

Anti-Atherogenic Effects of the Combination Therapy with Olmesartan and Azelnidipine in Diabetic Apolipoprotein E-Deficient Mice

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Many studies have aimed to identify anti-atherogenic agents in cardiovascular medicine. We have recently demonstrated that the combination therapy with olmesartan (OLM), an angiotensin II receptor blocker, and azelnidipine (AZL), a dihydropyridine calcium-channel blocker, improves endothelial function in diabetic Apolipoprotein-deficient (ApoE^{-/-}) mice. In the present study, we examined whether this combination therapy also inhibits atherosclerosis in mice. We used male control and streptozocin-induced diabetic ApoE^{-/-} mice. Diabetic ApoE^{-/-} mice were orally treated for 5 weeks with vehicle (Untreated), OLM (30 mg/kg/day), AZL (10 mg/kg/day), their combination (OLM+AZL), or hydralazine (HYD, 5 mg/kg/day) as an antihypertensive control. At 5 weeks, systolic blood pressure was significantly elevated in Untreated but was normalized in OLM+AZL and HYD. The atherosclerosis area in the thoracic aorta, perivascular fibrosis and medial thickness of the coronary arteries were increased in Untreated and were ameliorated in OLM+AZL but not in HYD. Staining with a fluorescent probe dihydroethidium showed that production of reactive oxygen species was increased in Untreated, and ameliorated in OLM+AZL. Consistent with these findings, macrophage infiltration in the kidney and the expression of receptor for advanced glycation end-products in the heart, kidney and liver were increased in Untreated and were all ameliorated in OLM+AZL, associated with up-regulation of endothelial NO synthases (eNOS). In conclusion, the combination therapy with OLM and AZL exerts anti-atherogenic effect in diabetic ApoE^{-/-} mice through suppression of oxidative stress and activation of eNOS, independent of its blood pressure-lowering effects. Clinically, this combination therapy may be useful for patients with hypertension, hyperlipidemia and diabetes.

Keywords: angiotensin II receptor blocker; atherosclerosis; calcium channel blocker; combination therapy; oxidative stress

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Endothelial dysfunction is one of the important mechanisms of atherosclerosis, for which reactive oxygen species (ROS) play a major role (Shimokawa 1999; Kawashima and Yokoyama 2004; Vanhoutte 2009). Atherosclerosis is a chronic inflammatory disease of the vascular wall, where pro-inflammatory cytokines are up-regulated and macrophages are activated with a resultant foam cell formation and ROS production (Libby et al. 2010). While many studies have been performed to identify anti-atherogenic agents, olmesartan (OLM), an angiotensin type 1 receptor (AT1R) blocker, has been found to decrease the expression of monocyte chemoattractant protein-1 (MCP-1), one of the major pro-inflammatory cytokines in animals (Jinno et al. 2004; Sukumaran et al. 2011) and humans (Fliser et al. 2004). On the other hand, we found the anti-atherogenic effect of azelnidipine (AZL), a dihydropyridine calcium

channel blocker with potent and long-acting blood pressure lowering effects without changing heart rate (Nakano et al. 2008). Indeed, we have previously demonstrated that the combination therapy with OLM and AZL markedly improve endothelium-dependent vasodilation via activation of the endothelial Akt-endothelial NO synthase (NOS) pathway in diabetic ApoE-deficient (ApoE^{-/-}) mice (Hosoya et al. 2010).

In diabetic state, advanced glycation end-products (AGEs) and its receptor, RAGE, are increased (Schmidt et al. 1999). This up-regulation of the AGEs-RAGE axis activates NADPH oxidase with a resultant ROS generation (Wautier et al. 2001) and atherosclerosis progression (Schmidt et al. 1999). It has been reported that some ARBs (including OLM) (Fujita et al. 2006) and nifedipine (a dihydropyridine CCB) reduce RAGE expression (Matsui et al.

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2009). Furthermore, it was recently reported that the combination therapy with OLM and AZL reduces ROS production via suppression of p22^{phox} and p47^{phox} expression and NADPH oxidase activity (Suzuki et al. 2005).

Renal failure is one of the important risk factors of cardiovascular diseases (Iwanaga and Miyazaki 2010). Because arteriosclerosis in the kidney, which could be caused by diabetes mellitus and dyslipidemia, induces ischemia and enhances fibrosis in the kidney (Lerman et al. 2009), it is important to protect renal function when treating life-style diseases, such as diabetes mellitus, dyslipidemia and hypertension. Similarly, liver fibrosis is also associated with life-style diseases, such as non-alcoholic steatohepatitis (NASH) and metabolic syndrome (Perseghin 2010).

In the present study, we thus examined whether the combination therapy with OLM and AZL exerts anti-atherogenic effects in diabetic ApoE^{-/-} mice in the heart, kidney and liver, and if so, what mechanisms are involved.

Methods

Animal Preparation

The present study was approved by the Research Committee of Tohoku University Graduate School of Medicine. We used male C57Bl/6N (control) and ApoE^{-/-} mice of 8-week-old, which were backcrossed to C57Bl/6 at least 10 times. They were fed a regular chow (Labo MR Stock, Nosan Corporation, Yokohama, Japan) throughout the experiment. The ApoE^{-/-} mice were made diabetic by 3 consecutive intraperitoneal injections of streptozocin (STZ) dissolved in citrate buffer, for a total cumulative dose of 210 mg/kg (85, 70 and 55 mg/kg on days 1, 2 and 3, respectively), while fasting (Kennedy and Zochodne 2000; How et al. 2006; Hosoya et al. 2010). Control mice received 3 consecutive intraperitoneal injections of the citrate buffer alone. Hyperglycemia was verified in a blood sample taken from a tail vein at 2 weeks after STZ injection, where fasting glucose levels more than 300 mg/dl were defined as diabetes (Morikawa et al. 2005). The whole-blood glucose test was carried out using Glutest-ace R (Sanwa Kagaku Kenkyusho Co, Nagoya, Japan).

The diabetic ApoE^{-/-} mice were orally administered either vehicle (Untreated), olmesartan (OLM, 30 mg/kg/day), azelnidipine (AZL, 10 mg/kg/day), their combination (OLM+AZL) or a vasodilator agent, hydralazine (HYD, 5 mg/kg/day), as an antihypertensive control, in their drinking water for the subsequent 5 weeks (Hosoya et al. 2010). OLM and AZL were suspended in carboxymethyl cellulose solution (WAKO). At the end of each treatment, body weight (BW) and systolic blood pressure (SBP, tail-cuff method) were measured. The animals were then anesthetized with intraperitoneal pentobarbital (50 mg/kg) and the animals were humanely killed.

Drugs

STZ and HYD were obtained from Sigma Chemical Co (St Louis, MO, USA). OLM and AZL were provided by Daiichi-Sankyo Pharmaceutical Co (Tokyo, Japan).

Western Blot Analysis

Western blot analyses were performed using antibodies that specifically recognize proteins, including eNOS, phospho-eNOS at Ser1177 (p-eNOS) and RAGE (Takaki et al. 2008; Tsoporis et al.

2010). The extent of eNOS phosphorylation at Ser1177 represents the eNOS activity (Bauer et al. 2003; Dudzinski and Michel 2007). The heart, kidneys and liver were homogenized and the proteins were extracted. The same amount of extracted protein (10–20 μ g) was loaded for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) immunoblot analysis. The regions containing proteins were visualized using ECL Plus Western blotting detection system (Amasham Biosciences, Buckinghamshire, UK). Each band was normalized by corresponding value of β -actin (Hosoya et al. 2010) or actin (Wang et al. 2011) as an internal control.

Histological and Immunohistochemical Analysis

Mice were anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg). The systemic circulatory system was perfused with Krebs buffer containing Na⁺ 144, K⁺ 5.9, Mg²⁺ 1.2, Ca²⁺ 2.5, H₂PO₄⁻ 1.2, HCO₃⁻ 24, Cl⁻ 129.7, and glucose 5.5 (mmol/L) via the left ventricle. Then, the aortic arch and the thoracic aorta were cut into 10 mm length from the top of the aortic arch, opened longitudinally, and were stained with Oil Red O. As a parameter of aortic atherosclerosis, the percentage of the plaque area stained by Oil Red O to the total luminal surface area was determined (Nakano et al. 2010). Histological studies were performed for determination of cardiomyocyte cross-sectional area, medial thickness (the ratio of medial thickness to internal diameter), perivascular fibrosis (the ratio of the fibrosis area surrounding the vessel to the total vessel area) and fibrosis area of the kidneys and liver (the ratio of fibrosis area to cross-sectional area), as previously reported (Lumeng et al. 2007; Rashid et al. 2009; Gandhi et al. 2012). The heart, kidneys and liver were fixed for 24–30 hours at room temperature in 4% PFA (paraformaldehyde) (Sigma Chemical Co) and embedded in paraffin. The sections were mounted on glass slides and depleted of paraffin with xylene. After blocking of endogenous biotin and avidin binding sites, sections were subjected to immunohistochemical staining overnight at 4°C with an anti-F4/80 monoclonal antibody (Abcam) (Nakajima et al. 2012). Immune complexes were detected with biotinylated secondary antibodies, HRP (horseradish peroxidase)-conjugated streptavidin and the peroxidase substrate diaminobenzidine. After staining with hematoxylin and eosin, mounting solution and cover slips were added to the sections. Slides were observed with a light microscope. All images were captured with an Olympus microscope equipped with a video camera (DP70, Tokyo, Japan) and analyzed using Adobe Photoshop.

Oxidative Fluorescent Microphotography

The heart, kidneys and liver were embedded in OCT without fixation, and immediately frozen by dry ice, then stored at -80°C. Each tissue was sectioned at 10- μ m thickness and stained using fluorescent probe dihydroethidium (DHE; Molecular Probes, Oregon, USA) as previously described (Yagi et al. 2010).

Statistical Analysis

Results are expressed as mean \pm SEM. Changes in BW, SBP and the plasma levels of glucose were analyzed by one-way ANOVA followed by Bonferroni/Dunn's post-hoc test for multiple comparisons. $P < 0.05$ was considered to be statistically significant.

Results

Body Weight and Systolic Blood Pressure

At 5 weeks after the treatment, body weight was significantly decreased in all ApoE^{-/-} mice groups compared

Table 1. Characteristics of the 6 Treatment Groups.

	Control (n = 6)	Untreated (n = 6)	OLM (n = 6)	AZL (n = 6)	HYD (n = 6)	OLM+AZL (n = 6)
Body weight (g)	29.4 ± 0.6	20.7 ± 0.9*	22.7 ± 0.7*	20.9 ± 1.4*	21.0 ± 0.7*	23.4 ± 1.2*
Systolic blood pressure (mmHg)	109 ± 3	124 ± 2*	112 ± 5 [†]	119 ± 6	113 ± 6 [†]	108 ± 4 [†]
Fasting glucose (mg/dl)	90 ± 6	477 ± 35*	470 ± 67*	417 ± 22*	439 ± 37*	402 ± 32*
Heart weight / Body weight	4.45 ± 0.04	4.62 ± 0.09*	4.59 ± 0.21*	4.56 ± 0.14*	4.58 ± 0.18*	4.55 ± 0.19*
Kidney weight / Body weight	12.6 ± 0.3	17.1 ± 0.7*	14.8 ± 0.57* [†]	15.9 ± 0.89*	18.7 ± 1.4*	15.2 ± 1.3*
Liver weight / Body weight	48.0 ± 0.9	67.9 ± 1.2*	62.9 ± 1.9*	63.0 ± 2.9*	64.4 ± 2.1*	68.0 ± 2.0*

Results are expressed as mean ± SEM. **P* < 0.05 vs. Control, [†]*P* < 0.05 vs. Untreated. Untreated, OLM, AZL, HYD, OLM+AZL; diabetic ApoE^{-/-} mice treated with OLM, AZL, HYD, and OLM plus AZL, respectively.

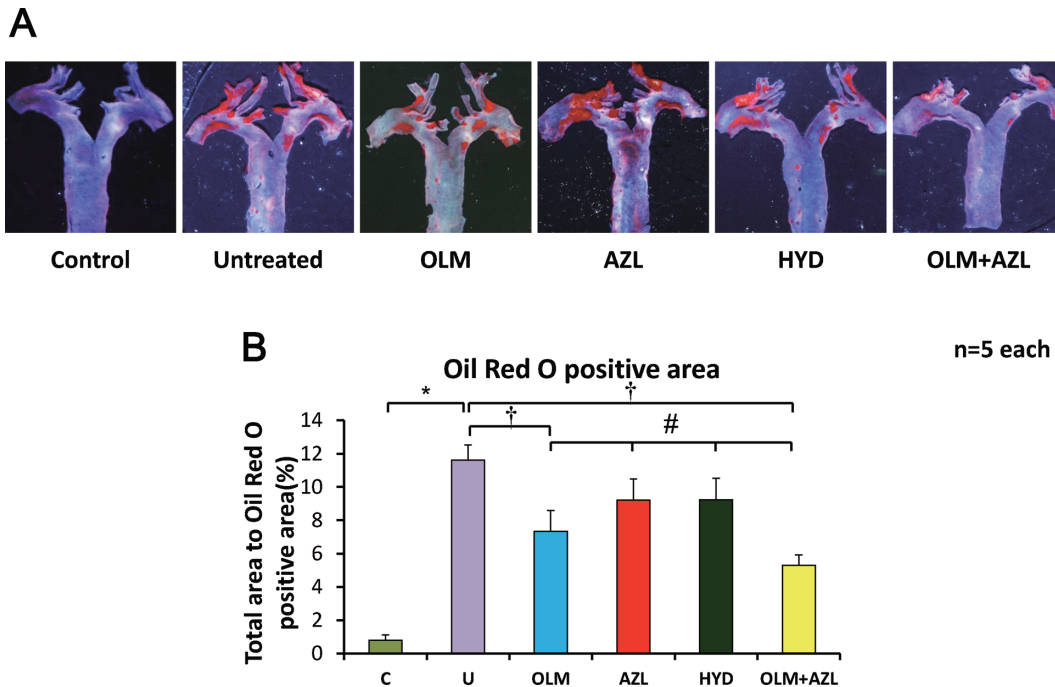


Fig. 1. Oil Red O staining of aortic arch and thoracic aorta.

(A) Representative photographs of Oil Red O staining of the aorta. Control, normal mice; Untreated, vehicle-treated diabetic ApoE^{-/-} mice; AZL, AZL-treated diabetic ApoE^{-/-} mice; OLM, OLM-treated diabetic ApoE^{-/-} mice; HYD, HYD-treated diabetic ApoE^{-/-} mice; and OLM+AZL, diabetic ApoE^{-/-} mice treated with AZL plus OLM. (B) Oil Red O positive area was significantly increased in Untreated compared with Control, but was normalized in OLM and OLM+AZL. Results are mean ± SEM (*n* = 5 each). **P* < 0.01 vs. Control, [†]*P* < 0.05 vs. Untreated, #*P* < 0.05 vs. OLM+AZL.

with Control. In contrast, systolic blood pressure was significantly increased in Untreated, which was significantly suppressed in OLM, HYD and OLM+AZL, to the same extent compared with Control (Table 1). The tissue weights of the heart, kidney and liver were comparable among the diabetic ApoE^{-/-} mice groups (Table 1).

Effects of the Drug Treatments on Atherosclerosis

Oil Red O staining demonstrated that the extent of aortic atherosclerosis was significantly increased in Untreated compared with Control, but was significantly suppressed in OLM and further suppressed in OLM+AZL (Fig. 1). Importantly, no significant suppression was detected in AZL or HYD. Atherosclerosis area in OLM+AZL was significantly decreased compared with that in AZL or HYD (Fig.

1).

Effects of the Drug Treatments on the Coronary Artery and Cardiomyocyte

Compared with Control, the extent of perivascular fibrosis of the coronary artery was significantly increased in Untreated, but was significantly suppressed in OLM and OLM+AZL (Fig. 2A, B). In contrast, no significant suppression was detected in AZL or HYD. Furthermore, the extent of medial thickening of the coronary artery was significantly increased in Untreated compared with Control, not significantly suppressed in OLM, AZL or HYD, but was significantly suppressed in OLM+AZL (Fig. 2C). We next evaluated cardiomyocyte hypertrophy as it is linked to cardiac hypertrophy (Rashid et al. 2009). The extent of cardiomyocyte cross-sectional area was also increased in Untreated and was significantly suppressed only in OLM+AZL, whereas systolic blood pressure was unaltered (Fig. 2D, E).

Effects of the Drug Treatments on Parenchymal Organ Damage

To evaluate the effect of the drug treatments on the kidney and liver, we analyzed the extent of fibrosis area. In the kidney, the fibrosis area was increased in Untreated compared with Control and was improved in OLM and OLM+AZL, but not in HYD despite the same blood pressure level (Fig. 2F, G). In the liver, the fibrosis area was significantly increased in Untreated compared with Control and was improved only in OLM+AZL (Fig. 2F, H).

Oxidative Stress

Because ROS was one of the major causes of atherosclerosis, we next evaluated the oxidative stress in each group. In the heart, kidney and liver, DHE staining showed that ROS production was significantly increased in Untreated compared with Control, but was significantly suppressed in OLM and OLM+AZL. In the kidney and liver, ROS production was also significantly inhibited in AZL, but not in HYD (Fig. 3A, B).

Macrophage Infiltration

In order to identify the source of ROS production, we examined macrophage infiltration by F4/80 staining as a marker of matured macrophages (Weisberg et al. 2003). In the kidney, the number of F4/80-positive cells was markedly increased in Untreated compared with Control, but was significantly suppressed in OLM and AZL, and was further suppressed in OLM+AZL (Fig. 4A, B). In the aorta, the number of F4/80 positive cells was markedly increased as in the kidney, but was significantly decreased to the same extent in OLM, AZL and OLM+AZL (Fig. 4A, C). In the liver, the infiltration of F4/80-positive cells was observed only in Untreated (data not shown). On the other hand, macrophages were not noted in the heart in any groups (data not shown).

RAGE Expression

In order to further identify other source of ROS production, we examined RAGE expression that is related to ROS production (Wautier et al. 2001). In all the organs examined (the heart, kidneys and liver), RAGE expression was significantly increased in Untreated compared with Control, and was significantly suppressed in AZL and OLM+AZL (Fig. 5A, B).

Expression and Activity of eNOS

As compared with Untreated, the expression of total eNOS protein was significantly increased in the kidneys and liver (but not in the hearts) in OLM+AZL (Fig. 6A, B). In contrast, the extent of eNOS phosphorylation, a marker of eNOS activation, was significantly increased in the heart (but not in the kidney or liver) in OLM+AZL as compared with Untreated (Fig. 6C, D).

Discussion

The novel finding of the present study is that the combination therapy with OLM and AZL exerts anti-atherogenic effects compared to each mono-therapy in diabetic ApoE^{-/-} mice through reduction of ROS production via at least 3 different effects: the increase in eNOS expression and activity, the decrease in RAGE expression, and the inhibition of macrophage infiltration (Fig. 7). On the other hand, reduced ROS production may also increase NO availability, decrease RAGE, and inhibit macrophage infiltration. Since HYD, which caused a similar extent of blood pressure-lowering effect as did OLM+AZL, failed to suppress the progression of atherosclerosis, the beneficial effects of the combination therapy with OLM and AZL appear to be independent of its blood pressure-lowering effects.

Pharmacological characteristic of OLM, AZL and HYD

OLM is a specific AT1 receptor antagonist characterized by its strong and long-acting blood pressure lowering effects (Mire et al. 2005). AZL is a dihydropyridine calcium channel blocker with potent and long-acting blood pressure lowering effects without changing heart rate (Kuramoto et al. 2003). HYD is a vasodilator agent with only a few pleiotropic effects besides blood pressure lowering effects (Kandler et al. 2011). This is why we used HYD as a control anti-hypertensive agent in the present study. We have previously demonstrated that the combination therapy with OLM plus AZL ameliorates endothelial function (Hosoya et al. 2010); however, it remains to be examined whether this combination therapy also suppresses atherosclerosis and parenchymal tissue damage such in the kidney and the liver. In the present study, we addressed this important point.

It has been reported that some ARBs, including OLM, improve endothelial function and reduce the expression of RAGE and pro-inflammatory cytokines (e.g. MCP-1) (Matsui et al. 2007). This suggests that the beneficial effects of OLM noted in the present study are class effects

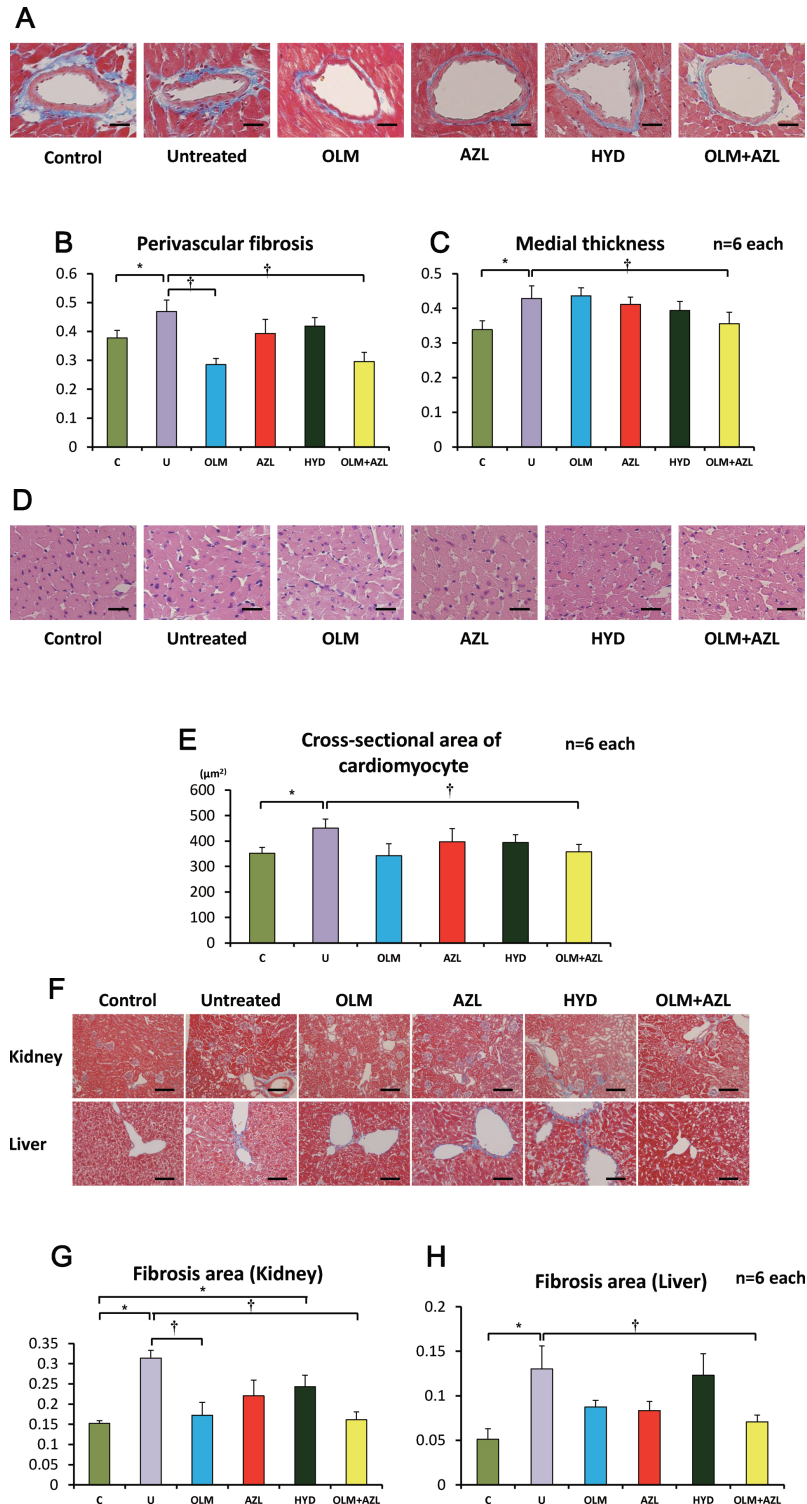


Fig. 2. Histological analysis of coronary arteries and cardiomyocytes.

(A) Representative microphotographs of the coronary arteries (Masson's trichrome staining) in the 6 treatment groups. See Figure 1 for the 6 treatment groups. Scale bar = 50 μm . (B) The perivascular fibrosis of the coronary arteries was significantly increased in Untreated compared with Control, but ameliorated in OLM and OLM+AZL. (C) Medial thickening of coronary artery was increased in Untreated compared with Control, and ameliorated only in OLM+AZL. (D) Representative microphotographs of cardiomyocytes (H&E) in the 6 treatment groups. Scale bar = 50 μm . (E) Compared with Control, cross-sectional area of cardiomyocyte was increased in Untreated but was ameliorated in OLM+AZL. (F) Representative microphotographs of the kidney and liver (Masson's trichrome staining) in the 6 treatment groups. Scale bar = 50 μm . (G) Fibrosis area was corrected by cross-sectional area of the kidney. Fibrosis area was significantly increased in Untreated and HYD and ameliorated in OLM and OLM+AZL. (H) Fibrosis area of the liver was increased in Untreated compared with Control, and was ameliorated only in OLM+AZL. Results are mean \pm SEM ($n = 6$ each). * $P < 0.05$ vs. Control, † $P < 0.05$ vs. Untreated.

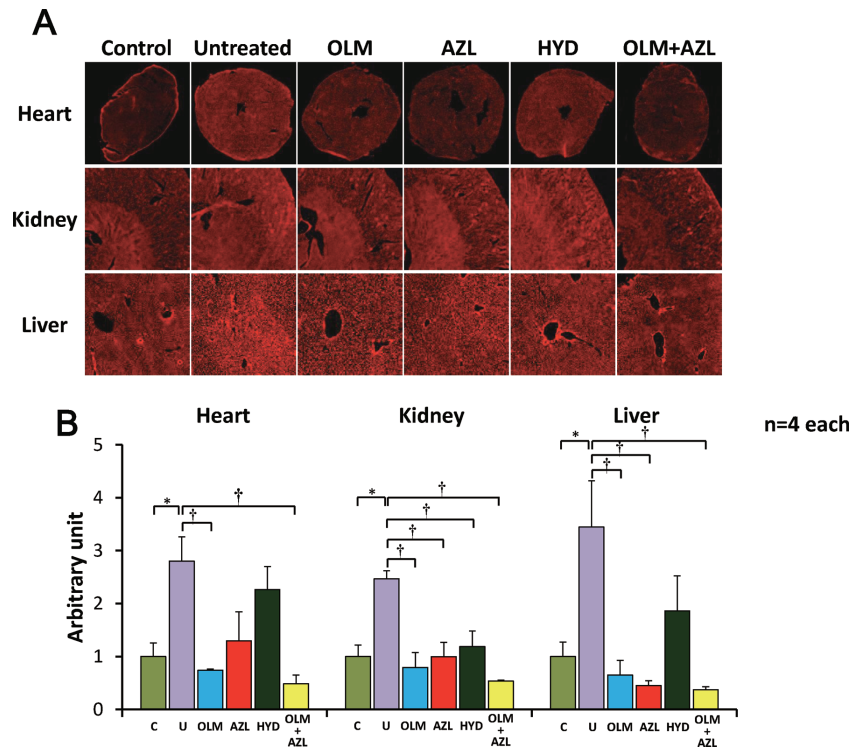


Fig. 3. DHE staining of the heart, kidney and liver.

(A) Representative DHE staining of the heart, kidney and liver in the 6 treatment groups. Unfixed frozen tissues were cut in 10- μ m thickness, and were then dyed using fluorescent probe dihydroethidium (0.5 μ M). (B) Superoxide production in the heart, kidney and liver. As compared with Control, the ROS production was increased in Untreated, and was markedly ameliorated by the OLM+AZL treatment. Results are expressed as mean \pm SEM ($n = 4$ each), * $P < 0.05$ vs. Control, $\dagger P < 0.05$ vs. Untreated.

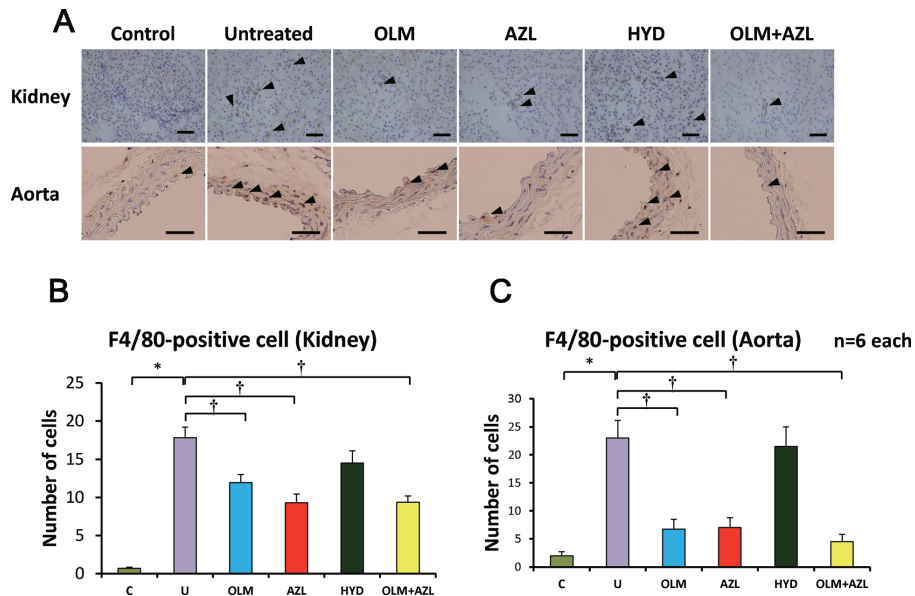


Fig. 4. Immunohistostaining for F4/80, a specific marker for mature macrophages, in the kidney and aorta.

(A) Representative immunohistostaining for F4/80 of the kidney and aorta in the 6 treatment groups. Scale bar = 50 μ m. (B) In the kidney, the number of F4/80-positive macrophages was markedly increased in Untreated compared with Control, and was significantly ameliorated in OLM, AZL and was further ameliorated by OLM+AZL. (C) In the aorta, the number of F4/80-positive cells was markedly increased in Untreated compared with Control, and was significantly decreased in OLM, AZL and OLM+AZL. Results are expressed as mean \pm SEM ($n = 6$ each), * $P < 0.05$ vs. Control, $\dagger P < 0.05$ vs. Untreated.

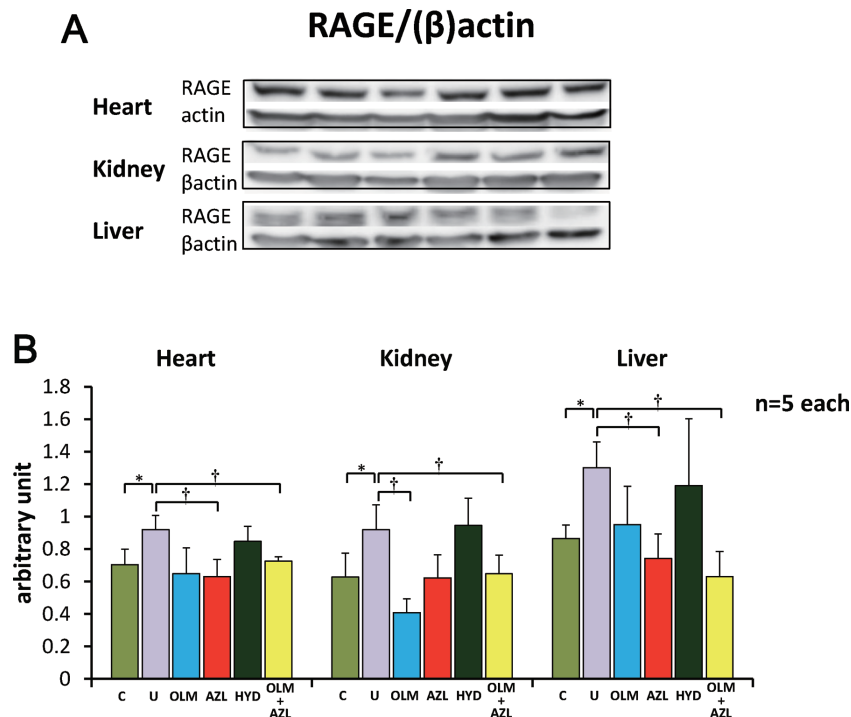


Fig. 5. Protein expression of Receptor for Advanced Glycation End-products (RAGE).

(A, B) The protein expressions of RAGE in the heart and kidney were increased in Untreated compared with Control, and was ameliorated in OLM+AZL. Similarly, the protein expressions of RAGE in the liver were increased in Untreated compared with Control, and was ameliorated in AZL and OLM+AZL. From the left, the order of the lanes was control, Untreated, OLM, AZL, HYD, and OLM+AZL. Results are expressed as mean \pm SEM ($n = 5$ each), * $P < 0.05$ vs. Control, † $P < 0.05$ vs. Untreated.

of ARBs. On the other hands, nifedipine, another dihydropyridine CCB, has previously been reported to maintain endothelial function (Luscher et al. 2009) and exert anti-atherogenic and anti-inflammatory effects (Matsui et al. 2009). This also suggests that the beneficial effects of AZL noted in the present study are also class effects of dihydropyridine CCBs.

Anti-atherogenic Effects of OLM plus AZL

OLM and AZL are new ARB and CCB with more potent vasodilator effects compared to other drugs in the same class (Kuramoto et al. 2003; Mire et al. 2005; Kojima et al. 2011). Furthermore, we have previously demonstrated that their combination ameliorate endothelial function to a greater extent than each monotherapy in ApoE^{-/-} mice (Hosoya et al. 2010); however, it remains to be examined whether this combination therapy also suppresses atherosclerosis and parenchymal tissue damage such in the kidney and the liver. In the present study, we addressed this important point. It is well known that enhanced ROS production accelerates atherosclerosis and mono-therapy with an ARB or a CCB decrease ROS production in animal models (Tsuda et al. 2005; Yamamoto et al. 2008). The present study further demonstrates that the combination therapy with OLM plus AZL exerts additive anti-atherogenic effects in diabetic ApoE^{-/-} mice, which could explain, at least in part, the beneficial effects of this combination therapy in

humans (Kojima et al. 2011). The beneficial effects of the combination therapy were also noted in the heart (coronary artery and cardiomyocytes), kidney and liver compared with each mono-therapy, independent of blood pressure. Indeed, it has been previously reported that this combination therapy is more effective in reducing albuminuria and oxidative stress in hypertensive diabetic patients with CKD (Abe et al. 2011). Thus, these lines of evidence support the beneficial effects of the combination therapy for the treatment of atherosclerotic cardiovascular diseases.

Anti-oxidant Effects of OLM plus AZL

ROS play a major role in the pathogenesis of atherosclerosis and fibrosis of parenchymal organs, where activated macrophages generate ROS and cause interstitial fibrosis (Ricardo et al. 2008). The present study demonstrates that the combination therapy with OLM plus AZL markedly reduces ROS production. It has been already reported that OLM and AZL decrease the expression of cytokines, such as MCP-1 and TNF- α , more than each mono-therapy (Inaba et al. 2009), which mechanism may be involved for the reduced macrophage infiltration in the present study. In diabetic state, AGEs and its receptor, RAGE, are increased though pro-inflammatory cytokines such as TNF- α (Tanaka et al. 2000). The activation of the AGEs-RAGE axis generates ROS through activation of NADPH oxidase (Wautier et al. 2001). It has been reported

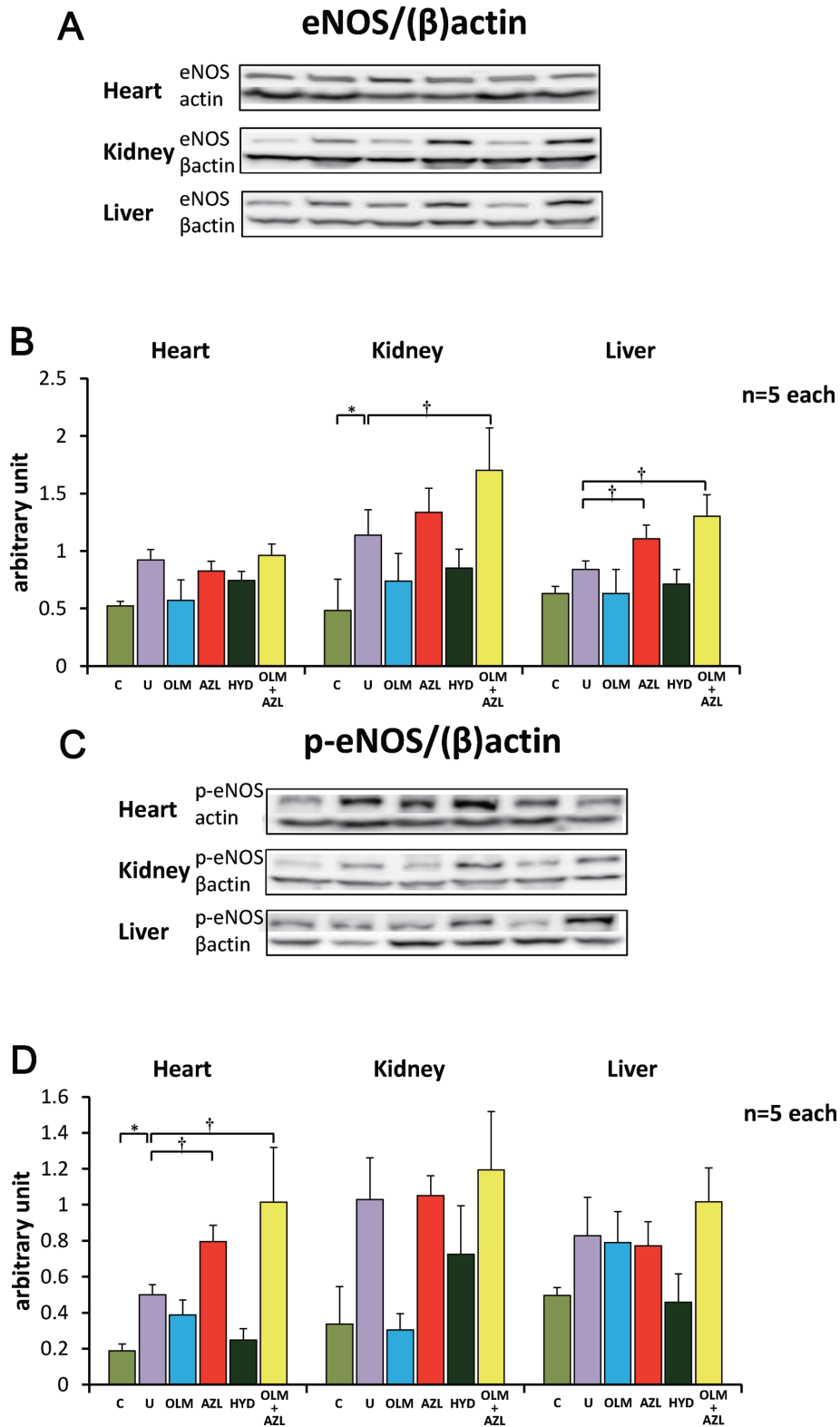


Fig. 6. Protein expression and phosphorylation of eNOS.

(A, B) As compared with the Untreated, the expression of total eNOS protein in the kidney and liver were significantly higher in OLM+AZL. (C, D) In contrast, the extent of eNOS phosphorylation in the heart, a marker of eNOS activation, was significantly increased in OLM+AZL as compared with the Untreated. See Figure 1 for the 6 treatment groups. Results are mean \pm SEM ($n = 5$ each), * $P < 0.05$ vs. Control, † $P < 0.05$ vs. Untreated.

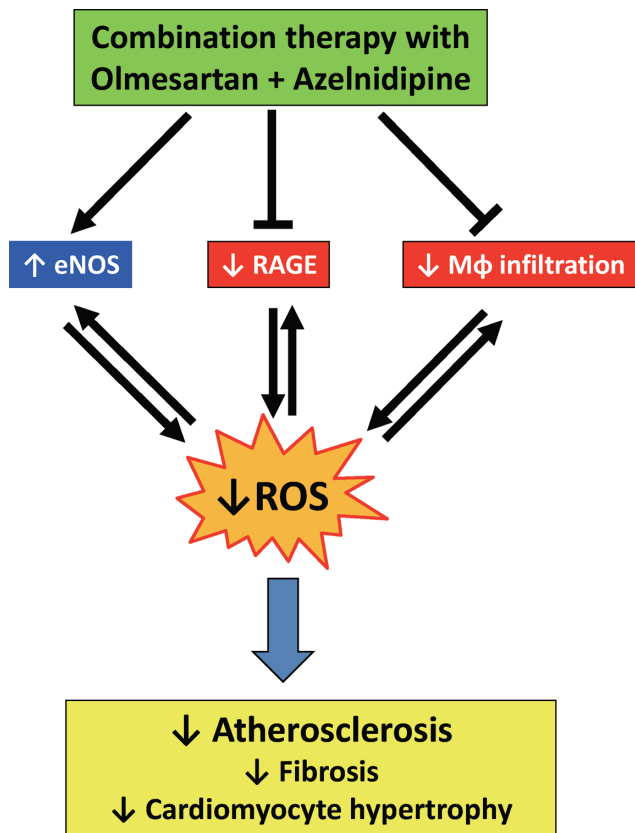


Fig. 7. Mechanisms for the beneficial effects of the combination therapy with OLM and AZL.

The combination therapy with OLM and AZL exerts at least 3 different effects, including increase in eNOS expression and activity and inhibition of RAGE expression and macrophage infiltration with a resultant decrease in ROS production. Reduced ROS production may also increase NO availability, decrease RAGE and inhibit macrophage infiltration. The arrowheads indicate stimulation and the bar heads inhibition.

that some ARBs (including OLM) (Yoshida et al. 2006; Yamagishi et al. 2008) and nifedipine, another dihydropyridine CCB, also suppress the AGEs-RAGE expressions (Matsui et al. 2010). It was previously reported that OLM reduces serum levels of the RAGE ligands, such as AGEs (Nangaku et al. 2003) and N-(epsilon)-carboxymethyllysine (CML) (Honda et al. 2012), and that AZL also reduces AGEs (Nakamura et al. 2011). Indeed, it has been recently demonstrated that the combination therapy with OLM and AZL reduces ROS production through suppression of p22^{phox} and p47^{phox} expression and NADPH oxidase activity (Suzuki et al. 2005). In the present study, we were able to demonstrate that AZL reduces RAGE expression and the combination therapy with OLM exerts additive effects, which may be involved in the beneficial effects of OLM plus AZL on ROS production in the present study.

Beneficial Effects of OLM plus AZL on Endothelial Function

NO produced by eNOS is a major substance that main-

tains endothelial functions, inhibiting leukocyte adhesion, vascular smooth muscle migration and proliferation, and platelet aggregation. We have previously demonstrated that the eNOS system has diverse vasodilator functions for NO-generating system in large arteries and EDHF/H₂O₂-generating system in microvessels (Takaki et al. 2008; Ohashi et al. 2012). Cardiovascular risk factors, such as diabetes mellitus, hypertension and hyperlipidemia impair endothelial function and suppress eNOS activity, thus promoting atherosclerosis and cardiovascular diseases (Shimokawa 1999; Vanhoutte 2009). In the present study, we were able to demonstrate that the combination therapy with OLM plus AZL increases the expression and activity of eNOS in the heart, kidney and liver.

The crosstalk among ROS, RAGE and eNOS

Although the combination therapy with OLM plus AZL exerted anti-atherogenic effects via suppression of ROS, the detailed mechanism(s) remains to be elucidated. It is, however, conceivable that the combination therapy with OLM and AZL decreases ROS production probably through eNOS activation and inhibition of RAGE and macrophage infiltration, while reduced ROS simultaneously increase NO availability, decrease RAGE and inhibit macrophage infiltration via suppression of pro-inflammatory cytokines (Fig. 7) (Tanaka et al. 2000; Charo and Taubman 2004). NO scavenges superoxide and causes up-regulation of superoxide dismutase (SOD) expression (Shimokawa 1999; Kawashima and Yokoyama 2004; Vanhoutte 2009). Thus, up-regulation of eNOS increases NO bioavailability, contributing to the maintenance of endothelial function and reduction of ROS. Conversely, reduced ROS production suppresses the RhoA/Rho-kinase signaling pathway (Knock and Ward 2011) that is known to decrease eNOS expression (Ming et al. 2002), thereby leading to eNOS up-regulation. Thus, the combination therapy is likely to inhibit the vicious cycle mentioned above.

Study Limitations

Several limitations should be mentioned for the present study. First, the present diabetic ApoE^{-/-} mouse model is an extreme animal model of atherosclerosis and relatively higher doses of OLM and AZL were used as compared with the clinical setting. Thus, the present findings remain to be confirmed in patients with diabetes and dyslipidemia in future studies with the clinical doses of the drugs. Second, although we examined several factors related to oxidative stress, including ROS production, RAGE expression, macrophage infiltration, and eNOS down-regulation, many other factors related to oxidative stress remain to be examined. Third, it remains to be examined whether the beneficial effects of the combination therapy with OLM and AZL actually ameliorates the long-term prognosis in diabetic ApoE^{-/-} mice. Fourth, we did not examine insulin secretion or insulin sensitivity in the present study in the present mouse model of type 1 diabetes mellitus.

Conclusion

The combination therapy with OLM plus AZL exerts anti-atherogenic effects compared to each mono-therapy in diabetic ApoE^{-/-} mice probably via reduction of oxidative stress and eNOS up-regulation, independent of its blood pressure lowering effects.

Acknowledgments

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Conflict of Interest

The authors declare that there is no conflict of interest to disclose regarding the present study.

References

- Abe, M., Maruyama, N., Okada, K., Matsumoto, S., Matsumoto, K. & Soma, M. (2011) Additive antioxidative effects of azelnidipine on angiotensin receptor blocker olmesartan treatment for type 2 diabetic patients with albuminuria. *Hypertens. Res.*, **34**, 935-941.
- Bauer, P.M., Fulton, D., Boo, Y.C., Sorescu, G.P., Kemp, B.E., Jo, H. & Sessa, W.C. (2003) Compensatory phosphorylation and protein-protein interactions revealed by loss of function and gain of function mutants of multiple serine phosphorylation sites in endothelial nitric-oxide synthase. *J. Biol. Chem.*, **278**, 14841-14849.
- Charo, I.F. & Taubman, M.B. (2004) Chemokines in the pathogenesis of vascular disease. *Circ. Res.*, **95**, 858-866.
- Dudzinski, D.M. & Michel, T. (2007) Life history of eNOS: partners and pathways. *Cardiovasc. Res.*, **75**, 247-260.
- Fliser, D., Buchholz, K. & Haller, H. (2004) Antiinflammatory effects of angiotensin II subtype 1 receptor blockade in hypertensive patients with microinflammation. *Circulation*, **110**, 1103-1107.
- Fujita, M., Okuda, H., Tsukamoto, O., Asano, Y., Hirata, Y.L., Kim, J., Miyatsuka, T., Takashima, S., Minamino, T., Tomoike, H. & Kitakaze, M. (2006) Blockade of angiotensin II receptors reduces the expression of receptors for advanced glycation end products in human endothelial cells. *Arterioscler. Thromb. Vasc. Biol.*, **26**, e138-142.
- Gandhi, S., Srinivasan, B.P. & Akarte, A.S. (2012) Effective blockade of RAAS by combination of aliskiren and olmesartan improves glucose homeostasis, glomerular filtration rate along with renal variables in streptozotocin induced diabetic rats. *Euro. J. Pharm. Sci.*, **46**, 32-42.
- Honda, H., Hosaka, N., Aoshima, Y., Hirai, Y., Michihata, T. & Akizawa, T. (2012) Olmesartan medoxomil is associated with decreased plasma AGEs, pentosidine, and N-(epsilon)-carboxymethyl-lysine levels in hemodialysis patients. *Clin. Exp. Hypertens.*, **34**, 17-23.
- Hosoya, M., Ohashi, J., Sawada, A., Takaki, A. & Shimokawa, H. (2010) Combination therapy with olmesartan and azelnidipine improves EDHF-mediated responses in diabetic apolipoprotein E-deficient mice. *Circ. J.*, **74**, 798-806.
- How, O.J., Aasum, E., Severson, D.L., Chan, W.Y., Essop, M.F. & Larsen, T.S. (2006) Increased myocardial oxygen consumption reduces cardiac efficiency in diabetic mice. *Diabetes*, **55**, 466-473.
- Inaba, S., Iwai, M., Tomono, Y., Senba, I., Furuno, M., Kanno, H., Okayama, H., Mogi, M., Higaki, J. & Horiuchi, M. (2009) Prevention of vascular injury by combination of an AT1 receptor blocker, olmesartan, with various calcium antagonists. *Am. J. Hypertens.*, **22**, 145-150.
- Iwanaga, Y. & Miyazaki, S. (2010) Heart failure, chronic kidney disease, and biomarkers—an integrated viewpoint—. *Circ. J.*, **74**, 1274-1282.
- Jinno, T., Iwai, M., Li, Z., Li, J.M., Liu, H.W., Cui, T.X., Rakugi, H., Ogihara, T. & Horiuchi, M. (2004) Calcium channel blocker azelnidipine enhances vascular protective effects of AT1 receptor blocker olmesartan. *Hypertension*, **43**, 263-269.
- Kandler, M.R., Mah, G.T., Tejani, A.M., Stabler, S.N. & Salzwedel, D.M. (2011) Hydralazine for essential hypertension. *Cochrane Database Syst Rev*, CD004934.
- Kawashima, S. & Yokoyama, M. (2004) Dysfunction of endothelial nitric oxide synthase and atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.*, **24**, 998-1005.
- Kennedy, J.M. & Zochodne, D.W. (2000) The regenerative deficit of peripheral nerves in experimental diabetes: its extent, timing and possible mechanisms. *Brain*, **123**, 2118-2129.
- Knock, G.A. & Ward, J.P. (2011) Redox regulation of protein kinases as a modulator of vascular function. *Antioxid. Redox Signal.*, **15**, 1531-1547.
- Kojima, T., Miyauchi, K., Yokoyama, T., Yokoyama, K., Kurata, T., Suwa, S., Kawamura, M., Tamura, H., Okazaki, S., Inoue, K., Fujiwara, Y., Sumiyoshi, M., Tanimoto, K., Nakazato, Y., Yamagami, S., Hiro, T., Komiyama, N. & Daida, H. (2011) Azelnidipine and amlodipine anti-coronary atherosclerosis trial in hypertensive patients undergoing coronary intervention by serial volumetric intravascular ultrasound analysis in Juntendo University (ALPS-J). *Circ. J.*, **75**, 1071-1079.
- Kuramoto, K., Ichikawa, S., Hirai, A., Kanada, S., Nakachi, T. & Ogihara, T. (2003) Azelnidipine and amlodipine: a comparison of their pharmacokinetics and effects on ambulatory blood pressure. *Hypertens. Res.*, **26**, 201-208.
- Lerman, L.O., Textor, S.C. & Grande, J.P. (2009) Mechanisms of tissue injury in renal artery stenosis: ischemia and beyond. *Prog. Cardiovasc. Dis.*, **52**, 196-203.
- Libby, P., Okamoto, Y., Rocha, V.Z. & Folco, E. (2010) Inflammation in atherosclerosis: transition from theory to practice. *Circ. J.*, **74**, 213-220.
- Lumeng, C.N., Bodzin, J.L. & Saltiel, A.R. (2007) Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *J. Clin. Invest.*, **117**, 175-184.
- Luscher, T.F., Pieper, M., Tendera, M., Vrolix, M., Rutsch, W., van den Branden, F., Gil, R., Bischoff, K.O., Haude, M., Fischer, D., Meinertz, T. & Munzel, T. (2009) A randomized placebo-controlled study on the effect of nifedipine on coronary endothelial function and plaque formation in patients with coronary artery disease: the ENCORE II study. *Eur. Heart J.*, **30**, 1590-1597.
- Matsui, T., Yamagishi, S., Takeuchi, M., Ueda, S., Fukami, K. & Okuda, S. (2009) Nifedipine, a calcium channel blocker, inhibits advanced glycation end product (AGE)-elicited mesangial cell damage by suppressing AGE receptor (RAGE) expression via peroxisome proliferator-activated receptor-gamma activation. *Biochem. Biophys. Res. Commun.*, **385**, 269-272.
- Matsui, T., Yamagishi, S., Takeuchi, M., Ueda, S., Fukami, K. & Okuda, S. (2010) Nifedipine inhibits advanced glycation end products (AGEs) and their receptor (RAGE) interaction-mediated proximal tubular cell injury via peroxisome proliferator-activated receptor-gamma activation. *Biochem. Biophys. Res. Commun.*, **398**, 326-330.
- Matsui, T., Yamagishi, S., Ueda, S., Nakamura, K., Imaizumi, T., Takeuchi, M. & Inoue, H. (2007) Telmisartan, an angiotensin II type 1 receptor blocker, inhibits advanced glycation end-product (AGE)-induced monocyte chemoattractant protein-1

- expression in mesangial cells through downregulation of receptor for AGEs via peroxisome proliferator-activated receptor-gamma activation. *J. Int. Med. Res.*, **35**, 482-489.
- Ming, X.F., Viswambharan, H., Barandier, C., Ruffieux, J., Kaibuchi, K., Ruconi, S. & Yang, Z. (2002) Rho GTPase/Rho kinase negatively regulates endothelial nitric oxide synthase phosphorylation through the inhibition of protein kinase B/Akt in human endothelial cells. *Mol. Cell. Biol.*, **22**, 8467-8477.
- Mire, D.E., Silfani, T.N. & Pugsley, M.K. (2005) A review of the structural and functional features of olmesartan medoxomil, an angiotensin receptor blocker. *J. Cardiovasc. Pharmacol.*, **46**, 585-593.
- Morikawa, K., Matoba, T., Kubota, H., Hatanaka, M., Fujiki, T., Takahashi, S., Takeshita, A. & Shimokawa, H. (2005) Influence of diabetes mellitus, hypercholesterolemia, and their combination on EDHF-mediated responses in mice. *J. Cardiovasc. Pharmacol.*, **45**, 485-490.
- Nakajima, S., Ohashi, J., Sawada, A., Noda, K., Fukumoto, Y. & Shimokawa, H. (2012) Essential role of bone marrow for microvascular endothelial and metabolic functions in mice. *Circ. Res.*, **111**, 87-96.
- Nakamura, T., Sato, E., Fujiwara, N., Kawagoe, Y., Koide, H., Ueda, Y., Takeuchi, M. & Yamagishi, S. (2011) Calcium channel blocker inhibition of AGE and RAGE axis limits renal injury in nondiabetic patients with stage I or II chronic kidney disease. *Clin. Cardiol.*, **34**, 372-377.
- Nakano, K., Egashira, K., Ohtani, K., Gang, Z., Iwata, E., Miyagawa, M. & Sunagawa, K. (2008) Azelnidipine has anti-atherosclerotic effects independent of its blood pressure-lowering actions in monkeys and mice. *Atherosclerosis*, **196**, 172-179.
- Nakano, M., Fukumoto, Y., Satoh, K., Ito, Y., Kagaya, Y., Ishii, N., Sugamura, K. & Shimokawa, H. (2010) OX40 ligand plays an important role in the development of atherosclerosis through vasa vasorum neovascularization. *Cardiovasc. Res.*, **88**, 539-546.
- Nangaku, M., Miyata, T., Sada, T., Mizuno, M., Inagi, R., Ueda, Y., Ishikawa, N., Yuzawa, H., Koike, H., van Ypersele de Strihou, C. & Kurokawa, K. (2003) Anti-hypertensive agents inhibit in vivo the formation of advanced glycation end products and improve renal damage in a type 2 diabetic nephropathy rat model. *J. Am. Soc. Nephrol.*, **14**, 1212-1222.
- Ohashi, J., Sawada, A., Nakajima, S., Noda, K., Takaki, A. & Shimokawa, H. (2012) Mechanisms for enhanced endothelium-derived hyperpolarizing factor-mediated responses in microvessels in mice. *Circ. J.*, **76**, 1768-1779.
- Rashid, M., Tawara, S., Fukumoto, Y., Seto, M., Yano, K. & Shimokawa, H. (2009) Importance of Rac1 signaling pathway inhibition in the pleiotropic effects of HMG-CoA reductase inhibitors. *Circ. J.*, **73**, 361-370.
- Ricardo, S.D., van Goor, H. & Eddy, A.A. (2008) Macrophage diversity in renal injury and repair. *J. Clin. Invest.*, **118**, 3522-3530.
- Schmidt, A.M., Yan, S.D., Wautier, J.L. & Stern, D. (1999) Activation of receptor for advanced glycation end products: a mechanism for chronic vascular dysfunction in diabetic vasculopathy and atherosclerosis. *Circ. Res.*, **84**, 489-497.
- Shimokawa, H. (1999) Primary endothelial dysfunction: atherosclerosis. *J. Mol. Cell. Cardiol.*, **31**, 23-37.
- Sukumaran, V., Watanabe, K., Veeraveedu, P.T., Gurusamy, N., Ma, M., Thandavarayan, R.A., Lakshmanan, A.P., Yamaguchi, K., Suzuki, K. & Kodama, M. (2011) Olmesartan, an AT1 antagonist, attenuates oxidative stress, endoplasmic reticulum stress and cardiac inflammatory mediators in rats with heart failure induced by experimental autoimmune myocarditis. *Int. J. Biol. Sci.*, **7**, 154-167.
- Suzuki, J., Iwai, M., Li, Z., Li, J.M., Min, L.J., Ide, A., Yoshii, T., Oshita, A., Mogi, M. & Horiuchi, M. (2005) Effect of combination of calcium antagonist, azelnidipine, and AT1 receptor blocker, olmesartan, on atherosclerosis in apolipoprotein E-deficient mice. *J. Hypertens.*, **23**, 1383-1389.
- Takaki, A., Morikawa, K., Tsutsui, M., Murayama, Y., Tekes, E., Yamagishi, H., Ohashi, J., Yada, T., Yanagihara, N. & Shimokawa, H. (2008) Crucial role of nitric oxide synthases system in endothelium-dependent hyperpolarization in mice. *J. Exp. Med.*, **205**, 2053-2063.
- Tanaka, N., Yonekura, H., Yamagishi, S., Fujimori, H., Yamamoto, Y. & Yamamoto, H. (2000) The receptor for advanced glycation end products is induced by the glycation products themselves and tumor necrosis factor-alpha through nuclear factor-kappaB, and by 17beta-estradiol through Sp-1 in human vascular endothelial cells. *J. Biol. Chem.*, **275**, 25781-25790.
- Tsoporis, J.N., Izhar, S., Leong-Poi, H., Desjardins, J.F., Huttunen, H.J. & Parker, T.G. (2010) S100B interaction with the receptor for advanced glycation end products (RAGE): a novel receptor-mediated mechanism for myocyte apoptosis postinfarction. *Circ. Res.*, **106**, 93-101.
- Tsuda, M., Iwai, M., Li, J.M., Li, H.S., Min, L.J., Ide, A., Okumura, M., Suzuki, J., Mogi, M., Suzuki, H. & Horiuchi, M. (2005) Inhibitory effects of AT1 receptor blocker, olmesartan, and estrogen on atherosclerosis via anti-oxidative stress. *Hypertension*, **45**, 545-551.
- Vanhoutte, P.M. (2009) Endothelial dysfunction: the first step toward coronary arteriosclerosis. *Circ. J.*, **73**, 595-601.
- Wang, W., Kagaya, Y., Asaumi, Y., Fukui, S., Takeda, M. & Shimokawa, H. (2011) Protective effects of recombinant human erythropoietin against pressure overload-induced left ventricular remodeling and premature death in mice. *Tohoku J. Exp. Med.*, **225**, 131-143.
- Wautier, M.P., Chappey, O., Corda, S., Stern, D.M., Schmidt, A.M. & Wautier, J.L. (2001) Activation of NADPH oxidase by AGE links oxidant stress to altered gene expression via RAGE. *Am. J. Physiol. Endocrinol. Metab.*, **280**, E685-694.
- Weisberg, S.P., McCann, D., Desai, M., Rosenbaum, M., Leibel, R.L. & Ferrante, A.W. Jr. (2003) Obesity is associated with macrophage accumulation in adipose tissue. *J. Clin. Invest.*, **112**, 1796-1808.
- Yagi, S., Akaike, M., Aihara, K., Ishikawa, K., Iwase, T., Ikeda, Y., Soeki, T., Yoshida, S., Sumitomo-Ueda, Y., Matsumoto, T. & Sata, M. (2010) Endothelial nitric oxide synthase-independent protective action of statin against angiotensin II-induced atrial remodeling via reduced oxidant injury. *Hypertension*, **55**, 918-923.
- Yamagishi, S., Matsui, T., Nakamura, K., Inoue, H., Takeuchi, M., Ueda, S., Fukami, K., Okuda, S. & Imaizumi, T. (2008) Olmesartan blocks advanced glycation end products (AGEs)-induced angiogenesis in vitro by suppressing receptor for AGEs (RAGE) expression. *Microvasc. Res.*, **75**, 130-134.
- Yamamoto, E., Dong, Y.F., Kataoka, K., Yamashita, T., Tokutomi, Y., Matsuba, S., Ichijo, H., Ogawa, H. & Kim-Mitsuyama, S. (2008) Olmesartan prevents cardiovascular injury and hepatic steatosis in obesity and diabetes, accompanied by apoptosis signal regulating kinase-1 inhibition. *Hypertension*, **52**, 573-580.
- Yoshida, T., Yamagishi, S., Nakamura, K., Matsui, T., Imaizumi, T., Takeuchi, M., Koga, H., Ueno, T. & Sata, M. (2006) Telmisartan inhibits AGE-induced C-reactive protein production through downregulation of the receptor for AGE via peroxisome proliferator-activated receptor-gamma activation. *Diabetologia*, **49**, 3094-3099.