biotin labeling reagent was applied for total protein detection. At the end of the run, the resulting chemiluminescent signal was displayed as a virtual blot-like image and electropherogram based on the

molecular weight using the Compass software (ProteinSimple Co). The protein expression of AT_1R, AT_2R, MasR, TRPV1, p-eNOS/eNOS, and p-p38 MAPK/p38 MAPK were normalized to the electropherogram peak areas of the total protein applied in each lane, and the results were expressed as ratios against the control.

2.7. Statistical analysis

The results are expressed as the mean ± standard error of the mean (SEM). All analyses were conducted using the GraphPad Prism software (La Jolla, CA, USA). Statistical analysis between two groups was evaluated by using Student's *t*-test. Statistical differences among groups were performed by one-way analysis of variance (ANOVA), followed by Dunnett's test or Tukey–Kramer's test for post-hoc analysis. Statistical significance was set at $P < 0.05$.

3. Results and discussion

3.1. Effects of hesperidin on NO production in HUVECs

In **Chapter II**, it has been revealed that the intake of hesperidin could ameliorate the impaired relaxation/constriction function of vessels *via* the activation of the aortic MasR vasorelaxation axis. Thus, **Chapter III** aims to reveal the underlying mechanism behind that hesperidin activated the aortic

MasR in HUVECs. Generally, MasR activation leads to NO generation [140]. Thus, we firstly investigated whether hesperidin increases MasR-related vasorelaxant NO levels in HUVECs. As shown in Fig. 3-1A&B, hesperidin exhibited a time-dependent and concentration-dependent NO production. Treatment with hesperidin at 1 μM concentration for 2 h caused a significant ($P < 0.01$) ca. two-fold increase in NO level compared to control (Fig. 3-1B). Therefore, further experiments were performed in HUVECs treated with 1 μM hesperidin for 2 h.

However, the intake of hesperidin did not change the plasma NO levels in SHRs (**Chapter II**). Two reasons might contribute to the controversy between *in vitro* and *in vivo* results on NO production. One might be that the plasma NO is not only sourced from endothelial cells, but also from red blood cells [141]. The plasma NO levels might not reflect the effect of hesperidin on the endothelial cells. The second might be that the effect of hesperidin on the plasma NO levels might disappear after fasting for 16 h. However, the finding that hesperidin enhanced NO production in HUVECs suggests that hesperidin may activate MasR and increase NO as a vasorelaxation trigger in endothelial cells.

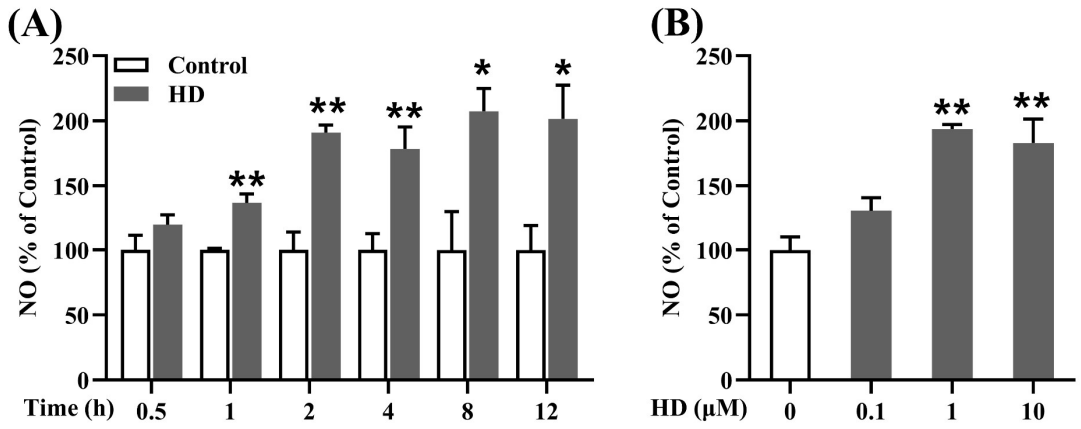

Fig. 3-1 Effect of treatment time (A) and concentration of hesperidin (B) on NO production in HUVECs.

(A) The HUVECs were treated with 1 μM hesperidin up to 12 h. (B)The HUVECs were treated with hesperidin at concentrations of 0.1, 1, or 10 μM for 2 h. Values are expressed as the mean ± SEM (n = 4). Statistical differences were evaluated using Dunnett's test and Student's t-test. *P < 0.05, **P < 0.01, vs. control.

3.2. Effects of hesperidin on Ang-related receptor expression in HUVECs

To further investigate the effect of hesperidin on the receptors regulating NO production, the protein expression of AT_1R, AT_2R, and MasR was also subsequently analyzed by the Wes analysis. As shown in Fig. 3-2, 1 μM hesperidin treatment for 2 h caused a significant increment of MasR expression ($P < 0.05$) in HUVECs, whereas no significant effect on both AT_1R and AT_2R expression was observed. Only MasR expression was upregulated among the aforementioned receptors, which was in line with the result of hesperidin-administered SHRs in **Chapter II**.

MasR is a 37-kDa seven-anchored G-protein coupled receptor found in various organs, including the brain, heart, kidneys, and blood vessels [142]. The preferred ligand of MasR is Ang (1-7), which is generated at the RAS from the hydrolysis of Ang (1-9) by ACE and Ang II by ACE2 [143]. Ang (1-7) plays a critical role in alleviating vascular dysfunction through MasR/NO activation [90]. The onset of cardiac and renal dysfunctions in MasR-knockout mice [123] revealed the essential role of the Ang (1-7)/MasR axis in blood pressure regulation in the RAS. Certain drugs that act as ACE2 activators, or MasR agonists, have been developed to prevent vascular dysfunction and hypertension, such as AVE0991 and CGEN-856S as MasR agonists [88], diminazene aceturate, xanthone, and NCP-2454 as ACE2 activators [91]. It has been reported that some natural compounds could activate the ACE2/Ang (1-7)/MasR axis, such as egg-derived tripeptide Ile-Arg-Trp [92], rapeseed protein-derived peptides, LY, RALP,

and GHS [93], and resveratrol [94] as an ACE2 activator to increase Ang (1-7) levels and MasR expression. In **Chapter II**, it has been revealed that upregulation of MasR by hesperidin is not through the Ang (1-7) produced by ACE2 activation. Further experiments were performed to confirm the mechanisms of the upregulation of MasR by hesperidin.

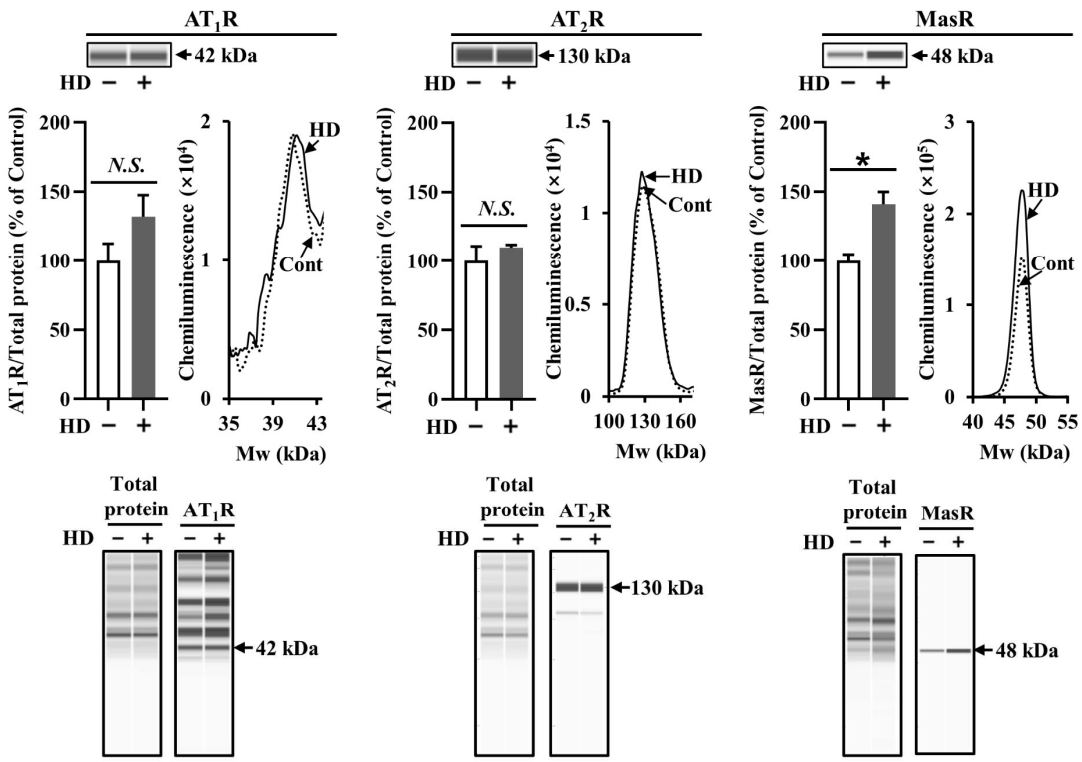

Fig. 3-2 Effects of hesperidin on the protein expression of Ang-related receptor in HUVECs.

The HUVECs were treated with 1 μM hesperidin for 2 h. The Protein expression of AT_1R, AT_2R, and MasR were determined using a Wes analysis. The expression was normalized by the electropherogram peak area of the applied total protein in each lane. The chemiluminescent signal is displayed as a virtual blot-like image, and an electropherogram was generated based on the molecular weight. Results are expressed as the mean ± SEM (n = 3–4). *$P < 0.05$ *vs.* control by Student's *t*-test. *N.S.*, no significance. HD, hesperidin.

3.3. A target receptor for hesperidin-induced NO production/MasR expression in HUVECs

Regarding hesperidin treatment upregulates the MasR expression in HUVECs, further studies were conducted to investigate whether the effect was attributable to the agonistic action of hesperidin on MasR. As shown in Fig. 3-3, the treatment with A-779 (MasR antagonist) did not affect the elevated MasR expression by hesperidin, whereas an elevated MasR expression by Ang (1-7) (an endogenous MasR ligand) [144] was significantly ($P < 0.05$) abolished by A-779. This finding strongly suggested that hesperidin was not an Ang (1-7)-like MasR agonist, and hesperidin did not directly bind to MasR to augment MasR expression. Additionally, the A-779 treatment did not reduce the increased hesperidin-induced NO production (Fig. 3-4), suggesting that hesperidin-induced NO production was not related to the MasR-mediated Akt/NO signaling pathway [145], but relied on an alternative pathway.

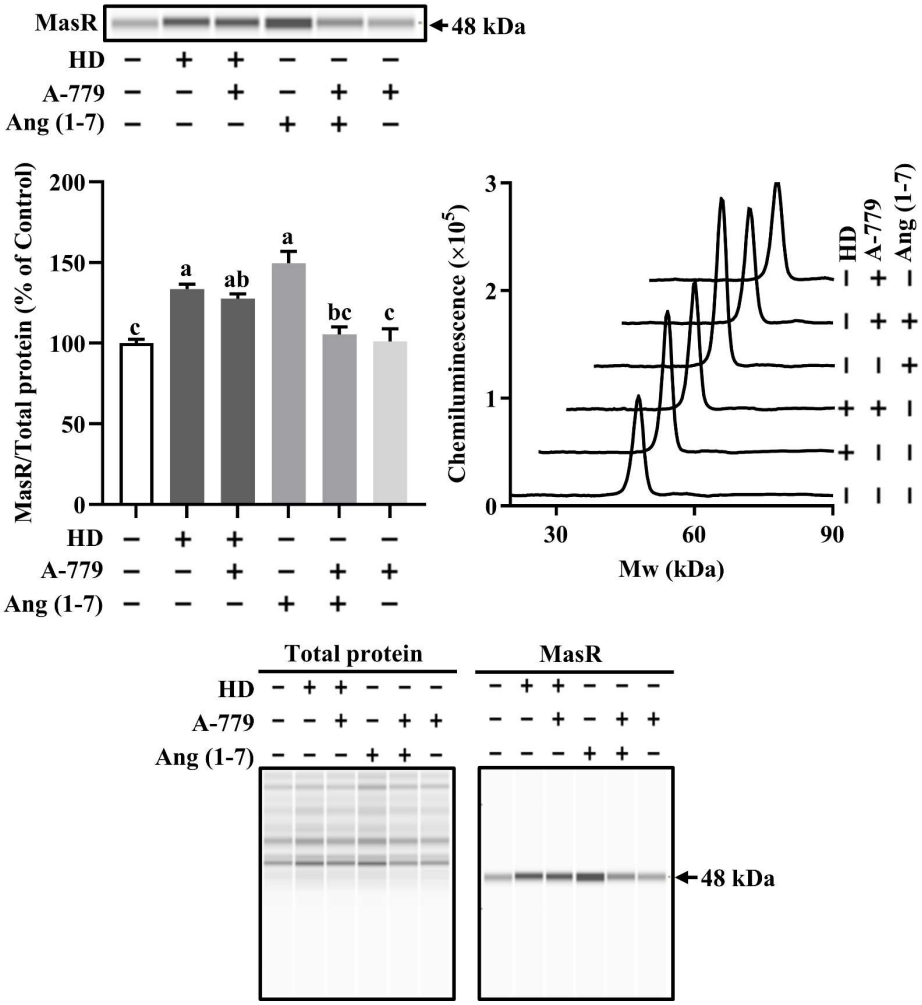

Fig. 3-3 Effects of MasR antagonist on hesperidin- or Ang (1-7)-induced MasR expression in HUVECs.

The HUVECs were treated with 1 μM hesperidin or 1 μM Ang (1-7) in the presence or absence of 1 μM MasR antagonist A-799 for 2 h. The protein expression of MasR was determined using a Wes analysis. The expression was normalized by the electropherogram peak area of the applied total protein in each lane. The chemiluminescent signal is displayed as a virtual blot-like image, and an electropherogram was generated based on the molecular weight. Results are expressed as the mean ± SEM (n = 4). Different letters represent the significant difference at $P < 0.05$ by Tukey–Kramer's test. HD, hesperidin.

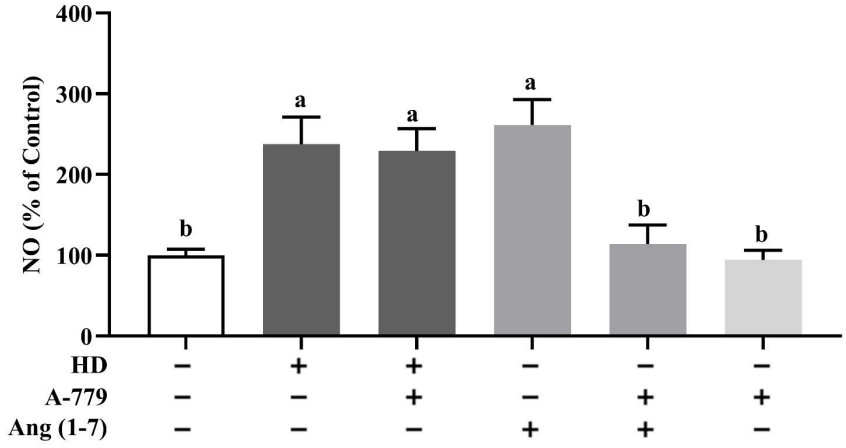

Fig. 3-4 Effects of MasR antagonist on hesperidin- or Ang (1-7)-induced NO production in HUVECs.

The HUVECs were treated with 1 μM hesperidin or 1 μM Ang (1-7) in the presence or absence of 1 μM MasR antagonist A-799 for 2 h. NO production was determined using an NO assay-FX kit. Results are expressed as the mean ± SEM (n = 4). Different letters represent the significant difference at $P < 0.05$ by Tukey–Kramer's test. HD, hesperidin.

Further investigations on trends in NO production and MasR expression upon hesperidin treatment were performed using the antagonists of receptors related to NO production in HUVECs. The activation of AT_2R by an AT_2R agonist results in increasing the ACE2 activity, Ang (1-7), and MasR levels in obese Zucker rats and HK-2 cells [146]. The stimulation of ER by its ligand (17 β-oestradiol) has been demonstrated to up-regulate the Mas receptor *via* the ACE2/Ang (1-7) activation in human pulmonary microvascular endothelial cells [147]. The activation of BK_2R can up-regulate the NO production and exert the vasodilation effect has been demonstrated [148]. TRPV1, which is expressed in endothelial cells, can activate eNOS to increase the NO production in HUVECs [149]. Therefore, the involvement of AT_2R, E_2R, BK_2R, and TRPV1 receptors in hesperidin-mediated NO production and Mas receptor expression were evaluated. As shown in Fig. 3-5, hesperidin-induced NO production was not affected by ER, AT_2R, or BK_2R antagonists, indicating that these receptors were not targeted by hesperidin. On the contrary, a TRPV1 antagonist, capsazepine, can significantly ($P < 0.05$) abolish the hesperidin-induced NO production (Fig. 3-5) and MasR expression (Fig. 3-6). Additionally, hesperidin, similar to capsaicin that can trigger Ca^{2+} influx, can also significantly increase the level of intracellular Ca^{2+}, and hesperidin-elevated the level of intracellular Ca^{2+} can be abolished by capsazepine (Fig. 3-7), strongly suggesting that these effects were initiated by the binding of hesperidin to TRPV1, following an increase in intracellular Ca^{2+} levels.

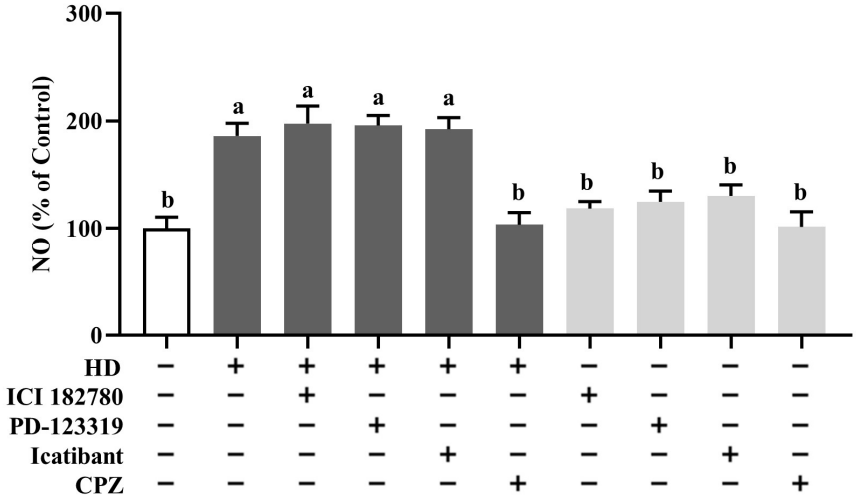

Fig. 3-5 Effects of receptor antagonists on hesperidin-induced NO production in HUVECs.

The HUVECs were treated with 1 μM hesperidin, in the presence or absence of either ICI 182780 (ER antagonist, 1 μM); PD-123319 (AT_2R antagonist, 1 μM); icatibant (BK_2R antagonist, 1 μM); or capsazepine (TRPV1 antagonist, 10 μM) for 2 h. The NO production was measured using an NO assay-FX kit. Results are expressed as the mean ± SEM (n = 4). Different letters represent the significant difference at $P < 0.05$ by Tukey–Kramer's test. CPZ, capsazepine; HD, hesperidin.

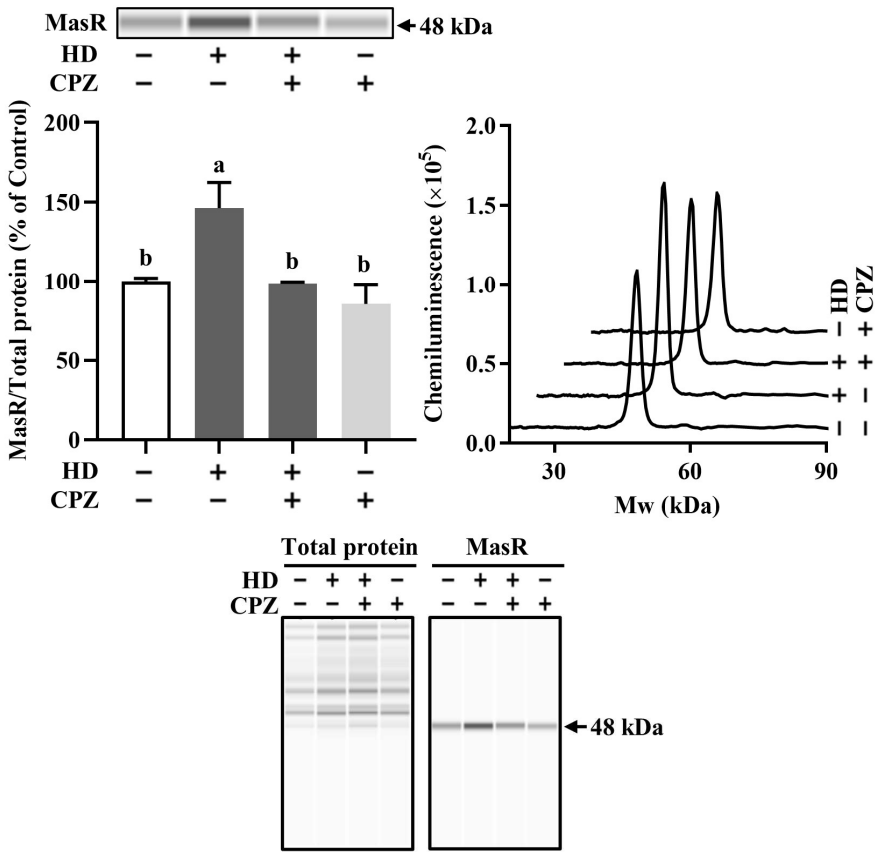

Fig. 3-6 Effects of TRPV1 antagonist (capsazepine, CPZ) on hesperidin-induced MasR expression in HUVECs.

The HUVECs were treated with 1 μM hesperidin in the presence or absence of capsazepine (TRPV1 antagonist, 10 μM) for 2 h. The protein expression of MasR was determined using a Wes analysis. The expression level of MasR was normalized by the electropherogram peak area of the applied total protein in each lane. The chemiluminescent signal is displayed as a virtual blot-like image, and an electropherogram was generated based on the molecular weight. Results are expressed as the mean ± SEM (n = 4). Different letters represent the significant difference at $P < 0.05$ by Tukey–Kramer's test. CPZ, capsazepine; HD, hesperidin.

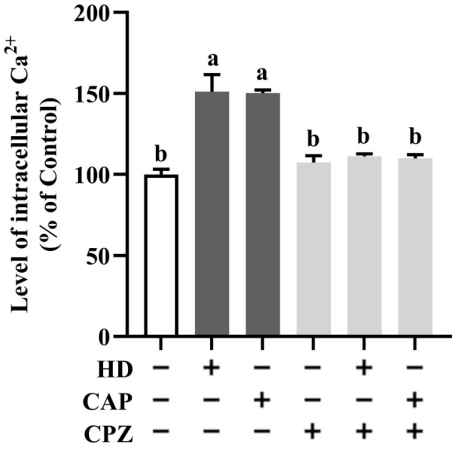

Fig. 3-7 Effects of hesperidin or capsaicin on the level of intracellular Ca^{2+} in HUVECs.

Fluo-8E AM NW-loading solution was added for 1 h at 37°C. The HUVECs were treated with HBSS containing 1 μM hesperidin or 1 μM capsaicin, in the presence or absence of capsazepine (TRPV1 antagonist, 10 μM). Values are expressed as the mean ± SEM (n = 3). Different letters represent significant differences at $P < 0.05$ by Tukey–Kramer's test. CPZ, capsazepine; CAP, capsaicin; HD, hesperidin.

3.4. Involvement of TRPV1 in hesperidin-induced NO production/MasR expression in HUVECs

Above-mentioned results pointed out that TRPV1 is involved in elevated NO production and MasR expression by hesperidin. To confirm that, TRPV1-knockdown HUVECs (Fig. 3-8) were used for hesperidin-induced NO production/MasR expression experiments. The increment of NO production by hesperidin was significantly ($P < 0.05$) abrogated in TRPV1-knockdown HUVECs (Fig. 3-9). The TRPV1-knockdown also significantly ($P < 0.05$) abolished the elevated MasR expression by hesperidin (Fig. 3-10). Moreover, the activation of TRPV1 by capsaicin (TRPV1 agonist, 0.1, 1, or 10 μM) also promotes the NO production and MasR expression (1 μM) in HUVECs (Fig. 3-11). These confirmed the capsazepine (TRPV1 antagonist)-assisted speculation (Fig. 3-5&6) that hesperidin elevates NO production and MasR expression *via* TRPV1 activation. TRPV1, a member of the transient receptor potential vanilloid superfamily of selective Ca^{2+}-permeable channels, was found to be widely expressed in the cardiovascular system, particularly in vascular endothelial cells and smooth muscle cells [150]. Recent studies have revealed that TRPV1 plays a vital role in regulating vascular function *via* releasing NO, neuropeptide substance P, and calcitonin gene-related peptide [95,96]. The present results show that hesperidin exerts a novel vasorelaxant potential *via* TRPV1-initiated MasR and NO activations in the HUVECs.

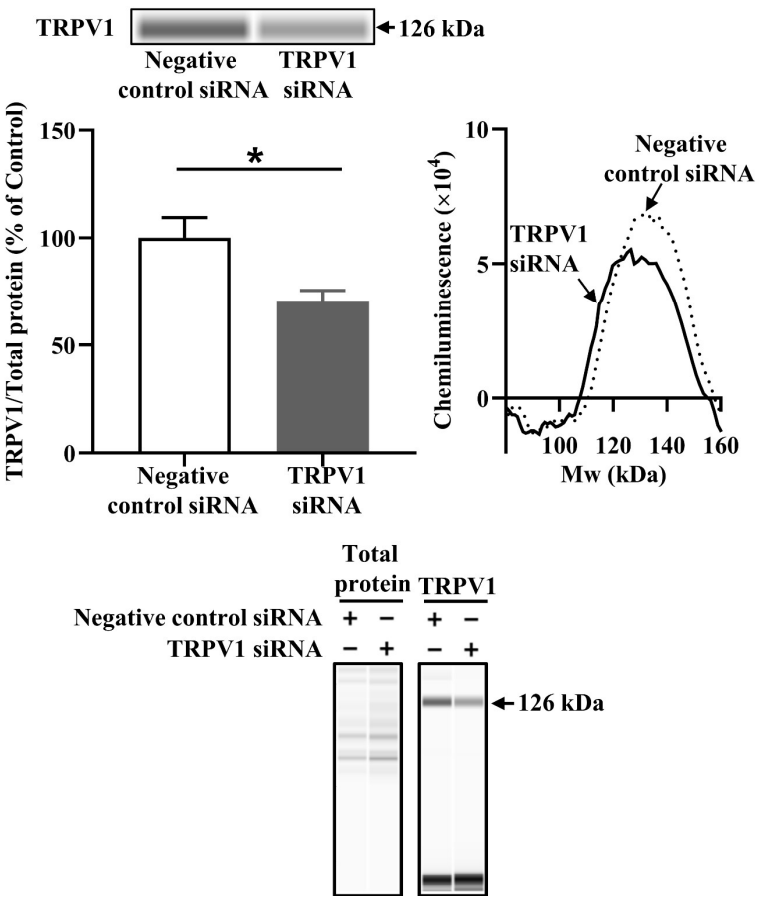

Fig. 3-8 The protein expression of TRPV1 in HUVECs transfected with TRPV1 siRNA and negative control siRNA.

The protein expression of TRPV1 was determined by Wes analysis. The expression was normalized by the electropherogram peak area of the applied total protein in each lane. The chemiluminescent signal is displayed as a virtual blot-like image, and an electropherogram was generated based on the molecular weight. Results are expressed as the mean ± SEM (n = 4). Statistical analysis between the two groups was performed by Student's t-test. *$P < 0.05$ *vs.* control.

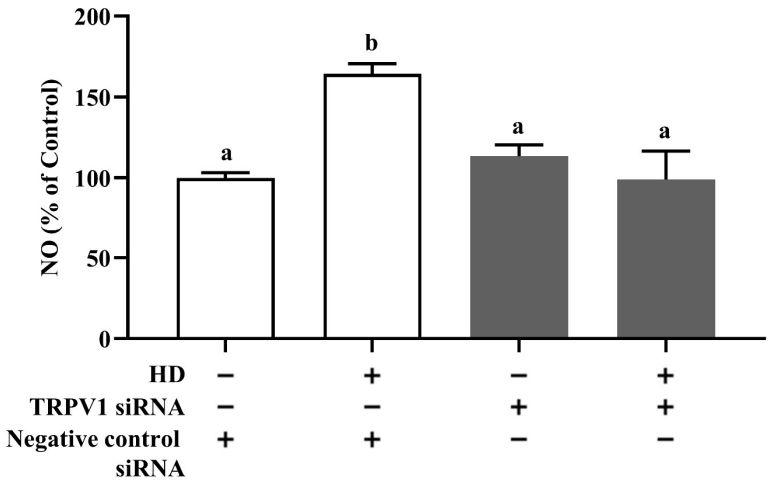

Fig. 3-9 Effect of TRPV1 knockdown on hesperidin-induced NO production.

TRPV1 or negative control siRNA-transfected HUVECs were treated with 1 μM hesperidin for 2 h. The NO production was measured using an NO assay-FX kit. Results are expressed as the mean ± SEM (n = 5). Different letters represent the significant difference at $P < 0.05$ by Tukey–Kramer's test. CPZ, capsazepine; HD, hesperidin.

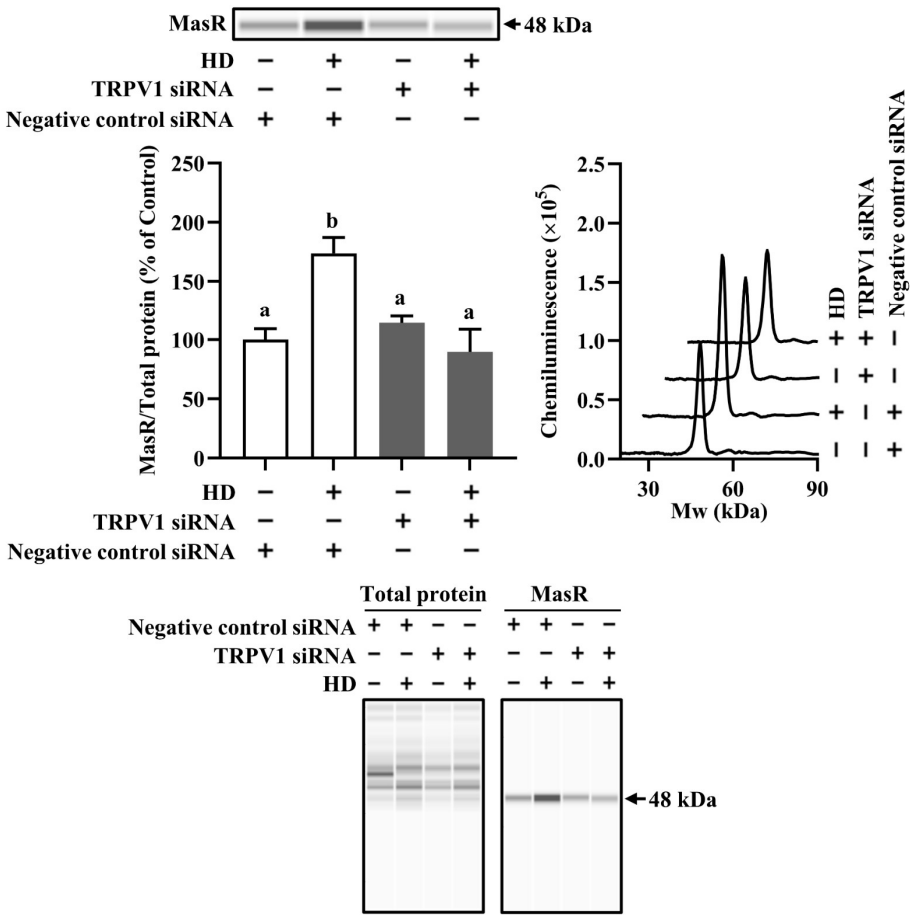

Fig. 3-10 Effect of TRPV1 knockdown on hesperidin-induced MasR expression.

TRPV1 or negative control siRNA-transfected HUVECs were treated with 1 μM hesperidin for 2 h. The protein expression of MasR was determined using a Wes analysis. The expression was normalized by the electropherogram peak area of the applied total protein in each lane. The chemiluminescent signal is displayed as a virtual blot-like image, and an electropherogram was generated based on the molecular weight. Results are expressed as the mean ± SEM (n = 5). Different letters represent the significant difference at $P < 0.05$ by Tukey–Kramer's test. HD, hesperidin.

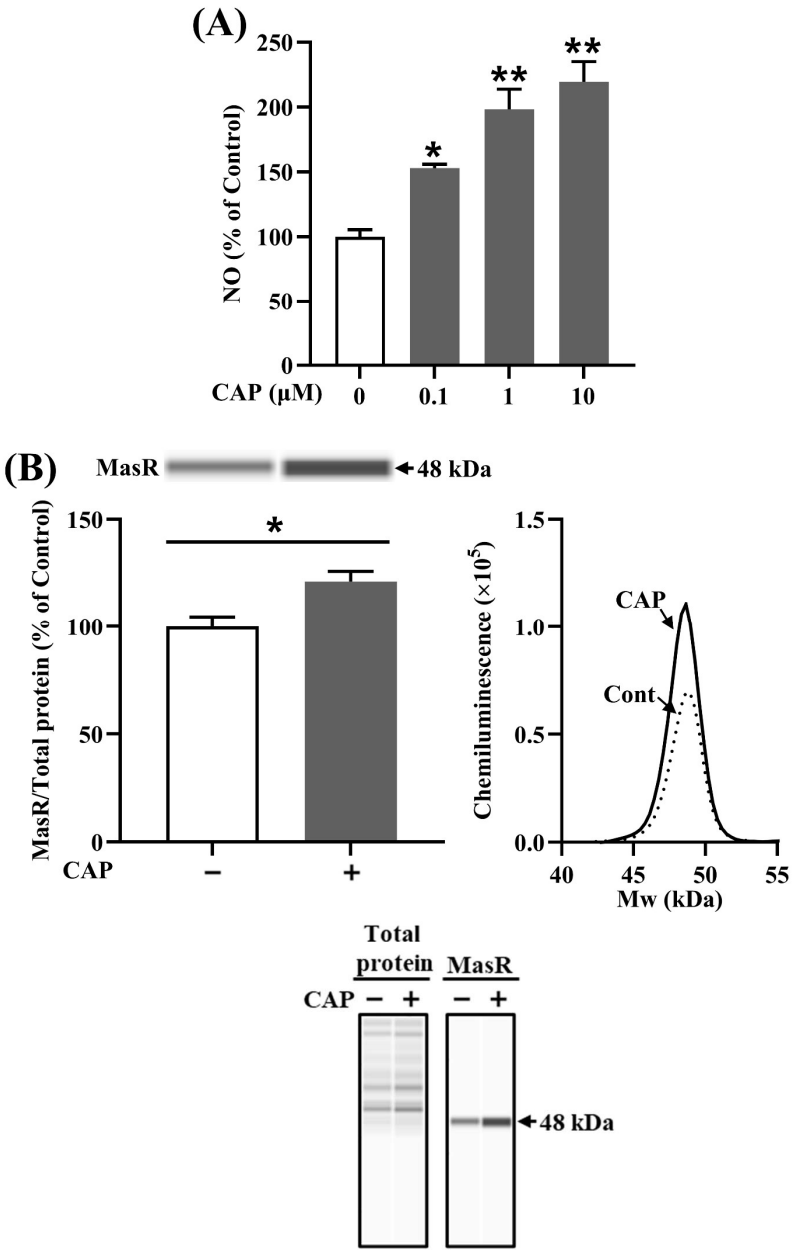

Fig. 3-11 Effects of capsaicin (CAP, TRPV1 agonist) on (A) NO production (CAP, 0.1, 1 or 10 μM) and (B) MasR expression (CAP, 1 μM) in HUVECs.

(A) The HUVECs cells were treated with capsaicin at concentrations of 0.1, 1, or 10 μM for 2h. NO production was determined using an NO assay-FX kit. (B) The HUVECs were treated with capsaicin at the concentration of 1 μM for 2 h. The protein expression analyzed by Wes analysis was normalized by the

electropherogram peak area of the applied total protein in each lane. The chemiluminescent signal is displayed as a virtual blot-like image, and an electropherogram was generated based on the molecular weight. Values are expressed as the mean ± SEM (n = 4). *$P < 0.05$, **$P < 0.01$ vs. control by using Dunnett's test and student's *t*-test.

Since TRPV1, a Ca^{2+} channel, is activated, an elevated Ca^{2+} influx in cells causes the activation of CaMKII [97,98,151]. Moreover, the signaling links between TRPV1 and CaMKII/p38 MAPK [152] and between p38 MAPK and MasR [125,153] have been previously reported. Thus, MasR expression experiments were conducted to clarify the link of TRPV1 with the MasR axis by using CaMKII inhibitor (KN-62) and p38 MAPK inhibitor (SB202190). As shown in Fig. 3-12, KN-62 significantly ($P < 0.05$) inhibited hesperidin-induced MasR expression in HUVECs. Furthermore, hesperidin significantly increased the phosphorylation of p38 MAPK, which was in the axis of the MasR expression (Fig. 3-13) [125,153]. The inhibition of p38 MAPK by SB202190 completely abrogated the increased hesperidin-induced MasR expression (Fig. 3-12). These findings suggested that the TRPV1/CaMKII/p38 MAPK signaling pathway may be involved in the axis of hesperidin-induced MasR expression in HUVECs. However, the precise signaling pathways between TRPV1 and MasR remain unclear. Further studies are warranted to clarify the signaling pathway(s) between TRPV1 and MasR in HUVECs, for example, by targeting transcription factors such as cAMP-response element-binding protein (CREB) [154], which is implicated in MasR upregulation by Ang (1-7).

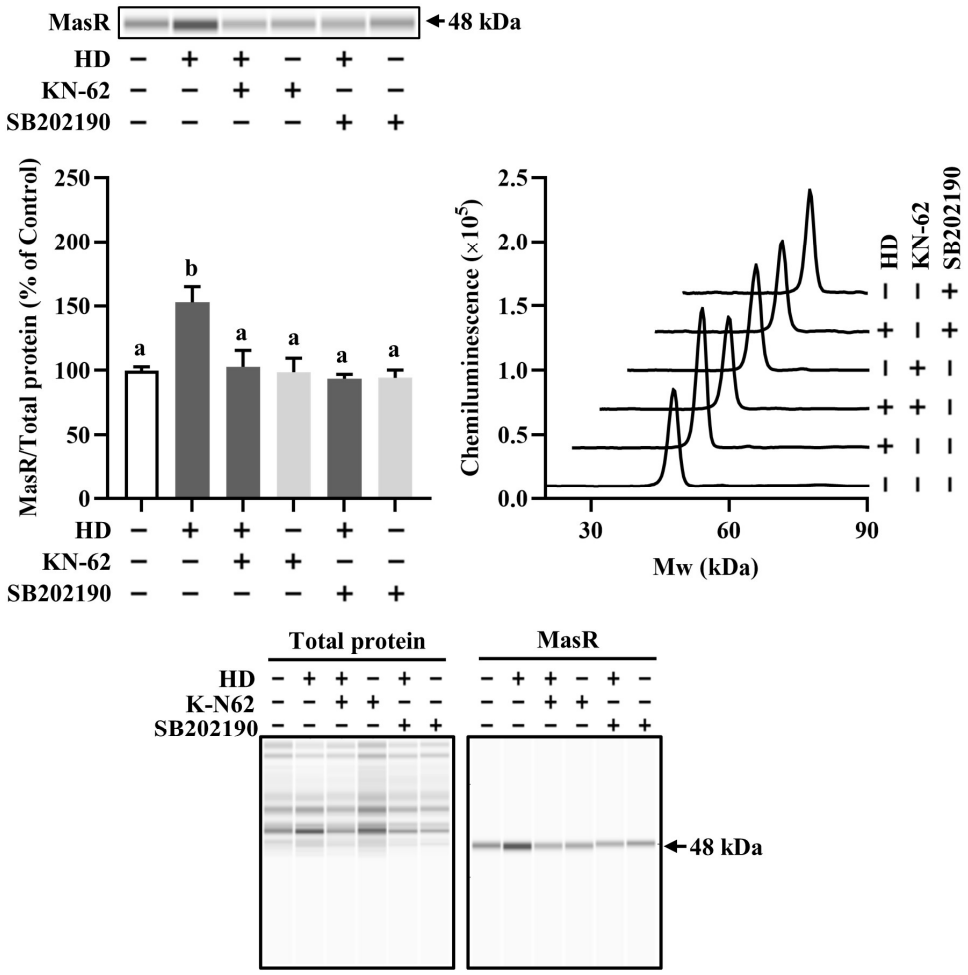

Fig. 3-12 Effects of CaMKII and p38 MAPK inhibitions on hesperidin-induced MasR expression in HUVECs.

The HUVECs were treated with 1 μM hesperidin in the presence or absence of KN-62 (CaMKII inhibitor, 10 μM) or SB202190 (p38 MAPK inhibitor, 10 μM) for 2 h. The protein expression of MasR was determined using a Wes analysis. The expression was normalized by the electropherogram peak area of the applied total protein in each lane. The chemiluminescent signal is displayed as a virtual blot-like image, and an electropherogram was generated based on the molecular weight. Results are expressed as the mean ± SEM (n = 3). Different letters

represent the significant difference at $P < 0.05$ by Tukey–Kramer's test. HD, hesperidin.

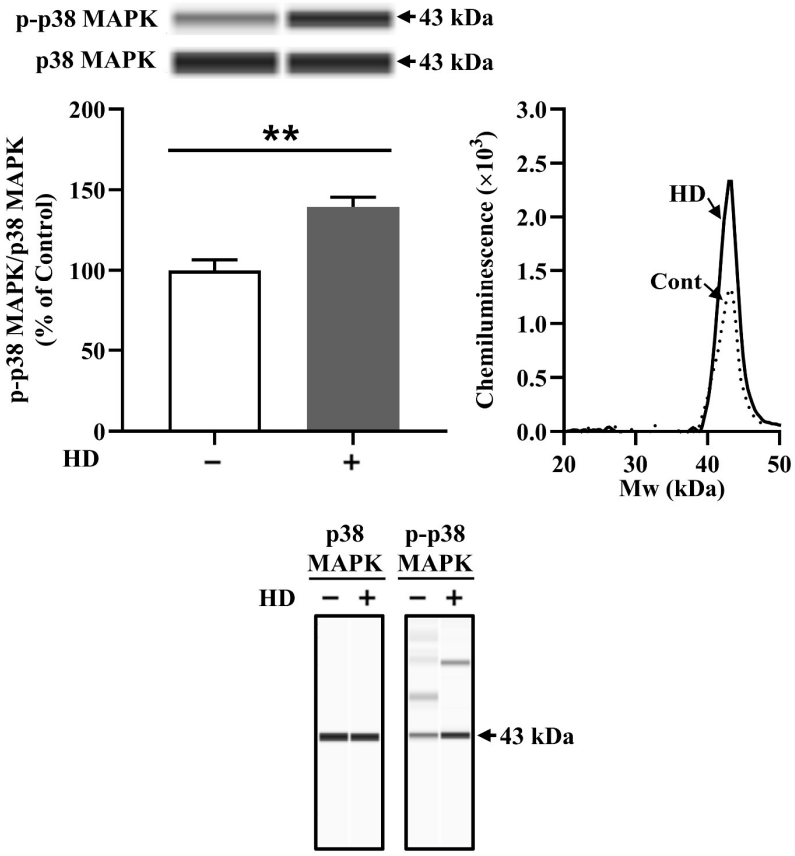

Fig. 3-13 Effect of hesperidin on the phosphorylation of p38 MAPK in HUVECs.

Effect of hesperidin on the phosphorylation of p38 MAPK. HUVECs were treated with 1 μM hesperidin for 2 h. The protein expression analyzed by Wes analysis was normalized by the electropherogram peak area of the applied total protein in each lane. The chemiluminescent signal is displayed as a virtual blot-like image, and an electropherogram was generated based on the molecular weight. Values are expressed as the mean ± SEM (n = 4). Statistical analysis between the two groups was performed by Student's *t*-test. $^{**}P < 0.01$ *vs*. control. HD, hesperidin.

Above-mentioned results have demonstrated that hesperidin-induced NO production was not attributable to the MasR-mediated signaling pathway (Fig. 3-4) but was initiated by binding to TRPV1 (Fig. 3-5). As shown in Fig. 3-14, the inhibition of CaMKII also resulted in the disappearance of NO production by hesperidin. Furthermore, hesperidin significantly increased the phosphorylation of eNOS (Fig. 3-15), and the inhibition of eNOS abolished the hesperidin-induced NO production (Fig. 3-14), indicating that the TRPV1/CaMKII/eNOS signaling pathway lies in the axis of hesperidin-induced NO production in HUVECs. This finding was consistent with the report that EGCG-activated TRPV1/CaMKII/eNOS/NO signaling pathway [97].

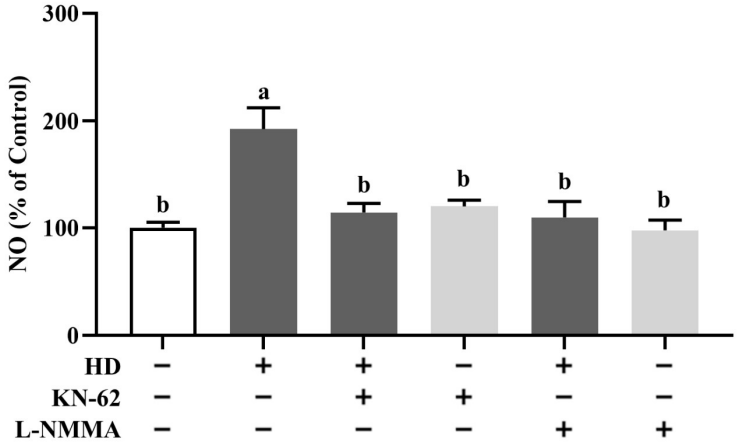

Fig. 3-14 Effect of CaMKII and eNOS inhibitions on hesperidin-induced NO production in HUVECs.

The HUVECs were treated with 1 μM hesperidin in the presence or absence of either KN-62 (CaMKII inhibitor, 10 μM) or L-NMMA (eNOS inhibitor, 100 μM) for 2 h. The NO production was measured using an NO assay-FX kit. Results are expressed as the mean ± SEM (n = 4). Different letters represent the significant difference at $P < 0.05$ by Tukey–Kramer's test. HD, hesperidin.

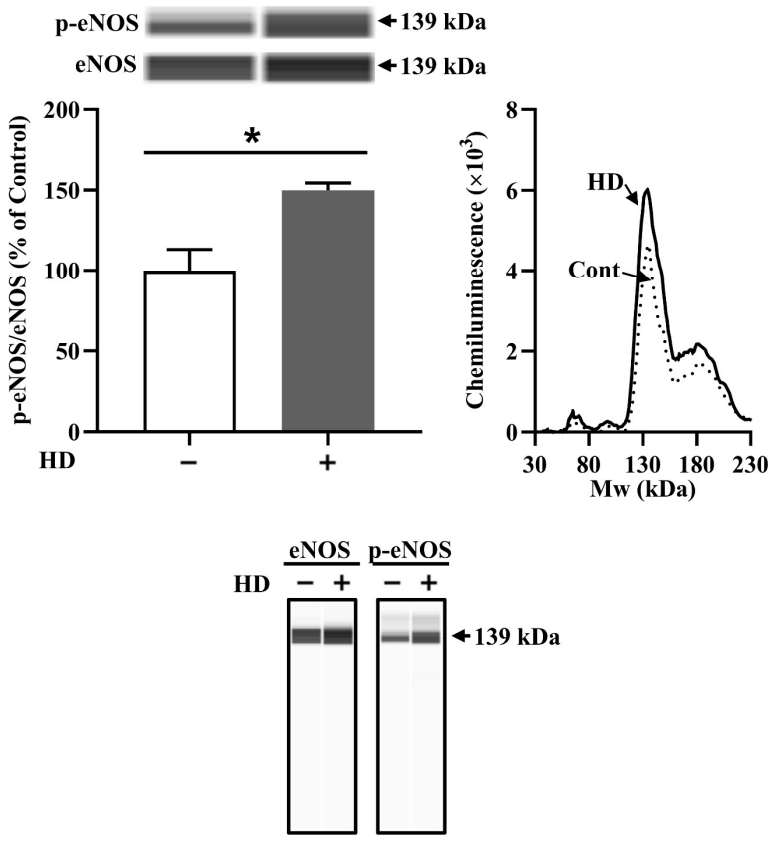

Fig. 3-15 Effect of hesperidin on the phosphorylation of eNOS in HUVECs. Effect of hesperidin on the phosphorylation of eNOS. HUVECs were treated with 1 μM hesperidin for 2 h. The protein expression analyzed by Wes analysis was normalized by the electropherogram peak area of the applied total protein in each lane. The chemiluminescent signal is displayed as a virtual blot-like image, and an electropherogram was generated based on the molecular weight. Values are expressed as the mean ± SEM (n = 4). Statistical analysis between the two groups was performed by Student's *t*-test. *$P < 0.05$ *vs.* control. HD, hesperidin.

3.5. Structural factors of hesperidin for the activation of TRPV1-mediated signaling pathways in HUVECs

Phytochemicals, such as EGCG [97] and sesamin [98] , have been reported to enhance NO generation *via* activation of the TRPV1/CaMKII/eNOS signaling pathway, similar to the present hesperidin study (Fig 3-15), although their structural factors for TRPV1 activation remain unclear. Thus, hesperetin, an aglycone of hesperidin, and its flavonoid analogs (eriodictyol, taxifolin, homoeriodictyol, naringenin, luteolin, and quercetin) (Fig. 3-16) were subjected to investigate the structural factors of hesperidin (hesperetin-7-*O*-rutinoside) for the activation of TRPV1-mediated signaling pathways. As shown in Fig. 3-17A, hesperetin exerted a significant ($P < 0.05$) increment of NO production, similar to that of its mother flavonoid, hesperidin. It suggested that its flavonoid skeleton was responsible for the potential of hesperidin to promote NO production in HUVECs. Among the hesperetin analogs, eriodictyol and taxifolin exhibited a significant promotion of NO production, whereas others failed to exert this effect (Fig. 3-17A). A criterion for NO production in the five flavanones was that they possessed an OH group at the 3' position of the B ring. The failure of luteolin and quercetin to promote NO production (although the two flavones have an OH group at the 3' position) (Fig. 3-17A) strongly suggested that a twisted configuration of the B ring may be an additional crucial factor in the ability of flavonoids to promote NO production in this study. Additionally, EGCG, with a twisted B ring and a hydroxy group at the 3' position, has been reported to

activate TRPV1 [97], suggesting that these two criteria were crucial factors for agonistic TRPV1 binding.

However, we could not conclude the structural factors of flavonoids as a specific ligand for TRPV1 based on the current limited experiments using hesperetin analogs (Fig.3-16). Since in TRPV1-knocked down HUVECs, NO production was promoted by eriodictyol and taxifolin, with the aforementioned structural factors (Fig. 3-17B), strongly suggesting that additional structural factors must be considered for the preferential binding of flavonoids to TRPV1.

Thus far, some *in silico* molecular docking studies[46,47] using TRPV1 ligands, such as capsaicin, capsazepine, and resiniferotoxin, have revealed that the TRPV1 ligands can be subdivided into three structural regions: head (aromatic ring), neck (polar entity), and tail (hydrophobic group). The head and neck are found to be important for the activity, but the tail is tolerated in modifications. However, the structural regions of flavonoids for TRPV1 binding remain unclear. Thus, an *in silico* analysis of the TRPV1-hesperetin complex using a CHARMM-GUI-aided molecular dynamics simulation in a virtual phospholipid membrane warrants further investigation to demonstrate the structural factors of flavonoids responsible for preferential TRPV1 binding.

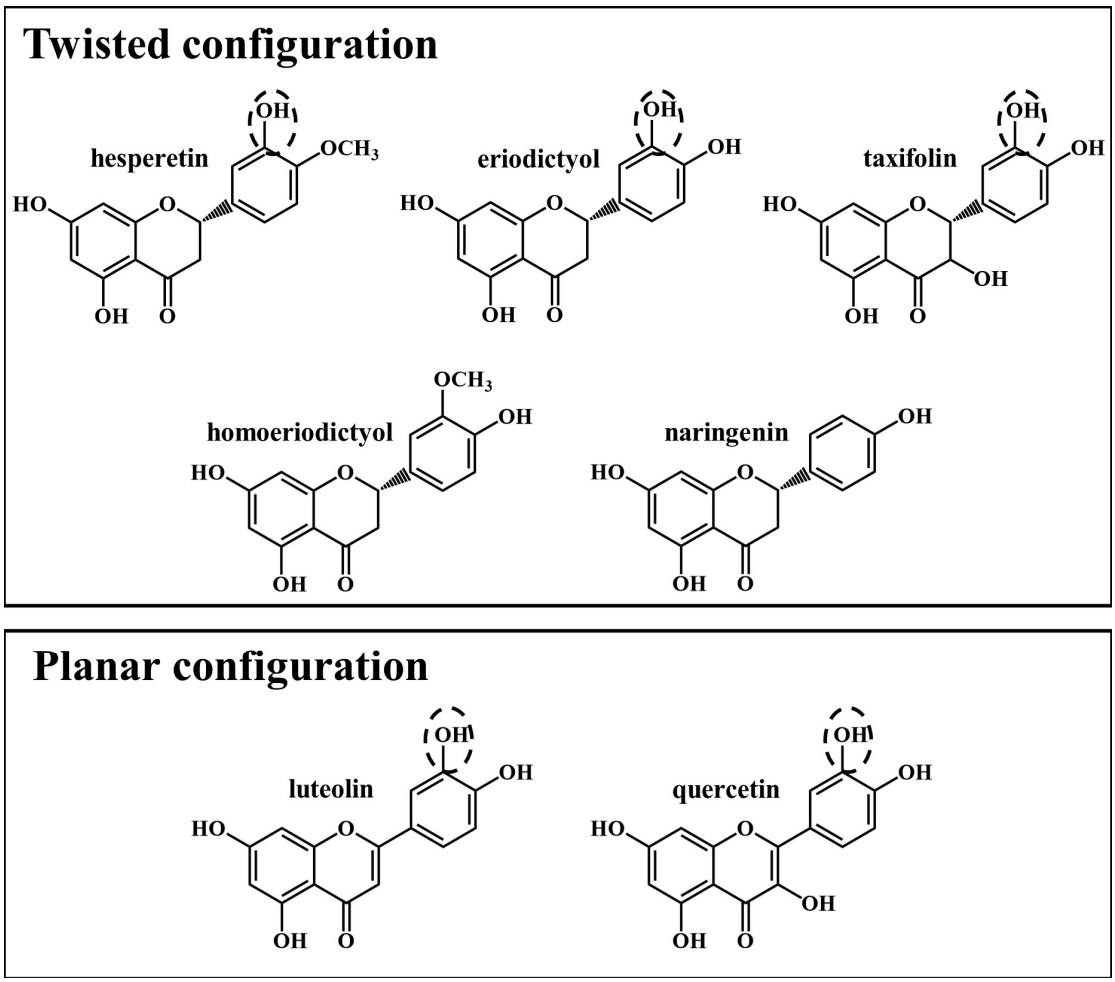

Fig. 3-16 Structures of hesperetin and its analogs (eriodictyol, taxifolin, naringenin, homoeriodictyol, quercetin, and luteolin) used in this study.

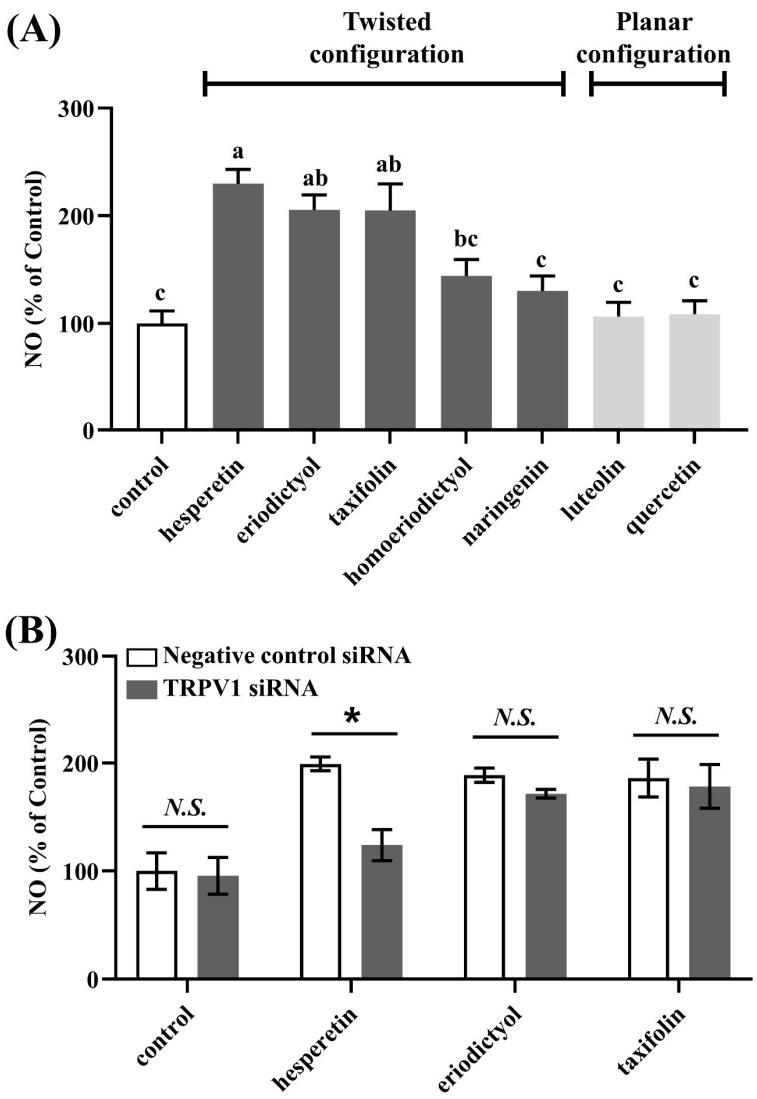

Fig. 3-17 Structure–activity relationship between hesperetin analogs and NO production in HUVECs.

The HUVECs were treated with hesperetin or its analogs at a concentration of 1 μM for 2 h. (A) Effect of hesperetin and its analogs on NO production in the HUVECs. Results are expressed as the mean ± SEM (n = 5). Different letters represent the significant different at $P < 0.05$ by Tukey–Kramer's test. (B) Effect of hesperetin, eriodictyol, and taxifolin on NO production in TRPV1-knocked down HUVECs. Results are expressed as the mean ± SEM (n = 4). Statistical

analysis between the two groups was performed by Student's *t*-test. *$P < 0.05$ *vs.* control. *N.S.*, no significance.

4. Summary

In conclusion, **Chapter III** demonstrates the following first findings (Fig. 3-18): 1) hesperidin (and hesperetin) activates TRPV1 in HUVECs. 2) The stimulation of hesperidin on TRPV1 caused the activation of two signaling axes mediated by CaMKII, namely, TRPV1/CaMKII/p38 MAPK/MasR and TRPV1/CaMKII/eNOS/NO. The TRPV1-initiated MasR and NO activations by hesperidin indicated a novel vasorelaxant potential in HUVECs. 3) The structure-activity relationship analysis of flavonoids demonstrated that the B ring of the twisted flavonoid skeleton with a hydroxy group at the 3' position was a crucial factor for agonistic TRPV1 binding.

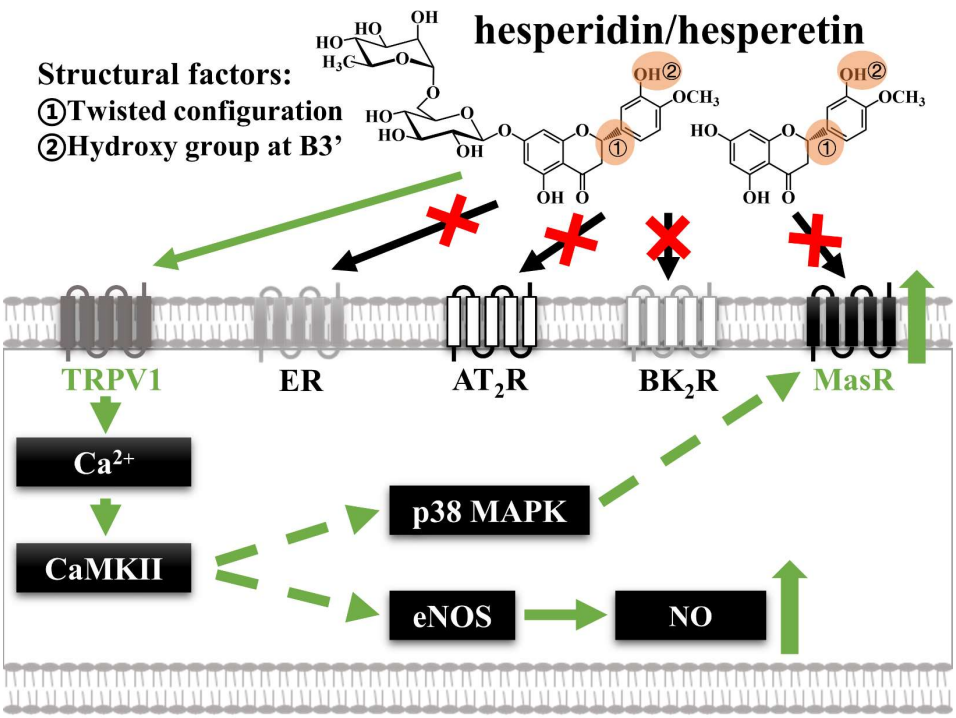

Fig. 3-18 Proposed signaling pathways of hesperidin and hesperetin induced MasR expression and NO production in HUVECs.

Chapter IV

Conclusion

Hypertension, the most prevalent chronic medical disorder in both developed and developing countries, is a major worldwide public-health challenge and the leading contributor to mortality and disability-adjusted life years lost [3]. Nowadays, medications targeting RAS can lower blood pressure (*e.g.*, captopril), but their long-term side effects, such as headaches, dizziness, constipation, coughing, and angioneurotic edema [65], should be noted. In this regard, diet and lifestyle changes are the first-line strategy for hypertension management. A high intake of plant-derived foods containing high naturally occurring polyphenols has been proved to be beneficial for lowering blood pressure. Thus, there is a growing interest in alternative-medicinal dietary food compounds, especially naturally occurring polyphenols that possess physiological potential in controlling blood pressure through, *e.g.*, inhibiting renin activity, inhibiting ACE activity, blockade of angiotensin receptor, or activation of ACE2/Ang (1-7)/MasR.

Hesperidin, a naturally occurring flavanone, is the predominant polyphenol consumed in dietary food. Previous studies revealed that daily consumption of hesperidin-containing Mikan tea significantly reduced the elevated SBP in mildly hypertensive volunteers [32]. However, the underlying anti-hypertensive mechanism(s) remains unclear.

The present study aimed to get insights into the anti-hypertensive effects and the underlying anti-hypertensive mechanisms of hesperidin by focusing on the RAS in animal and cell experiments using SHRs and HUVECs.

Chapter II Anti-hypertensive effect of hesperidin in spontaneously hypertensive rats

Although it was reported that the daily intake of hesperidin-containing Mikan tea significantly reduced the elevated SBP in mildly hypertensive volunteers, the underlying anti-hypertensive mechanism(s) remains unclear. In **Chapter II**, a long-term (20 weeks) administration study of hesperidin (50 mg/kg/day) and Mikan tea (50 mg/kg/day) was performed in 8-week-old SHRs. Daily intake of hesperidin or Mikan tea resulted in a significant SBP reduction of approximately 60 mmHg in SHRs. No change in the heart rate and the factors related to the circulating and local RAS were observed between groups. Hesperidin ameliorated the impaired vasomotor response in 28-week-old SHRs. Hesperidin or Mikan tea intake caused a significant upregulation of MasR expression in the aorta but did not affect $AT_{1/2}R$ expressions. An increase in

cAMP levels was also observed in the aorta of hesperidin-administered SHRs, indicating that hesperidin exerted an anti-hypertensive effect *via* the upregulated-MasR axis.

Chapter III Mechanism of hesperidin-induced MasR expression in human umbilical vein endothelial cells

As it has been disclosed in **Chapter II**, hesperidin exhibited an anti-hypertensive effect in SHRs, which appears to be involved in the vessel regulation *via* the activation of the aortic MasR axis. However, the mechanism(s) of elevated aortic MasR expression in SHR by hesperidin remains unclear. Thus, **Chapter III** focused on the investigation of the mechanism underlying hesperidin-stimulated aortic MasR expression using HUVECs.

In HUVECs, hesperidin treatment increased NO production and only caused a significant increment of MasR expression among the vasomotor-related receptors (AT_1R, AT_2R, and MasR). MasR antagonist did not alter the hesperidin-induced NO production and MasR expression. The hesperidin-induced MasR expression upregulation was entirely abrogated by TRPV1 knockout or inhibition of CaMKII and p38 MAPK. Meanwhile, the hesperidin-induced promotion of NO production was also abolished by TRPV1 knockout or inhibition of CaMKII and eNOS. These results indicated that hesperidin activates two signaling axes, CaMKII/p38 MAPK/MasR expression and CaMKII/eNOS/NO production through TRPV1 activation in HUVECs. The

TRPV1-initiated MasR and NO augmentation by hesperidin in the HUVECs indicated a novel vasorelaxant potential of hesperidin. Furthermore, the structure-activity relationship analysis of flavonoids revealed that the B ring of the twisted flavonoid skeleton with a hydroxy group at the 3' position was a crucial factor for agonistic TRPV1 binding.

Taking together, the present study demonstrates for the first time that hesperidin exerted an anti-hypertensive effect *via* the activation of the aortic MasR axis and were novel natural MasR activators *via* the TRPV1 binding (Fig. 4-1).

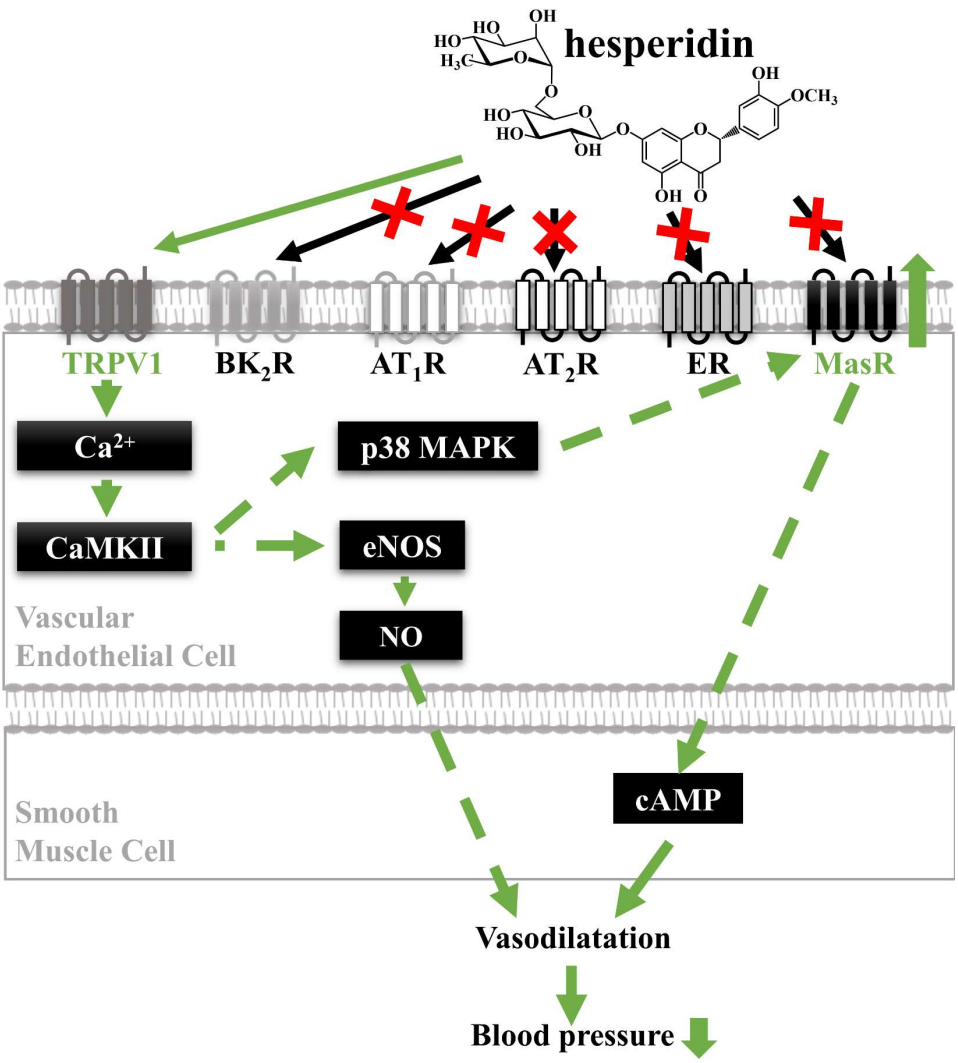

Fig. 4-1 Proposed mechanism of anti-hypertensive effect of hesperidin in spontaneously hypertensive rats and vascular endothelial cells

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