Hypertension is closely related to metabolic disorders such as insulin resistance, obesity, and type 2 diabetes. The coexistence of hypertension and metabolic disorders markedly increases the risk of cardiovascular events. Accumulating clinical and experimental evidence show that renin–angiotensin system (RAS) blockers, such as angiotensin-converting enzyme inhibitors or angiotensin receptor blockers (ARB), exert beneficial effects on insulin resistance, obesity, and type 2 diabetes. Thus, RAS blockers are regarded as the most recommended drugs for treatment of hypertension with type 2 diabetes or metabolic syndrome. Besides RAS blockers, calcium-channel blockers (CCB) are also used as the first-line drug for treatment of hypertension. Recently, the Avoiding Cardiovascular Events Through COMbination Therapy in Patients Living With Systolic Hypertension (ACCOMPLISH) trial, comparing a RAS blocker (benazepril), combined with a CCB (amlodipine) or a diuretic (hydrochlorothiazide), in the prevention of cardiovascular events in high-risk hypertensive patients, has demonstrated that the combination of a RAS blocker with a CCB is superior to a RAS blocker combined with a diuretic in preventing cardiovascular events in high-risk hypertensive patients. Furthermore, subgroup analyses of the ACCOMPLISH trial showed that the combination of a RAS blocker and a CCB was more effective in reducing cardiovascular events in patients with metabolic syndrome, suggesting that a combination of a RAS blocker and a CCB is a preferred treatment for patients with hypertension and metabolic syndrome.

**BACKGROUND**

The pharmacological advantage of combination of an angiotensin receptor blocker (ARB) and a calcium-channel blocker (CCB) is not fully defined. This study was undertaken to elucidate the potential benefit of their combination in metabolic syndrome.

**METHODS**

SHR/NDmcr-cp (SHRcp), a rat model of human metabolic syndrome, were divided into four groups, and were administered (i) vehicle, (ii) candesartan (an ARB) 0.3 mg/kg/day, (iii) amlodipine (a CCB) 3 mg/kg/day, and (iv) candesartan 0.3 mg/kg/day plus amlodipine 3 mg/kg/day, for 4 weeks.

**RESULTS**

Candesartan, amlodipine, or their combination significantly ameliorated the impairment of vascular endothelium-dependent relaxation with acetylcholine in SHRcp. However, the impairment of insulin-induced vasodilation in SHRcp was partially improved by candesartan alone, but not by amlodipine alone. Interestingly, amlodipine added to candesartan synergistically enhanced the improvement of impaired insulin-induced vasodilation by candesartan, indicating the synergistic improvement of vascular insulin resistance by the combination of these drugs. Candesartan alone, but not amlodipine alone, significantly attenuated vascular superoxide and NADPH oxidase subunit p22phox in SHRcp. Amlodipine added to candesartan synergistically enhanced the reduction of vascular p22phox levels and superoxide by candesartan in SHRcp, suggesting the association of vascular insulin resistance with oxidative stress. Furthermore, the combination of candesartan with amlodipine synergistically decreased the increase in visceral adipocyte size, serum free-fatty acid, and tumor necrosis factor-α in SHRcp.

**CONCLUSIONS**

ARB and CCB combination synergistically ameliorated vascular insulin resistance in metabolic syndrome, being associated with the synergistic attenuation of vascular oxidative stress and metabolic disorders.

**Keywords:** blood pressure; combination therapy; hypertension; inflammation; obesity; oxidative stress; vascular insulin resistance

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analysis of ACCOMPLISH trial showed that the combination of a RAS blocker with a CCB is also superior to a RAS blocker with a diuretic in diabetic hypertensive patients. However, the mechanisms underlying the above-mentioned clinical evidence on the benefits of combination of a RAS blocker and a CCB remain to be fully defined.

In the present study, we examined the potential benefit of combination of a RAS blocker, candesartan, and a CCB, amlodipine, in SHR/NDmcr-cp(+/+) (SHRcp) rats, a useful rat model of human metabolic syndrome. We obtained the evidence that their combination synergistically ameliorated vascular insulin resistance and metabolic disorders in this metabolic syndrome model.

METHODS

Animals. All procedures were performed in accordance with institutional guidelines for animal research approved by the Animal Care and Use Committee of Kumamoto University.

Male Wistar–Kyoto rats (WKY), spontaneously hypertensive rats (SHR), and SHRcp rats, a rat model of metabolic syndrome, were purchased from Japan SLC (Shizuoka, Japan). All rats were housed in an animal facility with a 12 h light–darkness cycle and were given standard chow and water ad libitum.

Drugs. Candesartan, an ARB, was kindly supplied from Takeda Pharma (Tokyo, Japan). Amlodipine, a CCB, was purchased from Wako Pure Chemical (Osaka, Japan). Nω-Nitro-l-arginine methylester (l-NAME) was purchased from Dojindo (Kumamoto, Japan).

Experimental protocol. Eleven-week-old SHRcp were divided into four groups (n = 6–9 per each group), and were orally given (i) vehicle (0.5% methylcellulose), (ii) candesartan (0.3 mg/kg), (iii) amlodipine (3 mg/kg), or (iv) candesartan (0.3 mg/kg) and amlodipine (3 mg/kg) for 4 weeks. Preliminary experiments showed that the above-mentioned dose of candesartan and amlodipine exerted comparable hypotensive effects in SHRcp. Age-matched WKY rats (n = 7) and SHR (n = 7) were used as the control and were orally given vehicle for 4 weeks. Body weight was periodically measured. Blood pressure and heart rate were measured by tail-cuff plethysmography (BP-98A; Softron, Tokyo, Japan) before, and 2, 3 and 4 weeks after the start of drug treatment. After 4 weeks of drug treatment, all rats were anesthetized with ether, and the heart, aorta, carotid artery, and subcutaneous and visceral adipose tissues were rapidly excised to perform biochemical and histological examinations, as described below in detail.

Vessel ring preparation and organ chamber experiments. Isometric tension studies were performed as previously described. In brief, carotid artery from rats were cut into 5-mm rings with special care to preserve the endothelium, and mounted in organ baths filled with modified Tyrode buffer (pH 7.4; NaCl 121 mmol/l, KCl 5.9 mmol/l, CaCl2 2.5 mmol/l, MgCl2 1.2 mmol/l, NaH2PO4 1.2 mmol/l, NaHCO3 15.5 mmol/l, and d-glucose 11.5 mmol/l) aerated with 95% O2 and 5% CO2 at 37 °C. The preparations were attached to a force transducer, and isometric tension was recorded on a polygraph. A resting tension of 1 g was maintained throughout the experiment. Vessel rings were primed with KCL (50 mmol/l) and then precontracted with l-phenylephrine (10−7 mol/l). After the plateau was attained, the rings were exposed to increasing concentrations of acetylcholine (10−9–10−4 mol/l), sodium nitroprusside (10−9–10−4 mol/l), or insulin (10−3–10−1 mU/ml) to obtain cumulative concentration response curves.

Measurement of vascular superoxide. Thoracic aortas, removed from rats, were immediately frozen in Tissue-Tek OCT embedding medium (Sakura Finetek, Tokyo, Japan). Dihydroethidium was used to evaluate tissue superoxide levels in situ, as previously described. In brief, dihydroethidium fluorescence was visualized by fluorescent microscopy using an excitation wavelength of 520–540 nm and a rhodamine emission filter. Dihydroethidium fluorescence of tissue was captured with the same exposure time (1.0 s), and it was quantified and expressed relative to values obtained from vehicle-treated group tissue.

Western blot analysis of aortic and adipose tissue proteins. The detailed method was previously described. Briefly, after aortic or adipose tissue protein extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electric transfer to polyvinylidene difluoride membrane, the membranes were probed with specific antibodies. Antibodies used were as follows; anti-p22phox (×2,000; Santa Cruz Biotechnology, Santa Cruz, CA), phospho-eNOS (×2,000; BD Biosciences, San Jose, CA), total-eNOS (×2,000; BD Biosciences) α-tubulin (×2,000; EMD Chemicals, Gibbstown, NJ), anti-tumor necrosis factor (TNF)-α (×2,000; R&D Systems, Minneapolis, MN), anti-adiponectin (×2,000; Abcam, Cambridge, MA), anti-manganese superoxide dismutase (SOD) (×10,000; Assay Designs, Farmingdale, NY), anti-copper-zinc SOD (×5,000; Assay Designs), and anti-extracellular-SOD (×20,000; Upstate, Charlottesville, VA).

The antibodies were visualized by using an enhanced chemiluminescence method (ECL Plus; GE Healthcare, Buckinghamshire, UK). The intensity of the bands was quantified by using analysis software (ImageJ; National Institute of Health, Bethesda, MD). In individual samples, each value was corrected for that of α-tubulin.

Measurement of adipocyte size. Epididymal adipose tissues were fixed with formalin, embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin–eosin staining. Adipocyte size was measured as adipocyte area in 10 sections per rat under a microscope. The cell size of at least 30 adipocytes per section was measured, and the average was used for the value of each sample.

HOMAIR and measurement of TNF-α, adiponectin, and serum free-fatty acid. Homeostasis model assessment of insulin resistance (HOMAIR), a simple assessment of insulin sensitivity, was calculated by using the following formula: (fasting plasma glucose (mmol/l) × insulin (pmol/l))/405. Plasma insulin levels
were quantified by using a commercial ELISA kit (Morinaga, Tokyo, Japan). Plasma glucose concentrations were measured with a kit (Wako Pure Chemical).

Plasma TNF-α concentrations were measured with a kit (R&D Systems). Serum adiponectin concentrations were measured with ELISA kit (Otsuka Pharmaceutical, Tokyo, Japan). Serum free-fatty acid concentrations were measured with a kit (Wako Pure Chemical).

**Statistical analysis.** All data are presented as means ± s.e.m. Statistical significance was determined with one-way analysis of variance followed by Fisher's protected least significant difference test, using StatView for Windows (SAS Institute, Cary, NC). The data on time course experiments were analyzed by two-way analysis of variance. In all tests, differences were considered statistically significant at a value of $P < 0.05$.

**RESULTS**

**Effects on body weight, organ weight, blood pressure, and heart rate**

**Supplementary Table S1** online shows that SHRcp exhibited greater body weight and greater adipose tissue weight than SHR. Treatment with candesartan, amlodipine, or their combination did not significantly affect body weight and adipose tissue weight of SHRcp.

As shown in **Supplementary Figure S1** online, SHRcp showed higher blood pressure than WKY rats. However, blood pressure of SHRcp was significantly lower than that of SHR ($P < 0.05$), being in consistent with previous reports.\(^1\)Candesartan or amlodipine alone significantly and comparably reduced blood pressure of SHRcp throughout the treatment.

The combination of candesartan with amlodipine exerted additive blood pressure lowering effect throughout the treatment. Heart rate in SHRcp was not significantly changed by treatment with candesartan alone, amlodipine alone, or their combination (see **Supplementary Figure S2** online).

**Effects on vascular relaxation with acetylcholine or sodium nitroprusside**

As shown in **Figure 1a**, vascular endothelium-dependent relaxation by acetylcholine was significantly impaired in SHRcp compared with SHR ($P < 0.05$). Candesartan monotherapy and candesartan in combination with amlodipine similarly normalized the impairment of acetylcholine-induced vascular relaxation in SHRcp. Amlodipine treatment also significantly ameliorated the impairment of vascular relaxation with acetylcholine in SHRcp. As shown in **Figure 1b**, pretreatment with L-NAME almost completely abolished acetylcholine-induced vascular relaxation in all groups of rats. **Figure 1c** showed that there was no significant difference in vascular endothelium-independent relaxation by sodium nitroprusside among all groups of rats.

**Effects on insulin-induced vascular relaxation (vascular insulin sensitivity)**

As shown in **Figure 2a**, SHRcp displayed much less vascular relaxation by insulin than SHR ($P < 0.01$). Candesartan monotherapy partially but significantly attenuated the impairment of insulin-induced vascular relaxation in SHRcp. On the other hand, amlodipine monotherapy did not significantly ameliorate it in SHRcp. However, amlodipine added to candesartan synergistically ameliorated the impairment of insulin-induced vascular relaxation in SHRcp 

![Figure 1](https://academic.oup.com/ajh/article-abstract/25/6/704/2743102)
vascular relaxation in SHRcp to the same levels to WKY rats. Vascular relaxation with insulin was almost completely abolished by pretreatment with l-NAME in all groups of rats (Figure 2b).

Effects on vascular superoxide, NADPH oxidase subunit p22phox, eNOS, and SOD

As shown in Figure 3, SHRcp showed greater aortic superoxide levels ($P < 0.01$) and greater NADPH oxidase subunit p22phox levels ($P < 0.01$) than SHR. Candesartan monotherapy significantly attenuated aortic superoxide ($P < 0.01$) and p22phox ($P < 0.01$) levels of SHRcp, whereas amlodipine monotherapy failed to attenuate them. However, the combination therapy of candesartan and amlodipine synergistically reduced aortic superoxide levels and p22phox levels of SHRcp (Figure 3).

As shown in Supplementary Figure S3 online, unexpectedly, SHRcp showed higher aortic phospho-eNOS levels than SHR ($P < 0.01$). Candesartan monotherapy and the combination of candesartan with amlodipine significantly and similarly prevented the increase in aortic phospho-eNOS levels in SHRcp ($P < 0.01$), whereas amlodipine monotherapy did not alter it.

Aortic extracellular-SOD, copper-zinc-SOD, and manganese-SOD levels in SHRcp were not significantly changed by candesartan, amlodipine, or their combination (see Supplementary Figure S4 online).

Effects on adipocyte size, serum free-fatty acid, and TNF-α

As shown in Figure 4a and b, SHRcp exhibited much larger visceral adipocyte size ($P < 0.01$) and higher serum free-fatty acid levels ($P < 0.01$) than SHR. Candesartan or amlodipine monotherapy did not significantly reduce visceral adipocyte size or serum free-fatty acid of SHRcp. However, the combination of these drugs significantly reduced visceral adipocyte size ($P < 0.05$) and serum free-fatty acid ($P < 0.05$) of SHRcp.

Figure 4c and d indicated higher TNF-α concentrations in adipose tissue ($P < 0.01$) and plasma ($P < 0.01$) of SHRcp than those of SHR. The combination of candesartan and amlodipine decreased adipose tissue and plasma TNF-α of SHRcp more than either monotherapy.

Effect on HOMAIR and adiponectin

As shown in Supplementary Figure S5 online, SHRcp displayed much higher HOMAIR than SHR. Candesartan monotherapy markedly reduced HOMAIR of SHRcp, whereas amlodipine monotherapy did not significantly reduce it. HOMAIR in their combination group tended to be lower than that in candesartan monotherapy group, although the difference did not reach statistical significance.

As shown in Supplementary Figure S6 online, adipose tissue adiponectin levels ($P < 0.01$) and serum adiponectin concentrations ($P < 0.01$) in SHRcp were significantly lower than those in SHR. Candesartan monotherapy and candesartan in combination with amlodipine significantly increased the decreased adiponectin in SHRcp to a similar extent. On the other hand, amlodipine failed to increase adiponectin.

Effect on cardiac hypertrophy and coronary arterial thickening

As shown in Supplementary Figure S7 online, candesartan alone significantly decreased cardiac left ventricular (LV) weight and coronary arterial thickening in SHRcp more than amlodipine alone. The addition of amlodipine to candesartan did not synergistically enhance the reduction of cardiac hypertrophy and vascular remodeling in SHRcp by candesartan.
Discussion

The major findings of this work were as follows: (1) amlodipine added to candesartan synergistically enhanced the amelioration by candesartan of impairment of insulin-induced vasodilation in a rat model of metabolic syndrome, and this synergistic improvement of insulin-induced vasodilation was associated with the synergistic amelioration of vascular oxidative stress; (2) add-on amlodipine synergistically enhanced the reduction by candesartan of adipocyte size, serum free-fatty acid, and TNF-α in a model of metabolic syndrome. Thus, our present work provided novel mechanisms involved in the benefit of the combination of an ARB plus a CCB in metabolic syndrome.

SHR cp is established to be a useful model of human metabolic syndrome.15,17 In the present work, we found that despite lower blood pressure in SHR cp than in SHR, SHR cp displayed more...
impairment of vascular relaxation by acetylcholine or insulin than SHR. Amlodipine or candesartan monotherapy as well as their combination almost normalized the impairment of vasodilation with acetylcholine. Furthermore, acetylcholine-induced vasodilation was abolished by pretreatment with l-NAME in all groups. These results show that amlodipine or candesartan alone normalized the capacity of endothelium in releasing NO in SHRCp. In contrast to the significant improvement of acetylcholine-induced vasodilation by treatment with amlodipine, amlodipine treatment did not at all ameliorate the impairment of insulin-induced vasodilation in SHRCp, indicating the failure of amlodipine to improve vascular insulin resistance. Of note, despite similar blood pressure lowering between amlodipine and candesartan treatment in SHRCp, candesartan treatment significantly ameliorated the impairment of insulin-induced vasodilation in SHRCp. These results demonstrate that candesartan, independently of blood pressure lowering, attenuated vascular insulin resistance. Interestingly, the addition of amlodipine to candesartan synergistically ameliorated the impairment of insulin-induced vasodilation (vascular insulin resistance) in SHRCp, indicating the specific benefit of the combination of candesartan with amlodipine in improvement of vascular insulin resistance.

Accumulating evidence indicates that amlodipine can exert pleiotropic vascular effects such as NO release through bradykinin-mediated mechanism and intrinsic antioxidant activity.18–20 Oxidative stress and endothelial nitric oxide synthase (eNOS)-generated NO play counter-regulatory roles in vascular endothelial function. Importantly, oxidative stress is shown to play a causative role in the impairment of insulin-induced vasodilation.21,22 In the present work, we found that vascular superoxide and NADPH oxidase subunit p22phox levels were greater in SHRCp than SHR, indicating the enhancement of vascular oxidative stress in SHRCp. Interestingly, candesartan, but not amlodipine, significantly decreased vascular superoxide and p22phox levels in SHRCp, indicating blood pressure-independent reduction of vascular oxidative stress by candesartan. It is noteworthy that add-on amlodipine to candesartan caused the synergistic attenuation of vascular oxidative stress in SHRCp. Taken together with the previous reports indicating major role of oxidative stress in impaired insulin-induced vasodilation,21,22 it is proposed that the above mentioned synergistic improvement of insulin-induced vasodilation by the combination of candesartan with amlodipine might be at least partially mediated by the synergistic attenuation of vascular oxidative stress, although further study is needed to demonstrate our proposal. Unexpectedly, SHRCp displayed the enhancement of vascular eNOS phosphorylation compared to SHR, and this result is in agreement with the previous report.23 Candesartan alone or combined with amlodipine reduced the enhancement of phospho-eNOS to similar levels to SHR, whereas amlodipine did not significantly affect it. Therefore, eNOS seems to play a minor role in the synergistic improvement of insulin-induced vasodilation by their combination. Furthermore, we also examined the role of SOD isoforms in the beneficial effects of amlodipine, since a dihydropyridine CCB (nifedipine) is reported to upregulate endothelial SOD via vascular smooth muscle cell-dependent pathways.24 However, in this study, amlodipine alone or in combination with candesartan did not affect vascular SOD isoforms, providing no evidence for the role of SOD in the beneficial effects observed in this study.

Accumulating evidence indicate that pathophysiological crosstalk exists between vascular function and adipose tissue.25,26 Obesity causes the increased release from adipose tissues of free-fatty acid and TNF-α which are causative factors involved in vascular endothelial dysfunction, while obesity causes the decreased release of adiponectin which protects against vascular endothelial dysfunction.27,28 In this study, as expected, obesity in SHRCp was accompanied by the increased serum free-fatty acid and TNF-α, and the decreased serum adiponectin. Interestingly, amlodipine in addition to candesartan synergistically reduced adipocyte size, serum free-fatty acid and TNF-α in SHRCp, indicating the benefit of combination of candesartan with amlodipine in treatment of metabolic disorders. Taken together with the fact that free-fatty acid and TNF-α are responsible for vascular endothelial dysfunction,25,26 our present work suggests that the synergistic improvement of vascular insulin resistance in SHRCp by the combination therapy might be partially mediated by the synergistic amelioration of free-fatty acid and TNF-α.

Study limitation

The present work has several study limitations with respect to the plausible mechanism of the synergistic vascular protective effects of amlodipine added to candesartan. First, the addition of amlodipine to candesartan produced additive blood pressure lowering. Therefore, the present work did not allow us to exclude the possibility that the synergistic beneficial effects of their combination might be partially attributed to attenuation of oxidative stress caused by additive blood pressure lowering. Second, in this study, we tested the effect of amlodipine only with an insufficient dose because the main purpose was to test whether amlodipine added to candesartan can exert synergistic beneficial effects on metabolic syndrome. Thus, further study is needed to define the efficacy of higher dose of amlodipine in metabolic syndrome.29 Third, amlodipine is reported to have intrinsic mineralocorticoid receptor antagonist activity.30 However, the present study did not address the possibility of role of mineralocorticoid receptor in the synergistic effects of the combination therapy. Finally, it has been recently suggested that not all ARBs may exert the same beneficial effects on metabolic syndrome.31 Therefore, it is unclear whether the present observations can apply to all ARBs.

In conclusion, our present work provided the first evidence that the combination of an ARB plus a CCB exerted a synergistic protective effect against vascular insulin resistance and metabolic disorders in a rat model of metabolic syndrome. Given that ARB and CCB are recommended for treatment of human hypertension, our work has an important clinical implication and highlights the combination of an ARB and a CCB as a promising therapeutic strategy for hypertension with metabolic syndrome.
Supplementary material is linked to the online version of the paper at http://www.nature.com/ajh

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