Circulation Journal
Official Journal of the Japanese Circulation Society
http://www.j-circ.or.jp

ORIGINAL ARTICLE
Vascular Medicine

Combination Therapy With Olmesartan and Azelnidipine Improves EDHF-Mediated Responses in Diabetic Apolipoprotein E-Deficient Mice

Maki Hosoya, MD; Junko Ohashi, MD; Ayuko Sawada, BS;
Aya Takaki, PhD; Hiroaki Shimokawa, MD

Background: The endothelium modulates vascular tone by synthesizing and releasing several vasodilating factors, including vasodilator prostaglandins, nitric oxide (NO) and endothelium-derived hyperpolarizing factor (EDHF). In the present study, we examined whether an angiotensin-receptor blocker, a calcium-channel blocker or their combination improved EDHF-mediated responses in diabetic apolipoprotein E-deficient ($ApoE^{-/-}$) mice.

Methods and Results: We used male C57BL/6N (control) and streptozocin-induced diabetic $ApoE^{-/-}$ mice. The diabetic $ApoE^{-/-}$ mice were administered oral vehicle (untreated), olmesartan (OLM, 30 $mg \cdot kg^{-1} \cdot day^{-1}$), azelnidipine (AZL, 10 $mg \cdot kg^{-1} \cdot day^{-1}$), their combination (OLM+AZL), or hydralazine (HYD 5 $mg \cdot kg^{-1} \cdot day^{-1}$) for 5 weeks. In the untreated group, systolic blood pressure was significantly higher and both EDHF-mediated relaxation and endothelium-dependent hyperpolarization were markedly reduced as compared with the control group. Although EDHF-mediated relaxation was not significantly improved in the HYD, OLM and AZL groups, it was significantly improved in the OLM+AZL group, as was also the case with phosphorylation of Akt and endothelial NO synthase (eNOS). In contrast, the endothelium-independent relaxation response to sodium nitroprusside or NS-1619 (a direct opener of K_{Ca} channels) was unaltered in any group.

Conclusions: OLM+AZL may improve the severely impaired EDHF-mediated responses in diabetic $ApoE^{-/-}$ mice, in which activation of the endothelial Akt–eNOS pathway may be involved. (*Circ J* 2010; **74:** 798–806)

Key Words: Angiotensin-receptor blocker; Calcium-channel blocker; Endothelium-dependent relaxation; Endothelium-derived hyperpolarizing factor; Microcirculation

The endothelium plays an important role in maintaining vascular homeostasis by synthesizing and releasing several vasodilating factors, including vasodilator prostaglandins (PGs), nitric oxide (NO), and endothelium-derived hyperpolarizing factor (EDHF).[1–4] Several candidates for EDHF have been proposed, including cytochrome P450 metabolites,[5,6] potassium ions,[7,8] gap junctions,[9,10] and, as we have identified, hydrogen peroxide (H_2O_2).[11–14] We have demonstrated that endothelium-derived H_2O_2 is an EDHF in mouse[11] and human[12] mesenteric arteries, and in porcine coronary microvessels.[13] It is now widely accepted that EDHF plays an important role in modulating vascular tone, especially at the microcirculation level.[1,4,15–17]

Various risk factors, such as diabetes mellitus, hypercholesterolemia and hypertension, cause endothelial dysfunction, leading to atherosclerosis and microvascular disorders.[1,18,19] The influence of these risk factors, as well as the effects of their treatments, on NO-mediated responses have been extensively studied.[1–4,20] However, although these risk factors are also known to impair EDHF-mediated responses,[21,22] especially when diabetes and hypercholesterolemia are combined, as we have previously demonstrated,[23] effective treatments to ameliorate the reduced EDHF-mediated response remains to be developed.[1–4] We have demonstrated that blockade of the renin–angiotensin system with an angiotensin-converting enzyme inhibitor (ACEI) enhances EDHF-mediated responses in normal mice, independent of its blood pressure-lowering effect.[24] However, the effects of angiotensin type 1 receptor blockers (ARB), calcium-channel blockers (CCB) and their combination on impaired EDHF-mediated responses remain to be examined.

Thus, in this study, we examined whether long-term therapy with an ARB, a CCB, and their combination could ameliorate the severely impaired EDHF-mediated responses in diabetic apolipoprotein E-deficient ($ApoE^{-/-}$) mice.

Received November 6, 2009; revised manuscript received December 17, 2009; accepted December 24, 2009; released online February 13, 2010 Time for primary review: 31 days
Department of Cardiovascular Medicine, Tohoku University Graduate School of Medicine, Sendai, Japan
The Guest Editor for this article was Koichi Node, MD.
Mailing address: Hiroaki Shimokawa, MD, Department of Cardiovascular Medicine, Tohoku University Graduate School of Medicine, 1-1 Seiryo-machi, Aoba-ku, Sendai 980-8574, Japan. E-mail: shimo@cardio.med.tohoku.ac.jp
ISSN-1346-9843 doi:10.1253/circj.CJ-09-0862

Methods

Animals and Tissue Preparation

This study was approved by the Research Committee of Tohoku University Graduate School of Medicine. We used male C57BL/6N (Control) and ApoE$^{-/-}$ mice aged 8 weeks. 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 day 1, 2 and 3, respectively), while fasting.[25,26] Control mice received 3 consecutive intraperitoneal injections of the citrate buffer alone. Hyperglycemia was verified at 2 weeks after the STZ injection in blood samples taken from the tail vein, whereby fasting blood glucose levels >300 mg/dl were defined as indicating diabetes.[23] The whole-blood glucose test was carried out using Glutest-ace R (Sanwa Kagaku Kenkyusho Co, Nagoya, Japan).

The diabetic ApoE$^{-/-}$ mice were administered oral vehicle (Untreated), olmesartan (OLM, 30 mg·kg^{-1}·day^{-1}, an ARB), azelnidipine (AZL, 10 mg·kg^{-1}·day^{-1}, a CCB), their combination (OLM+AZL), or hydralazine (HYD, 5 mg·kg^{-1}·day^{-1}) in their drinking water for the subsequent 5 weeks. In a preliminary study, we confirmed that these doses of OLM and AZL exert the maximum effect on EDHF-mediated responses (data not shown). At the end of each treatment, body weight (BW), systolic blood pressure (SBP: tail-cuff method) and plasma levels of glucose and lipids were measured.[11,27] Next, the animals were anesthetized with intraperitoneal pentobarbital (50 mg/kg), blood was collected from the heart using a plastic microsyringe coated with heparin, and the animals were humanely killed. The mesenteric arteries were carefully isolated and were cut into 0.8–1 mm and 1.5 mm rings for the measurement of isometric tension and membrane potentials, respectively. The remaining mesenteric arteries were used later for Western blot analysis.

Measurements of Plasma Levels of Lipids and Glucose

Blood samples were separated by high-speed centrifugation and the plasma was removed, flash-frozen, and stored at −80°C. Plasma levels of glucose, total cholesterol, and triglycerides were measured using assay kits based on enzymatic methods (Wako Pure Chemical Industries Ltd, Osaka, Japan).[28,29]

Organ Chamber Experiments

Experiments were performed in 37°C Krebs solution bubbled with 95% O_2 and 5% CO_2. Isometric tension was recorded using isolated small mesenteric arteries (200–250 μm) as previously described.[11,24,27,30] The contributions of vasodilator PGs, NO, and EDHF to acetylcholine (ACh)-induced endothelium-dependent relaxation were determined by the inhibitory effect of indomethacin (10^{-5} mol/L), N$^{\omega}$-nitro-L-arginine (L-NNA, 10^{-4} mol/L) in the presence of indomethacin, and a combination of 100 nmol/L charybdotoxin [an inhibitor of large- and intermediate-conductance calcium-activated potassium (K_{Ca}) channels] and 1 μmol/L apamin (an inhibitor of small-conductance K_{Ca} channels) in the presence of indomethacin and L-NNA, respectively.[11,24,27,30] The endothelium-dependent vasodilator responses under these 4 conditions were examined in different blood vessels in parallel. In addition, endothelium-independent relaxation responses to sodium nitroprusside (SNP), a donor of NO, and NS-1619, a direct opener of K_{Ca} channels, were also examined in arterial rings without endothelium.[30] EDHF causes vascular smooth muscle hyperpolarization and vasodilatation through activation of K_{Ca} channels.[1–4] All agents were applied to organ chambers 30 min before precontraction with prostaglandin $F_{2\alpha}$. The extent of the contraction by prostaglandin $F_{2\alpha}$ was adjusted to 50–70% of the contractions induced by 60 mmol/L KCl.[11,24,27,30] To compare the relaxation curves, we used the area under the curve.[11,24,27,30]

Electrophysiological Experiments

Rings of small mesenteric arteries were placed in experimental chambers perfused with 37°C Krebs solution containing indomethacin (10^{-5} mol/L) and L-NNA (10^{-4} mol/L) bubbled with 95% O_2 and 5% CO_2.[11,24,27,30] A vascular smooth muscle cell was impaled on a fine glass capillary microelectrode inserted from the adventitial side of the artery and changes in membrane potentials produced by ACh were continuously recorded.[11,24,27,30]

Drugs and Solution

The ionic composition of the Krebs solution was as follows (mmol/L): Na^+ 144, K^+ 5.9, Mg^{2+} 1.2, Ca^{2+} 2.5, $H_2PO_4^-$ 1.2, HCO_3^- 24, Cl^- 129.7, and glucose 5.5. STZ, ACh, NS1619, indomethacin, HYD and L-NNA were obtained from Sigma Chemical Co (St Louis, MO, USA) and SNP from Maruishi Seiyaku (Osaka, Japan). Indomethacin was dissolved in 10^{-2} mol/L Na_2CO_3 and NS1619 in dimethyl sulfoxide. Other drugs were dissolved in distilled water. OLM and AZL were donated by Daiichi-Sankyo Pharmaceutical Co (Tokyo, Japan).

Western Blot Analysis

Western blot analysis was performed using antibodies that specifically recognize proteins, including endothelial NO synthase (eNOS), phospho-eNOS at Ser1177 (p-eNOS), Akt, phospho-Akt at Ser473 (p-Akt), and Cu,Zn-superoxide dismutase (SOD).[27,30] The extent of eNOS phosphorylation at Ser1177[31,32] and that of Akt phosphorylation at Ser473[33] represent the activation of each molecule. All of the mesenteric tissues were homogenized and the proteins were extracted. The same amount of extracted protein (10–20 μg) 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 as an internal control.[34]

Statistical Analysis

Results are expressed as mean±SEM. Changes in BW, SBP and the plasma levels of glucose and lipids during the treatments were compared by 1-way ANOVA followed by Bonferroni/Dunn's post-hoc test for multiple comparisons. Serial changes in the characteristics of the 6 treatment groups from baseline values at 0 week were analyzed by 1-way repeated ANOVA followed by Bonferroni/Dunn's post-hoc test for multiple comparisons. Dose-response curves were analyzed by 2-way ANOVA followed by Bonferroni/Dunn's post-hoc test for multiple comparisons.

Other values were analyzed by 1-way ANOVA followed by Bonferroni/Dunn's post-hoc test for multiple comparisons. $P<0.05$ was considered to be statistically significant.

Results

BW and SBP

Before the experiment and at 2 weeks after STZ or vehicle injection, BW and SBP were comparable among the 6 groups (**Table**). At week 7, BW was significantly increased in the

Table. 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)						
Week 0	23.0±0.6	22.9±0.7	23.0±0.6	23.1±0.4	22.8±0.5	23.1±0.6
Week 2	23.6±0.5	20.5±0.5	21.0±0.6	20.9±0.7*	20.6±0.4*	20.8±0.5*
Week 7	26.4±0.6*	21.6±0.6‡	23.4±0.5†	23.0±0.6†	23.4±0.4†	23.8±0.7†,¶
Systolic blood pressure (mmHg)						
Week 0	115±1	117±2	115±1	115±2	114±1	116±2
Week 2	117±1	120±2	119±3	120±3	120±3	121±3
Week 7	118±2	124±2**,†	113±2¶	114±2¶	105±2*,†,¶	108±1*,†,¶
Fasting glucose (mg/dl)						
Week 0	108±12	115±7	107±12	111±8	109±10	106±10
Week 2	120±14	482±28**,†	457±28**,†	462±35**,†	461±18**,†	490±25**,†
Week 7	128±17	495±19**,†	448±35**,†	454±43**,†	443±28**,†	452±29**,†
Total cholesterol (mg/dl)						
Week 7	100±4	466±54†	438±26†	428±31†	418±51†	500±66†
Triglyceride (mg/dl)						
Week 7	81±7	180±15†	172±15†	167±27†	186±21†	165±20†

Results are mean±SEM, *P<0.05, **P<0.01 vs week 0, †P<0.05 vs Control, ¶P<0.05 vs Untreated.
Untreated, vehicle-treated diabetic ApoE$^{-/-}$ mice; OLM, olmesartan-treated diabetic ApoE$^{-/-}$ mice; AZL, azelnidipine-treated diabetic ApoE$^{-/-}$ mice; HYD, hydralazine-treated diabetic ApoE$^{-/-}$ mice; OLM+AZL, diabetic ApoE$^{-/-}$ mice treated with azelnidipine and olmesartan.

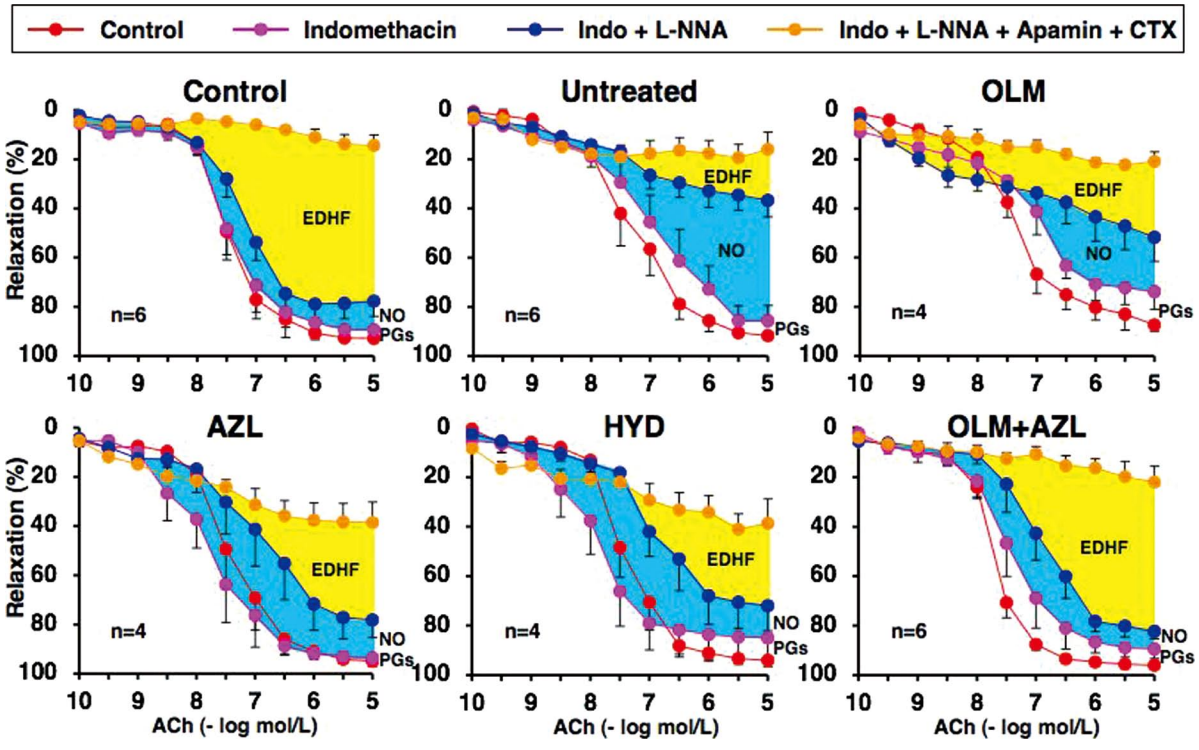

Figure 1. Endothelium-dependent relaxation of mesenteric arties in the 6 treatment groups. Control, normal mice; Untreated, vehicle-treated diabetic ApoE$^{-/-}$ mice; AZL, azelnidipine-treated diabetic ApoE$^{-/-}$ mice; OLM, olmesartan-treated diabetic ApoE$^{-/-}$ mice; HYD, hydralazine-treated diabetic ApoE$^{-/-}$ mice; OLM+AZL, diabetic ApoE$^{-/-}$ mice treated with azelnidipine plus olmesartan; Indo, indomethacin; L-NNA, N$^{\omega}$-nitro-L-arginine; CTX, charybdotoxin. Results are mean±SEM. It is evident that EDHF-mediated relaxation (in the presence of Indo and L-NNA (shown in yellow) was markedly reduced in the untreated group, not improved in the OLM, AZL or HYD group, but markedly improved in the OLM+AZL group.

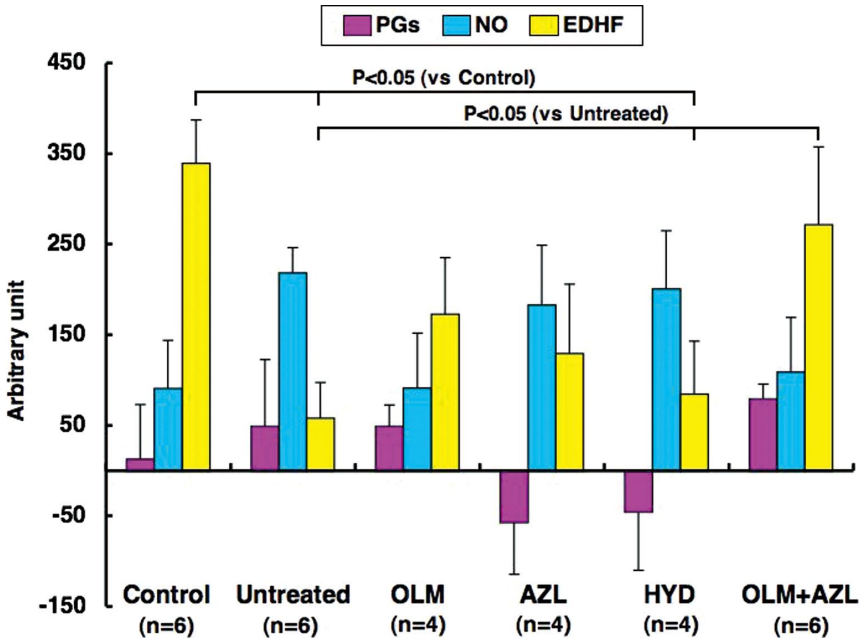

Figure 2. Relative contributions of vasodilator prostaglandins (PGs), nitric oxide (NO) and endothelium-derived hyperpolarizing factor (EDHF) to endothelium-dependent relaxation responses to acetylcholine (ACh) in mouse mesenteric arteries. Results are mean ± SEM. See **Figure 1** for the 6 treatment groups. Relative contributions of PGs, NO, and EDHF to ACh-induced endothelium-dependent relaxation were determined by the inhibitory effect of indomethacin, L-NNA (in the presence of indomethacin), and a combination of charybdotoxin and apamin (in the presence of indomethacin and L-NNA), respectively. The EDHF component was markedly impaired in the untreated group and was almost normalized in the OLM+AZL group. L-NNA, N^{ω}-nitro-L-arginine.

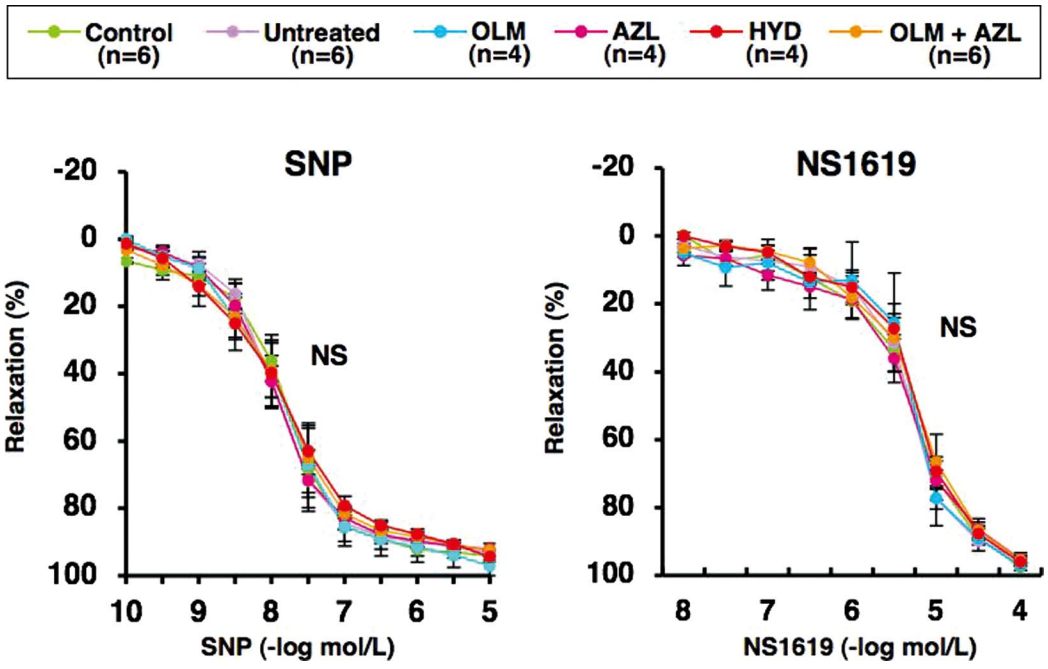

Figure 3. Endothelium-independent relaxation of mouse mesenteric arteries. Results are mean ± SEM. See **Figure 1** for the 6 treatment groups. Endothelium-independent relaxation responses to sodium nitroprusside (SNP, an NO donor) and those to NS1619 (a direct opener of calcium-activated K_{Ca} channel) were comparable among the 6 treatment groups. NS, not statistically significant; NO, nitric oxide.

control group and tended to be reduced in the untreated group, but was more or less maintained in the other groups (**Table**). At week 7, SBP was significantly elevated in the untreated group as compared with the other groups, but was significantly and equally decreased in the HYD and OLM+AZL groups (**Table**).

Plasma Levels of Glucose and Lipids

At weeks 2 and 7 after STZ injection, the fasting glucose levels were markedly and significantly elevated in the un-

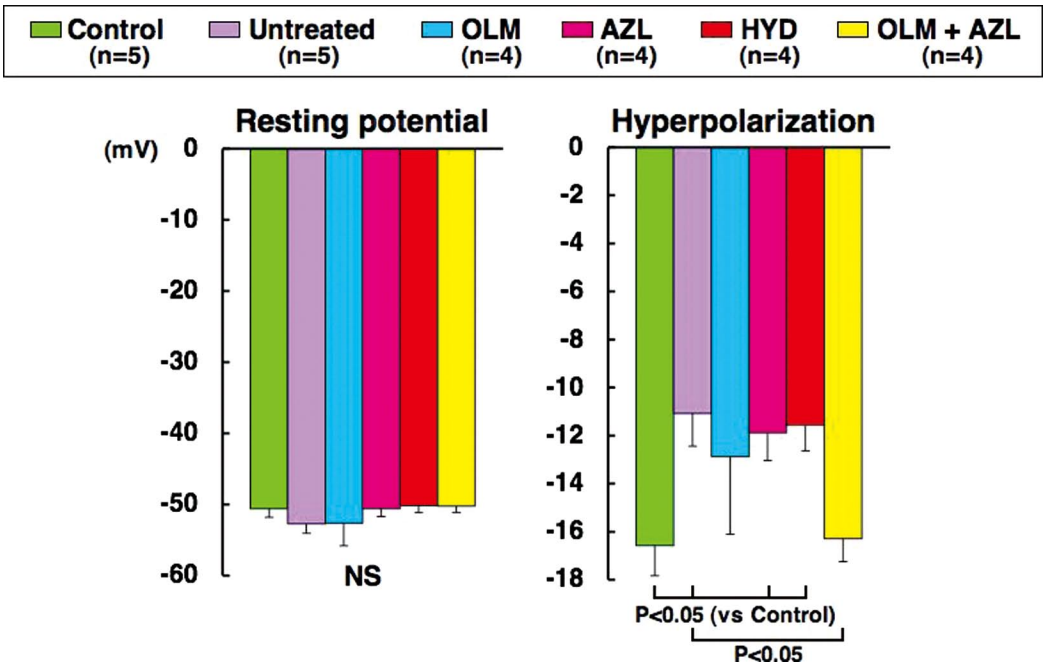

Figure 4. Endothelium-dependent hyperpolarization to acetylcholine (ACh) in mouse mesenteric arteries Results are mean ± SEM. See **Figure 1** for the 6 treatment groups. While resting membrane potentials were comparable among the 6 groups (**Left**), endothelium-dependent hyperpolarization to ACh (10^{-5}mol/L) was significantly impaired in the untreated group, not improved in the AZL, OLM, or HYD group, but was markedly improved in the OLM+AZL group (**Right**).

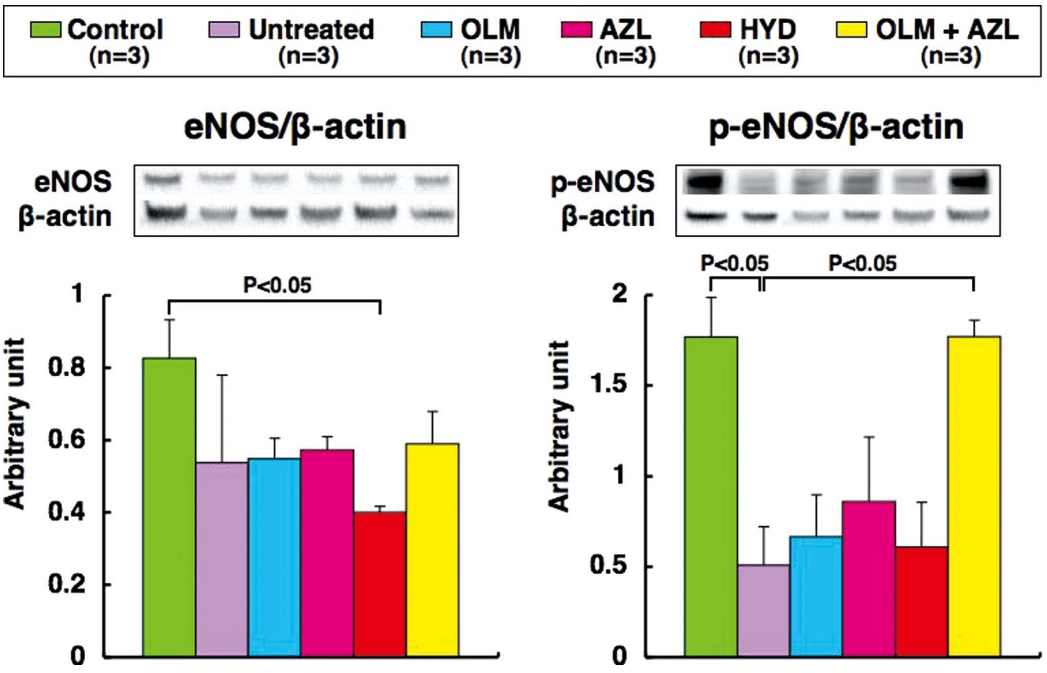

Figure 5. Protein expression of eNOS and phosphorylated-eNOS. Results are mean ± SEM. See **Figure 1** for the 6 treatment groups. Although the protein expression of total eNOS is significantly reduced only in the HYD group (**Left**), that of phosphorylated eNOS (p-eNOS), a marker of eNOS activation, is significantly reduced in the untreated group, not improved in the OLM, AZL or HYD group, and markedly improved in the OLM+AZL group (**Right**). NS, not statistically significant; eNOS, endothelial nitric oxide synthase.

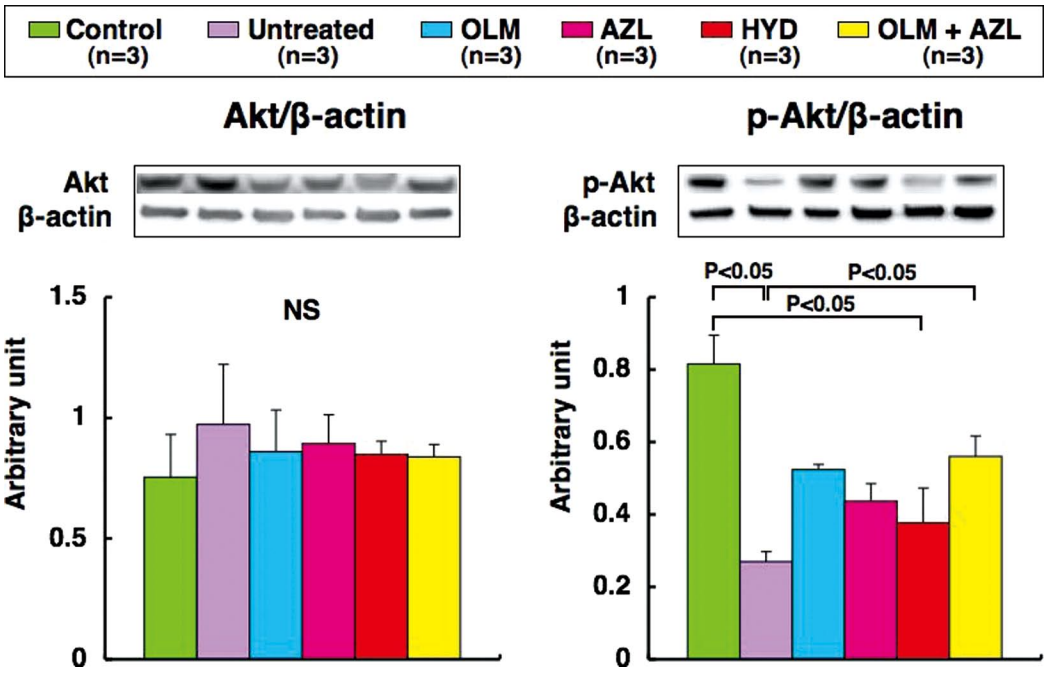

Figure 6. Protein expression of Akt and phosphorylated-Akt. Results are mean ± SEM. See **Figure 1** for the 6 treatment groups. Although the protein expression of total Akt is comparable among the 6 groups (**Left**), that of phosphorylated Akt (p-Akt), a marker of Akt activation, is significantly reduced in the untreated or HYD group, not improved in the OLM or AZL group, and significantly improved in the OLM+AZL groups (**Right**). NS, not statistically significant.

treated group as compared with the control group and were not affected by any treatment (**Table**). Similarly, at week 7, the plasma levels of total cholesterol and triglycerides were also significantly higher in the 5 diabetic ApoE$^{-/-}$ mouse groups than in the control group (**Table**).

Endothelium-Dependent and -Independent Relaxation

In the control group, ACh elicited concentration-dependent relaxation, which was resistant to indomethacin (vasodilator PGs component) or indomethacin plus L-NNA (NO component), but was highly sensitive to the combination of indomethacin, L-NNA, apamin, and charibdotoxin (EDHF component) (**Figure 1**). This EDHF component was markedly reduced in the untreated group, not improved in the OLM, AZL or HYD group, but was markedly improved in the OLM+AZL group (**Figure 1**). Quantitative analysis of the relative contribution of vasodilator PGs, NO, and EDHF to endothelium-dependent relaxation responses to ACh demonstrated that as compared with the control group, the EDHF component was significantly reduced in the untreated group, not significantly improved in the OLM, AZL or HYD group, but was significantly improved only in the OLM+AZL group (**Figure 2**). In contrast, as compared with the control group, the PGs or NO component was not significantly altered in the other groups (**Figure 2**). Importantly, endothelium-independent relaxation reponses to SNP or NS1619 were comparable among the 6 groups, indicating that the vasodilator properties of vascular smooth muscle cells were unaltered in all groups (**Figure 3**).

Endothelium-Dependent Hyperpolarization

Membrane potentials of vascular smooth muscle cells were recorded in the presence of indomethacin and L-NNA to exclude the possible contribution of vasodilator PGs and NO, respectively.[11,24,27,30] Resting membrane potential was comparable among the 6 groups (**Figure 4**). In contrast, the endothelium-dependent hyperpolarization resonse to ACh (10^{-6} mol/L) was significantly impaired in the untreated group, not significantly improved in the OLM, AZL or HYD group, but was significantly improved in the OLM+AZL group (**Figure 4**).

Expression and Phosphorylation of eNOS, Akt and Cu,Zn-SOD

As compared with the control group, the expression of total eNOS protein in the mesenteric arteries was significantly lower only in the HYD group (**Figure 5**). In contrast, the extent of eNOS phosphorylation, a marker of eNOS activation,[31,32] was significantly reduced in the untreated group as compared with the control group, not significantly improved in the OLM, AZL or HYD groups, but was significantly improved in the OLM+AZL group (**Figure 5**). Similarly, although the expression of total Akt protein was comparable among the 6 groups, the extent of Akt phosphorylation, a marker of Akt activation,[33] was significantly reduced in the untreated or HYD group, not significantly improved in the OLM or AZL group, but was significantly improved in the OLM+AZL group (**Figure 6**). In contrast, the expression of Cu,Zn-SOD protein was comparable among the 6 groups (**Figure 7**).

Discussion

The major findings of the present study are as follows; (1) the severely impaired EDHF-mediated responses under the

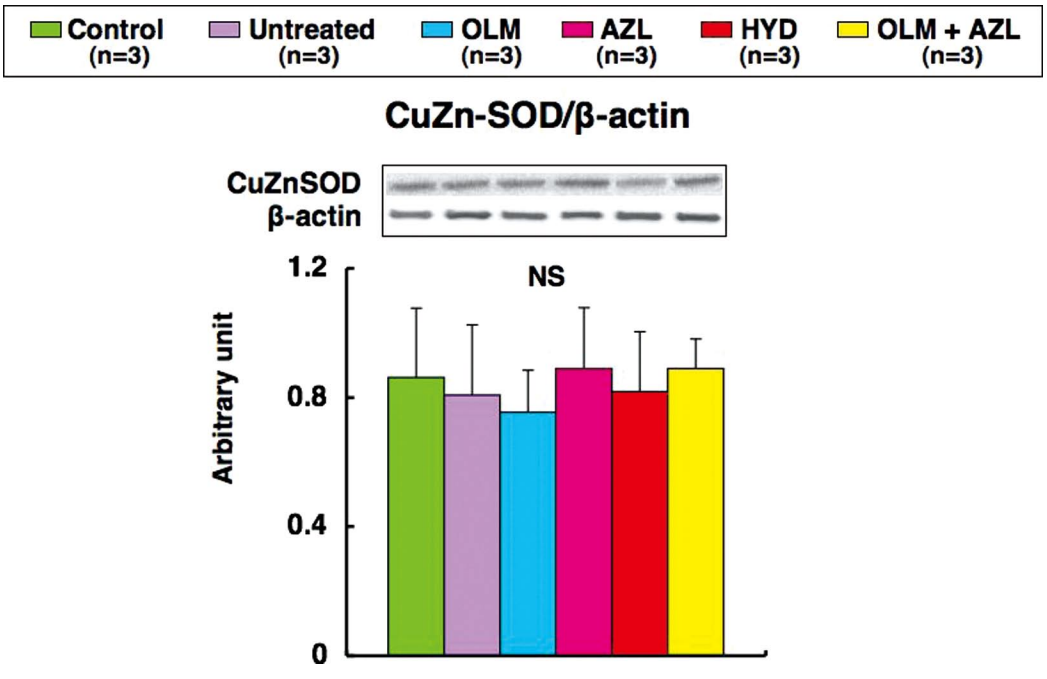

Figure 7. Protein expression of Cu, Zn-superoxide dismutase (Cu, Zn-SOD). Results are mean ± SEM. The protein expression of Cu, Zn-SOD was comparable among the 6 groups (see **Figure 1** for the 6 treatment groups). NS, not statistically significant.

combined condition of diabetes and dyslipidemia were not significantly improved by OLM or AZL alone but were markedly improved by their combination, (2) the beneficial effects of combined therapy with OLM+AZL were not due to its blood pressure-lowering effect, and (3) the beneficial effects of the combination therapy were mediated, at least in part, by activation of the endothelial eNOS/Akt pathway. To the best of our knowledge, this is the first report of the beneficial effects of combination therapy with an ARB and a CCB on impaired EDHF-mediated responses.

Impaired EDHF-Mediated Responses in Diabetic ApoE$^{-/-}$ Mice

It is widely known that diabetes mellitus and hypercholesterolemia alone or in combination impair NO-mediated endothelium-dependent relaxation.[35–39] Similarly, EDHF-mediated endothelium-dependent relaxation is impaired by diabetes mellitus,[40] hypercholesterolemia[15] and hypertension.[41] Furthermore, we have previously demonstrated that the combination of diabetes mellitus and hypercholesterolemia markedly impairs EDHF-mediated relaxation in diabetic ApoE$^{-/-}$ mice.[23] When considering the extent of the impairment of EDHF-mediated relaxation and hyperpolarization, the present diabetic ApoE$^{-/-}$ mouse model may represent an advanced stage of endothelial dysfunction in humans, such as advanced metabolic syndrome. We were able to confirm our previous finding in the present diabetic ApoE$^{-/-}$ mouse model.[23]

Beneficial Effect of OLM+AZL on EDHF Responses in Diabetic ApoE$^{-/-}$ Mice

In the present study, only the combination therapy (OLM+AZL), not the monotherapies, significantly ameliorated the severely impaired EDHF-mediated relaxation and hyperpolarization in diabetic ApoE$^{-/-}$ mice. Because the endothelium-independent relaxation response to SNP or NS1619 was unaltered by the combination therapy, it is the effects of the therapy on endothelial function, not those on vascular smooth muscle function, that appear to be involved. Furthermore, treatment with HYD, although it achieved a comparable antihypertensive effect to the combination therapy, failed to improve either EDHF-mediated relaxation or hyperpolarization, indicating that the beneficial effects of the combination therapy were not mediated by a simple blood pressure-lowering effect. We have previously demonstrated that long-term treatment with temocapril, an ACEI, enhances EDHF-mediated responses in normal mice.[42] However, in the present model of severely impaired EDHF-mediated responses, monotherapy with OLM or AZL failed to significantly improve the responses, whereas the combination significantly and markedly improved them, suggesting the usefulness of the combination therapy for the treatment of microvascular endothelial dysfunction. In the present study, we examined the vascular responses of small mesenteric arteries, resistance vessels that are known to be unaffected by atherosclerotic changes, such as medial thickening. Thus, we consider that the functional improvement in the EDHF-mediated responses in the present study with the combination treatment of OLM+AZL is not related to their preventive effects on the progression of atherosclerosis.

Possible Mechanism of the Beneficial Effect of OLM+AZL on EDHF-Mediated Responses

It is known that long-term treatment with an ARB improves endothelial dysfunction in patients with diabetes mellitus or[43,44] hypertension,[45–47] and in the aged.[42] It has also been reported that the mechanisms of the beneficial effects of OLM are mediated in part through inhibition of reactive oxygen species generation and eNOS uncoupling,[48] as is the case with CCBs.[47] In addition, the beneficial effects of com-

bination therapy with an ARB and a CCB have been reported in terms of glucose intolerance in diabetic mice,[49] vascular dysfunction in hypertensive rats,[47] and for atherosclerosis and oxidative stress in ApoE$^{-/-}$ mice.[50] However, it remains to be examined whether an ARB, a CCB and their combination improve EDHF-mediated responses and if so, what mechanism(s) is involved. In the present study, although the total protein expression of eNOS or Akt was unaltered by the combination therapy, the extent of phosphorylation of both eNOS and Akt was significantly improved. We have recently demonstrated that the endothelial NOS system has diverse vasodilator functions depending on the vessel size, mainly contributing to EDHF/H_2O_2 responses in microvessels while serving as a NO-generating system in large arteries.[30] The present results suggest that the improvement in EDHF-mediated responses by OLM+AZL was mediated, at least in part, by activation of the endothelial Akt–eNOS pathway. This finding is consistent with a recent report that OLM+AZL suppressed the development of atherosclerosis in ApoE$^{-/-}$ mice.[50] Although in the present study we did not further examine the molecular mechanism(s) for the enhanced phosphorylation of Akt and eNOS, it has been reported that combination therapy with OLM and a CCB (including AZL), but not their monotherapies, significantly decreased oxidative stress (eg, pathological superoxide anion production) independent of their blood pressure-lowering effects.[51,52] Indeed, excessive oxidative stress has been implicated in the pathogenesis of endothelial dysfunction partly because of eNOS dysfunction.[53,54]

The antihypertensive effect of the combination therapy may not play a major role in the activation of the endothelial Akt–eNOS pathway, because HYD treatment, which has a similar antihypertensive effect, failed to do so. It is conceivable that the combination of OLM+AZL exerts beneficial effects not only on endothelial function (as shown in the present study) but also on angiogenesis and glucose metabolism, because the Akt–eNOS pathway also plays an important role in those functions.[55]

We have previously demonstrated that endothelial Cu,Zn-SOD plays an important role in producing H_2O_2 as an EDHF synthase in mouse and human mesenteric arteries.[27] However, in the present study, there was no significant upregulation of Cu,Zn-SOD in the treatment groups, suggesting that Cu,Zn-SOD upregulation may not be involved in the beneficial effects of OLM+AZL therapy.

Study Limitations

First, we only examined the long-term effects of the combined therapy with OLM+AZL on the EDHF-mediated responses in normal and STZ-induced diabetic ApoE$^{-/-}$ mice. In order to further explore the present findings, the effects of combination therapy with other ARBs and CCBs need to be examined in other animal models of advanced atherosclerosis with endothelial dysfunction. Second, as we have previously demonstrated that endothelium-derived H_2O_2 is an EDHF in mouse mesenteric arties,[11–14] it remains to be examined whether endothelium-derived H_2O_2 is actually involved in the beneficial effects of OLM+AZL treatment in the present study. Third, because we have previously demonstrated the beneficial effects of an ACEI on EDHF/H_2O_2-mediated responses,[24] the effects of ACEIs remain to be examined in the present diabetic ApoE$^{-/-}$ mouse model. Fourth, we only examined the role of eNOS and the role of other NO synthases (inducible and neuronal NOSs) remains to be examined. Finally, the beneficial effects of combination therapy with an ARB and a CCB on impaired EDHF-mediated responses remain to be confirmed in patients with metabolic syndrome.

Conclusion

The present study demonstrates that combination therapy with OLM and AZL effectively improves the severely impaired EDHF-mediated responses in diabetic ApoE$^{-/-}$ mice, in which activation of endothelial Akt–eNOS pathway may be involved.

Acknowledgments

The authors thank F. Tatebayashi for excellent technical assistance in this study, which was supported in part by the grants-in-aid (Nos. 16209027, 18659218, 20659122), those for scientific research on innovative areas (signaling functions of reactive oxygen species), and those for Tohoku University Global COE for Conquest of Signal Transduction Diseases with Network Medicine from the Japanese Ministry of Education, Culture, Sports, Science and Technology, Tokyo, Japan.

References

1. Shimokawa H. Primary endothelial dysfunction: Atherosclerosis. *J Mol Cell Cardiol* 1999; **31:** 23–37.
2. Busse R, Edwards G, Félétou M, Fleming I, Vanhoutte PM, Weston AH. EDHF: Bringing the concepts together. *Trends Pharmacol Sci* 2002; **23:** 374–380.
3. Félétou M, Vanhoutte PM. Endothelium-derived hyperpolarizing factor: Where are we now? *Arterioscler Thromb Vasc Biol* 2006; **26:** 1215–1225.
4. Vanhoutte PM. Endothelial dysfunction: The first step toward coronary arteriosclerosis. *Circ J* 2009; **73:** 595–601.
5. Fisslthaler B, Popp R, Kiss L, Potente M, Harder DR, Fleming I, et al. Cytochrome P450 2C is an EDHF synthase in coronary arteries. *Nature* 1999; **401:** 493–497.
6. Michaelis UR, Fleming I. From endothelium-derived hyperpolarizing factor (EDHF) to angiogenesis: Epoxyeicosatrienoic acids (EETs) and cell signaling. *Pharmacol Ther* 2006; **111:** 584–595.
7. Edwards G, Dora KA, Gardener MJ, Garland CJ, Weston AH. K^+ is an endothelium-derived hyperpolarizing factor in rat arteries. *Nature* 1998; **396:** 269–272.
8. Brähler S, Kaistha A, Schmidt VJ, Wölfle SE, Busch C, Kaistha BP, et al. Genetic deficit of SK3 and IK1 channels disrupts the endothelium-derived hyperpolarizing factor vasodilator pathway and causes hypertension. *Circulation* 2009; **119:** 2323–2332.
9. Taylor HJ, Chaytor AT, Evans WH, Griffith TM. Inhibition of the gap junctional component of endothelium-dependent relaxations in rabbit iliac artery by 18-alpha glycyrrhetinic acid. *Br J Pharmacol* 1998; **125:** 1–3.
10. Mather S, Dora KA, Sandow SL, Winter P, Garland CJ. Rapid endothelial cell-selective loading of connexin 40 antibody blocks endothelium-derived hyperpolarizing factor dilation in rat small mesenteric arteries. *Circ Res* 2005; **97:** 399–407.
11. Matoba T, Shimokawa H, Nakashima M, Hirakawa Y, Mukai Y, Hirano K, et al. Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in mice. *J Clin Invest* 2000; **106:** 1521–1530.
12. Matoba T, Shimokawa H, Kubota H, Morikawa K, Fujiki T, Kunihiro I, et al. Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in human mesenteric arteries. *Biochem Biophys Res Commun* 2002; **290:** 909–913.
13. Matoba T, Shimokawa H, Morikawa K, Kubota H, Kunihiro I, Urakami-Harasawa L, et al. Electron spin resonance detection of hydrogen peroxide as an endothelium-derived hyperpolarizing factor in porcine coronary microvessels. *Arterioscler Thromb Vasc Biol* 2003; **23:** 1224–1230.
14. Shimokawa H, Matoba T. Hydrogen peroxide as an endothelium-derived hyperpolarizing factor. *Pharmacol Res* 2004; **49:** 543–549.
15. Shimokawa H, Yasutake H, Fujii K, Owada MK, Nakaike R, Fukumoto Y, et al. The importance of the hyperpolarizing mechanism increases as the vessel size decreases in endothelium-dependent relaxations in rat mesenteric circulation. *J Cardiovasc Pharmacol* 1996; **28:** 703–711.
16. Urakami-Harasawa L, Shimokawa H, Nakashima M, Egashira K, Takeshita A. Importance of endothelium-derived hyperpolarizing factor in human arteries. *J Clin Invest* 1997; **100:** 2793–2799.
17. de Wit C, Wölfle SE. EDHF and gap junctions: Important regulators

of vascular tone within the microcirculation. *Curr Pharm Biotechnol* 2007; **8:** 11–25.
18. De Vriese AS, Verbeuren TJ, Van de Voorde J, Lameire NH, Vanhoutte PM. Endothelial dysfunction in diabetes. *Br J Pharmacol* 2000; **130:** 963–974.
19. d'Uscio LV, Smith LA, Katusic ZS. Hypercholesterolemia impairs endothelium-dependent relaxations in common carotid arteries of apolipoprotein E-deficient mice. *Stroke* 2001; **32:** 2658–2664.
20. Higashi Y, Noma K, Yoshizumi M, Kihara Y. Endothelial function and oxidative stress in cardiovascular diseases. *Circ J* 2009; **73:** 411–418.
21. Feletou M, Vanhoutte PM. EDHF: New therapeutic targets? *Pharmacol Res* 2004; **49:** 565–580.
22. Zhang H, Park Y, Wu J, Chen X, Lee S, Yang J, et al. Role of TNF-α in vascular dysfunction. *Clin Sci* 2009; **116:** 219–230.
23. Morikawa K, Matoba T, Kubota H, Hatanaka M, Fujiki T, Takahashi S, et al. Influence of diabetes mellitus, hypercholesterolemia, and their combination on EDHF-mediated responses in mice. *J Cardiovasc Pharmacol* 2005; **45:** 485–490.
24. Fujiki T, Shimokawa H, Morikawa K, Kubota H, Hatanaka M, Talukder MA, et al. Endothelium-derived hydrogen peroxide accounts for the enhancing effect of an angiotensin-converting enzyme inhibitor on endothelium-derived hyperpolarizing factor-mediated responses in mice. *Arterioscler Thromb Vasc Biol* 2005; **25:** 766–771.
25. How OJ, Aasum E, Severson DL, Chan WY, Essop MF, Larsen TS. Increased myocardial oxygen consumption reduces cardiac efficiency in diabetic mice. *Diabetes* 2006; **55:** 466–473.
26. Kennedy JM, Zochodne DW. The regenerative deficit of peripheral nerves in experimental diabetes: Its extent, timing and possible mechanisms. *Brain* 2000; **123:** 2118–2129.
27. Morikawa K, Shimokawa H, Matoba T, Kubota H, Akaike T, Talukder MA, et al. Pivotal role of Cu,Zn-superoxide dismutase in endothelium-dependent hyperpolarization. *J Clin Invest* 2003; **112:** 1871–1879.
28. Hojjati MR, Li Z, Zhou H, Tang S, Huan C, Ooi E, et al. Effect of myriocin on plasma sphingolipid metabolism and atherosclerosis in apoE-deficient mice. *J Biol Chem* 2005; **280:** 10284–10289.
29. Gonzalez-Navarro H, Vila-Caballer M, Pastor MF, Vinue A, White MF, Burks D, et al. Plasma insulin levels predict the development of atherosclerosis when IRS2 deficiency is combined with severe hypercholesterolemia in apolipoprotein E-null mice. *Front Biosci* 2007; **12:** 2291–2298.
30. Takaki A, Morikawa K, Tsutsui M, Murayama Y, Tekes E, Yamagishi H, et al. Crucial role of nitric oxide synthases system in endothelium-dependent hyperpolarization in mice. *J Exp Med* 2008; **205:** 2053–2063.
31. Bauer PM, Fulton D, Boo YC, Sorescu GP, Kemp BE, Jo H, et al. 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* 2003; **278:** 14841–14849.
32. Dudzinski DM, Michel T. Life history of eNOS: Partners and pathways. *Cardiovasc Res* 2007; **75:** 247–260.
33. Alessi DR, Andjelkovic M, Caudwell B, Cron P, Morrice N, Cohen P, et al. Mechanism of activation of protein kinase B by insulin and IGF-1. *EMBO J* 1996; **15:** 6541–6551.
34. Abe K, Shimokawa H, Morikawa K, Uwatoku T, Oi K, Matsumoto Y, et al. Long-term treatment with a Rho-kinase inhibitor improves monocrotaline-induced fatal pulmonary hypertension in rats. *Circ Res* 2004; **94:** 385–393.
35. d'Uscio LV, Smith LA, Katusic ZS. Hypercholesterolemia impairs endothelium-dependent relaxations in common carotid arteries of apolipoprotein E-deficient mice. *Stroke* 2001; **32:** 2658–2664.
36. Williams SB, Goldfine AB, Timimi FK, Ting HH, Roddy MA, Simonson DC, et al. Acute hyperglycemia attenuates endothelium-dependent vasodilation in humans in vivo. *Circulation* 1998; **97:** 1695–1701.
37. Matsumoto T, Kobayashi T, Wachi H, Seyama Y, Kamata K. Vascular NAD(P)H oxidase mediates endothelial dysfunction in basilar arteries from Otsuka Long-Evans Tokushima Fatty (OLETF) rats. *Atherosclerosis* 2007; **192:** 15–24.
38. Laursen JB, Somers M, Kurz S, McCann L, Warnholtz A, Freeman BA, et al. Endothelial regulation of vasomotion in apoE-deficient mice: Implications for interactions between peroxynitrite and tetrahydrobiopterin. *Circulation* 2001; **103:** 1282–1288.
39. Mullen MJ, Kharbanda RK, Cross J, Donald AE, Taylor M, Vallance P, et al. Heterogenous nature of flow-mediated dilatation in human conduit arteries in vivo: Relevance to endothelial dysfunction in hypercholesterolemia. *Circ Res* 2001; **88:** 145–151.
40. Pannirselvam M, Ding H, Anderson TJ, Triggle CR. Pharmacological characteristics of endothelium-derived hyperpolarizing factor-mediated relaxation of small mesenteric arteries from db/db mice. *Eur J Pharmacol* 2006; **551:** 98–107.
41. Dal-Ros S, Bronner C, Schott C, Kane MO, Chataigneau M, Schini-Kerth VB, et al. Angiotensin II-induced hypertension is associated with a selective inhibition of endothelium-derived hyperpolarizing factor-mediated responses in the rat mesenteric artery. *J Pharmacol Exp Ther* 2009; **328:** 478–486.
42. Mukai Y, Shimokawa H, Higashi M, Morikawa K, Matoba T, Hiroki J, et al. Inhibition of renin-angiotensin system ameliorates endothelial dysfunction associated with aging in rats. *Arterioscler Thromb Vasc Biol* 2002; **22:** 1445–1450.
43. Fan Q, Liao J, Kobayashi M, Yamashita M, Gu L, Gohda T, et al. Candesartan reduced advanced glycation end-products accumulation and diminished nitro-oxidative stress in type 2 diabetic KK/Ta mice. *Nephrol Dial Transplant* 2004; **19:** 3012–3020.
44. Diederich D, Skopec J, Diederich A, Dai F. Endothelial dysfunction in mesenteric resistance arteries of diabetic rats: Role of free radicals. *Am J Physiol* 1994; **266:** H1153–H1161.
45. Ferrario C. Effect of angiotensin receptor blockade on endothelial function: Focus on olmesartan medoxomil. *Vasc Health Risk Manag* 2009; **5:** 301–314.
46. Ono Y, Nakaya Y, Bando S, Soeki T, Ito S, Sata M. Telmisartan decreases plasma levels of asymmetrical dimethyl-L-arginine and improves lipid and glucose metabolism and vascular function. *Int Heart J* 2009; **50:** 73–83.
47. Yao K, Sato H, Sonoda R, Ina Y, Suzuki K, Ohno T. Effects of benidipine and candesartan on kidney and vascular function in hypertensive Dahl rats. *Hypertens Res* 2003; **26:** 569–576.
48. Yamamoto E, Dong YF, Kataoka K, Yamashita T, Tokutomi Y, Matsuba S, et al. Olmesartan prevents cardiovascular injury and hepatic steatosis in obesity and diabetes, accompanied by apoptosis signal regulating kinase-1 inhibition. *Hypertension* 2008; **52:** 573–580.
49. Iwai M, Li HS, Chen R, Shiuchi T, Wu L, Min LJ, et al. Calcium channel blocker azelnidipine reduces glucose intolerance in diabetic mice via different mechanism than angiotensin receptor blocker olmesartan. *J Pharmacol Exp Ther* 2006; **319:** 1081–1087.
50. Suzuki J, Iwai M, Li Z, Li JM, Min LJ, Ide A, et al. Effect of combination of calcium antagonist, azelnidipine, and AT$_1$ receptor blocker, olmesartan, on atherosclerosis in apolipoprotein E-deficient mice. *J Hypertens* 2005; **23:** 1383–1389.
51. Inaba S, Iwai M, Tomono Y, Senba I, Furuno M, Kanno H, et al. Prevention of vascular injury by combination of an AT1 receptor blocker, olmesartan, with various calcium antagonists. *Am J Hypertens* 2009; **22:** 145–150.
52. Jinno T, Iwai M, Li Z, Li JM, Liu HW, Cui TX, et al. Calcium channel blocker azelnidipine enhances vascular protective effects of AT$_1$ receptor blocker olmesartan. *Hypertension* 2004; **43:** 263–269.
53. Griendling KK, FitzGerald GA. Oxidative stress and cardiovascular injury: Part I: Basic mechanisms and in vivo monitoring of ROS. *Circulation* 2003; **108:** 1912–1916.
54. Cai H, Harrison DG. Endothelial dysfunction in cardiovascular diseases: The role of oxidant stress. *Circ Res* 2000; **87:** 840–844.
55. Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, Zeiher AM. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature* 1999; **399:** 601–605.