Original Article

Inhibition of Balloon Injury–Induced Neointimal Formation by Olmesartan and Pravastatin in Rats with Insulin Resistance

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The combined effect of an angiotensin II type 1 receptor blocker and a 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor on vascular lesion formation in the insulin-resistant state has not been examined. We tested whether or not combined treatment is superior to single-drug treatment for inhibiting vascular lesion formation in insulin-resistant rats. The rats were maintained on a fructose-rich diet for 4 weeks and then treated with olmesartan (1 mg/kg/day) and/or pravastatin (10 mg/kg/day) for 3 weeks. After 1 week of drug treatment, balloon injury of the carotid arteries was performed. Two weeks later, the injured arteries were harvested for morphometry and immunostaining. Olmesartan and pravastatin each modestly attenuated neointimal formation without significant changes in blood pressure or serum lipid levels. The combination of olmesartan and pravastatin significantly suppressed the neointimal formation compared with either monotherapy. The number of terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL)–positive cells was increased by olmesartan but not by pravastatin. Olmesartan and pravastatin each decreased the number of Ki-67–positive cells, which indicates cell proliferation, to the same extent. The combined treatment increased the number of TUNEL-positive cells but did not affect the number of Ki-67–positive cells. The combined treatment decreased the insulin level and increased the number of circulating endothelial progenitor cells. These results suggest that the combination of olmesartan and pravastatin is beneficial for the treatment of vascular diseases in the insulin-resistant state independently of blood pressure or cholesterol levels. (*Hypertens Res* 2007; 30: 971–978)

***Key Words*: olmesartan, pravastatin, neointimal formation, balloon injury, insulin resistance**

Introduction

Coronary heart disease (CHD) is the leading cause of cardiac mortality and morbidity in the developed countries. Hypertension, hypercholesterolemia, and diabetes mellitus (DM) cause endothelial dysfunction and consequently lead to atherosclerosis. The prevalence of DM is epidemic (*1*). The risk of cardiovascular diseases is two to four times higher in diabetic patients than in the non-diabetic population. Recent studies on the metabolic syndrome (MetS), which is characterized by hypertension, elevated levels of cholesterol and triglyceride, insulin resistance, and central obesity have suggested that these conditions are linked and synergistically

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enhance the development of DM and atherosclerosis (*2*, *3*). Insulin resistance is believed to play a pivotal role in the development of MetS.

The renin-angiotensin system (RAS) is crucially involved in the development of cardiovascular diseases. Angiotensin (Ang) II is the principal final effector molecule of the RAS. The physiological effects of Ang II are mediated through Ang II receptors (*4*). Several studies have suggested that the beneficial effects of Ang II receptor blocker (ARB) are due to inhibition of the type 1 receptor (AT_1R) function as well as to enhanced stimulation of the type 2 receptor, whose effects are generally believed to be opposite those of AT_1R. Clinical trials have shown that ARB improves the prognosis of patients with acute myocardial infarction (*5*), heart failure (*6*), and renal disease (*7*). And these effects are believed to be independent, at least in part, of their blood pressure (BP)–lowering effects. In addition, most of the recent clinical trials have revealed that ARB treatment decreases the incidence of new-onset diabetes (*8*).

3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors (statins) have been found useful for both primary and secondary prevention of CHD (*9*). In addition to their powerful lipid-lowering effect, it is generally accepted that statins have pleiotropic effects independent of cholesterol-lowering effects, such as enhancement of nitric oxide production, inhibition of smooth muscle proliferation, and anti-inflammatory and antioxidative actions (*10*).

A few reports have suggested that a combination of statins and RAS inhibitors may be more effective than single drug treatment in preventing vascular remodeling after angioplasty. Horiuchi *et al*. reported that fluvastatin enhances the inhibitory effects of valsartan on cuff injury–induced neointimal formation in mice (*11*) and in apolipoprotein E knockout mice (*12*). Recently, Nishikawa *et al*. reported that combination treatment with statin and ARB after coronary stenting is useful for reducing in-stent restenosis (*13*). A recent study showed that ARB and statin improved the anti-atherosclerotic gene expression profiles of internal mammary arteries from patients undergoing coronary artery bypass graft (*14*). However, it has not been examined whether a combination of ARB and statin is effective for inhibiting neointimal formation in an insulin-resistant state. We therefore examined whether or not a combination of ARB and statin is beneficial for preventing balloon injury (BI)–induced neointimal formation in insulin-resistant rats.

Methods

Materials

Olmesartan (Olm), an ARB, and pravastatin (Pra), an HMG-CoA reductase inhibitor, were gifts from Sankyo Co. (Tokyo, Japan). A 2F Fogarty balloon catheter was purchased from Baxter (Deerfield, USA). Olm was dissolved in methylcellulose and diluted in distilled water (final concentration 1 mg/mL). Pra was dissolved in distilled water (final concentration 10 mg/mL).

Animal Model

All procedures and animal care were approved by the Committee on Ethics of Animal Experiments, Kyushu University, and were conducted according to the animal care guidelines of the American Physiological Society. Male Sprague-Dawley (SD) rats at 7 weeks old were divided into two groups. One group was fed a standard rat chow containing 60% vegetable starch, 5% fat, and 24% protein (control group). The other group was fed a fructose-rich chow containing 60% fructose, 5% fat, and 20% protein for 4 weeks, which induced an insulin-resistant state (fructose group) (*15*). The fructose group rats were further divided into a sham operation (Sham) group, a BI group, a BI+Olm group, a BI+Pra group, and a BI+Olm+Pra group. The drugs were given orally by gastric gavage once a day, which was started 1 week before BI and continued for 2 weeks after BI. The rats were fed a fructose-rich diet during drug treatment. Olm was given at a dose of 1 mg/kg/day, which is reported not to affect BP level (*16*). Pra was given at a dose of 10 mg/kg/day, which does not affect serum lipid level (*17*). On the last day of the experiments, rats were kept fasting for 12 h and then sacrificed.

Serum concentrations of glucose, triglyceride (TG), total and low-density lipoprotein cholesterol (TC and LDL-C, respectively), and insulin were measured. Systolic BP (SBP) and heart rate (HR) were measured using the tail-cuff method (UR-5000; Ueda Industries, Tokyo, Japan).

BI of Rat Carotid Artery

The rats were anesthetized by intraperitoneal injection of pentobarbital sodium at the age of 12 weeks. The left common carotid artery was denuded of the endothelium with a 2F Fogarty balloon catheter introduced through the external carotid artery (*18*). Inflation and retraction of the balloon catheter were conducted three times. Then the balloon was removed and the external carotid artery was ligated. Sham operation was performed without BI.

Morphometry and Immunostaining

Two weeks after BI, rats were euthanized with a lethal dose of pentobarbital, and the carotid arteries were fixed by perfusion at 100 mmHg with 4% formaldehyde *via* an 18G intravenous cannula placed retrogradely in the abdominal aorta. The arteries were additionally fixed by immersion in the same fixative used for perfusion. The arteries were excised and then embedded in paraffin. Sections were stained with hematoxylin and eosin. The balloon-injured carotid arteries with intact internal elastic lamina were subjected to morphometry for assessing the intima/media (I/M) ratio. Immunohistochemistry was performed using a denoted primary antibody and a commercially

Table 1. BW, BP and HR before and after Fructose Rich Diet

Group	Weeks	*n*	BW (g)	SBP (mmHg)	HR (/min)
Control	7	16	222±15	111±8	352±21
Control	11	8	353±10†	110±7	345±16
Fructose	11	8	413±22†,‡	112±8	342±16

Data are expressed as mean±SD. †$p<0.05$ *vs*. control at 7-weeks. ‡$p<0.05$ *vs*. control at 11-weeks. BW, body weight; SBP, systolic blood pressure; HR, heart rate.

Table 2. Biochemical Analysis of Serum in Control and DM Rats

Group	Weeks	*n*	Glucose (mg/dL)	TC (mg/dL)	LDL-C (mg/dL)	TG (mg/dL)	Insulin (ng/mL)
Control	7	8	128±7	54±6	5±0.5	22±7	0.7±0.2
Control	11	8	134±12	60±10	9±4	37±18	1.0±0.3
Fructose	11	8	177±23‡	398±32‡	87±30‡	75±26‡	4.4±0.7‡

Data are expressed as mean±SD. ‡$p<0.05$ *vs*. control at 11-weeks. TC, total cholesterol; LDL-C, low density lipoprotein cholesterol; TG, triglyceride.

Table 3. BW, BP and HR at 14 Weeks

Group	*n*	BW (g)	SBP (mmHg)	HR (/min)
Control+Sham	8	405±20	113±9	340±18
Control+BI	8	410±23	112±7	336±20
Fructose+Sham	8	416±18	114±9	336±19
Fructose+BI	8	428±18	113±7	344±35
Fructose+BI+Olm	8	405±20	110±6	337±14
Fructose+BI+Pra	8	422±16	114±8	333±13
Fructose+BI+Olm+Pra	8	406±25	108±8	335±19

Data are expressed as mean±SEM. BW, body weight; SBP, systolic blood pressure; HR, heart rate; BI, balloon injury; Olm, olmesartan; Pra, pravastatin.

Table 4. Biochemical Analysis of Serum at 14 Weeks

Group	*n*	Glucose (mg/dL)	TC (mg/dL)	LDL-C (mg/dL)	TG (mg/dL)	Insulin (ng/mL)
Control+Sham	8	133±13	63±12	8±5	39±12	1.0±0.4
Control+BI	8	138±5	61±10	9±5	42±13	1.1±0.4
Fructose+Sham	8	186±25‡	401±42‡	79±21‡	77±18‡	3.8±.6‡
Fructose+BI	8	174±46‡	382±63‡	75±23‡	83±14‡	3.9±.8‡
Fructose+BI+Olm	8	198±30‡	409±74‡	83±31‡	77±14‡	3.4±.9‡
Fructose+BI+Pra	8	222±34‡	357±55‡	69±25‡	70±27‡	3.2±.8‡
Fructose+BI+Olm+Pra	8	176±25‡	324±42‡	56±18‡	59±18‡	1.1±0.4#

Data are expressed as mean±SEM. ‡$p<0.05$ *vs*. control (Sham or BI), #$p<0.01$ *vs*. other fructose groups. TC, total cholesterol; LDL-C, low density lipoprotein cholesterol; TG, triglyceride; BI, balloon injury; Olm, olmesartan; Pra, pravastatin.

available detection system (Dako Glostrup, Denmark). The extent of neointimal formation was quantified by computed planimetry of histologically stained sections. The cross-sectional areas of the blood vessel layers including the lumen area, intima area, and medial area were quantified at three different sections (proximal, middle, and distal). The ratio of Ki-67–positive cells to total nucleated cells was expressed as the Ki-67 index.

Detection of Apoptosis

Apoptotic cells were detected by the terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL) method with an Apoptosis *in situ* Detection Kit (Wako Pure

Chemical Industries, Osaka, Japan). The counterstain was done with hematoxylin. Quantitative analysis was performed in five independent sections in each rat ($n=5$). The ratio of TUNEL-positive cells to total nucleated cells was expressed as the TUNEL index.

Isolation of Peripheral Blood Mononuclear Cells and Identification of Endothelial Progenitor Cells

Rat mononuclear cells (MNCs) were initially isolated from peripheral buffy coat blood in a Histopaque-1083 (Sigma-Aldrich, St. Louis, USA). MNCs were then suspended in EGM-2 (Cambrex Bio Science, East Rutherford, USA), placed on a plate coated with collagen type I (Becton Dickinson, Franklin Lakes, USA) and incubated for 4 days. To detect the uptake of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine–labeled acetylated low-density lipoprotein (DiLDL; Biomedical Technologies, Stoughton, USA), cells were incubated with DiLDL (10 μg/mL) at 37°C for 4 h. Cells were then fixed with 0.5% paraformaldehyde for 10 min, and lectin staining was performed by incubation with fluorescein isothiocyanate (FITC)–labeled Bandeiraea simplicifolia agglutinin BS-I (lectin, 20 μg/mL; Sigma-Aldrich) at 4°C overnight. After the staining, samples were viewed with an inverted fluorescent microscope (IX71; Olympus, Tokyo, Japan). Dual-stained cells positive for both lectin and DiLDL were judged to be endothelial progenitor cells (EPCs) (*19*) and the number of dual-stained cells was counted per dish with a blinded investigator by 15 randomly selected high-power fields (×200).

Statistical Analysis

Statistical analysis was performed with one-way ANOVA and Fisher's test if appropriate. $p<0.05$ was considered to be statistically significant. Data are shown as means±SEM.

Results

Effect of Fructose-Rich Diet

BP and HR were measured before and after the rats were maintained on normal or fructose-rich chow for 4 weeks (Table 1). The body weight (BW) of the fructose group was significantly higher than that of the control group. However, BP and HR were not significantly different between the control and fructose groups. Biochemical analysis of serum showed that levels of TC, LDL-C, and TG were higher in the fructose group than in the control group (Table 2). It was supposed that the majority of the increased cholesterol was high-density lipoprotein (HDL). The increase in insulin level with an increase in blood glucose level suggests that these rats are insulin-resistant.

At the end of the experiments (14 weeks of age), BW, BP, and HR did not differ significantly among the groups (Table 3). The biochemical analysis at 14 weeks of age revealed that drug treatment did not significantly affect serum levels of glucose or lipids (Table 4). The combination of Olm and Pra, however, significantly decreased serum insulin levels compared with the other fructose-fed groups, suggesting that insulin sensitivity was improved.

BI of Rat Carotid Artery

The hematoxylin-eosin (HE) staining showed that the extent of neointimal formation after BI in fructose-fed rats was the same as that of control rats (Fig. 1). Olm or Pra modestly inhibited neointimal formation. The combination of Olm and Pra was more effective than single drug treatment at inhibiting neointimal formation.

Apoptosis and Proliferation

The TUNEL positively stained cells, which indicate apoptotic cells, was increased in the neointima (Fig. 2A, white bars) of the Olm-treated group. Pra did not affect the number of TUNEL-positive cells. However, the addition of Pra to Olm significantly increased the number of TUNEL-positive cells (Fig. 2A). The same tendency was observed in the media (filled bars), although the absolute number of TUNEL-positive cells in the media was small.

The number of Ki-67–positive cells, which indicates cell proliferation, was increased in the neointima of fructose-fed rats. The number of Ki-67–positive cells was decreased in Olm to the same extent as in Pra (Fig. 2B). However, a synergistic decrease in Ki-67–positive cells was not observed in the combination treatment group. Very few Ki-67–positive cells were observed in the media, and there was no significant difference in the number of Ki-67–positive cells among the groups (data not shown). These data may suggest that the reduction in the neointimal area by the combination treatment is due to an increase in the number of apoptotic cells.

Effect on the Number of EPCs

EPCs are believed to participate in the recovery of endothelium of injured artery. We therefore measured the number of EPCs in the MNCs from these control and fructose-fed rats at the end of the experiments (Fig. 3A). The number of EPCs in the fructose-fed rats was significantly decreased (Fig. 3B). BI significantly increased the number of EPCs in both the control and fructose groups. Neither Olm nor Pra alone had any effect on the number of EPCs in the fructose group. However, the combination treatment significantly increased the number of EPCs.

Discussion

Although we hypothesized that the extent of neointimal formation after BI would be exaggerated in the insulin-resistant

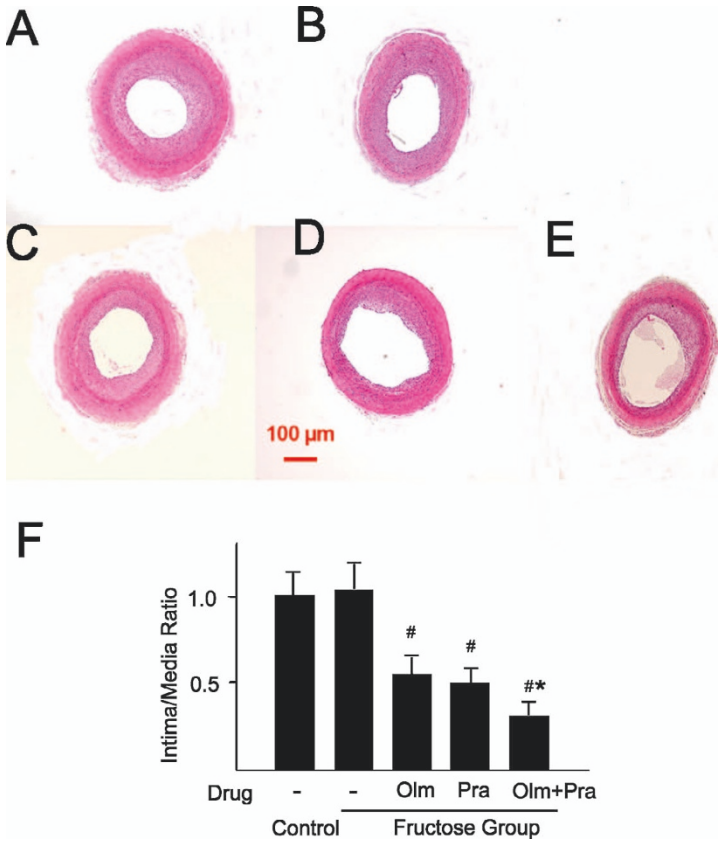

Fig. 1. *Neointimal formation in the carotid artery of control and fructose-fed rats. Representative microphotographs of hematoxylin-eosin staining of carotid artery after 14 days of balloon injury are shown. A: control; B: fructose group; C: fructose+Olm group; D: fructose+Pra group; E: fructose+Olm+Pra group, F: Bar-graph indicates I/M ratio. The values are expressed as means±SEM. n=8. $^{\#}p<0.05$ vs. control. *$p<0.05$ vs. Olm or Pra group and $p<0.01$ vs. control.*

state, this was not the case. The present data and previous results (*11*) suggest that insulin resistance induced by fructose feeding has little effect on BI-induced neointimal formation, which ARB and statin synergistically suppressed to the same extent in the control and fructose-fed rats.

It has been reported that feeding rats a fructose-rich diet induces insulin resistance and hypertension (*15*). Our data showed that both blood glucose and insulin levels were elevated in rats after they were fed a fructose-rich diet for 4 weeks, suggesting that an insulin-resistant state was established. However, the BP level was not changed. A recent paper showed that a fructose-rich diet did not affect BP level (*20*), which is consistent with our results. The mechanism explaining this difference is not yet clear. It is also difficult to explain the increased lipid levels in the fructose-fed rats. The ingredients of the fructose-rich diet used in our experiment are almost the same as those used in the previous studies (*20*, *21*), which reported no significant increase in total cholesterol level but an increase in triglyceride level. Although the mechanism for the increase in cholesterol levels is not clear, Pra did not show a significant effect on cholesterol level in the fructose-fed rats, indicating that a comparison among the fructose groups is possible.

Olm at doses that decrease BP was reported to improve insulin resistance in fructose-fed rats (*21*). Our data, however, indicated that Olm at a dose without BP change did not affect insulin or glucose levels. Intriguingly, the combination of Olm and Pra significantly decreased insulin levels, although Pra alone did not affect insulin levels. The mechanism underlying this combination effect is not clear. One of the possible common target molecules of ARB and statin is Rho-kinase. Ang II is reported to activate the Rho–Rho kinase pathway (*22*, *23*). Thus, ARB prevents Ang II–induced Rho-kinase activation. And a recent report suggested that Rho-kinase phosphorylates the serine residue of IRS-1, which inhibits insulin signaling and results in insulin resistance (*23*). Statins are known to inhibit activation of Rho by inhibiting geranylgeranylation (*24*). Actually, it was reported that fluvastatin, another HMG-CoA reductase inhibitor, prevented Ang II–induced cardiac hypertrophy through the inhibition of Rho-kinase (*25*). Therefore, it may be possible that a combination of Olm and Pra synergistically suppresses the Rho–

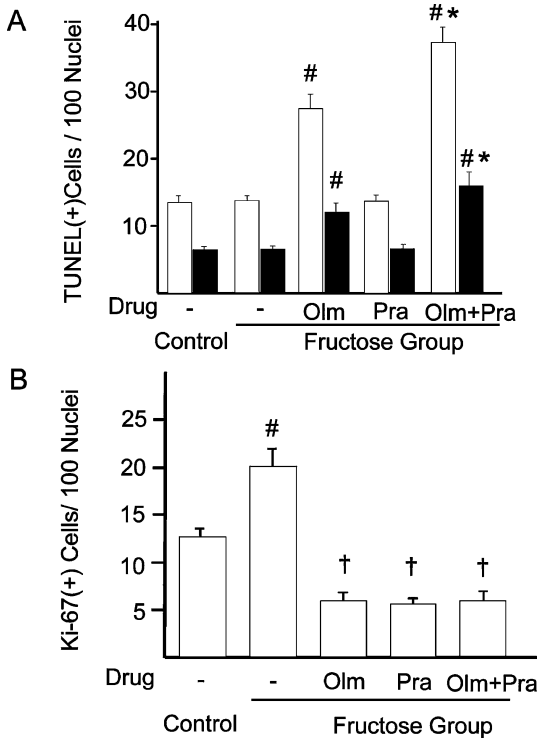

Fig. 2. *Apoptosis and proliferation of VSMC in the injured artery. TUNEL staining and immunohistochemical analysis for Ki-67 staining were performed in cross sections of carotid artery 14 days after balloon injury. A: TUNEL index of the intima (white bars) or media (black bars) is indicated (n = 5). B: Ki-67–positive index of intima (white bars) is indicated. Because there were so few Ki-67–positive cells in the media, these are not indicated in the graph. The values are expressed means ± SEM.* $^{\#}p < 0.05$ *vs. control.* $*p < 0.05$ *vs. Olm.* $^{\dagger}p < 0.05$ *vs. control and* $p < 0.01$ *vs. fructose group without drug. n = 5.*

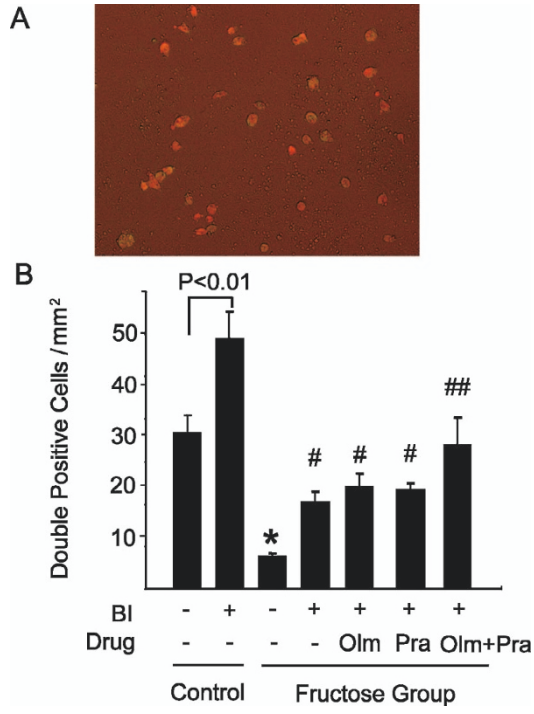

Fig. 3. *The number of endothelial progenitor cells. A: A representative microphotograph of attached mononuclear cells stained with FITC-lectin (green) and incorporating DiI-LDL (red). Part of the microscopic field is enlarged. B: The number of double-positive cells is counted as EPCs. The bar graph indicates the number of EPCs in each group. The values are expressed as means ± SEM.* $p < 0.05$ *vs. fructose without BI.* $*p < 0.05$ *vs. control without BI.* $^{\#}p < 0.05$ *vs. fructose group without BI.* $^{\#\#}p < 0.01$ *vs. fructose group without BI and* $p < 0.05$ *vs. other fructose with BI groups. n = 5–8.*

Rho kinase pathway and improves insulin resistance.

Contradictory results were reported about the effects of Pra, a hydrophilic statin that penetrates the cell membrane poorly, on vascular smooth muscle cells (VSMC) proliferation and apoptosis. Pra was found to have a minimal effect on the inhibition of VSMC proliferation and the induction of apoptosis, whereas substantial effects were observed with fluvastatin or simvastatin (*26*). However, a recent report showed that Pra at a relatively high dose induces apoptosis and growth inhibition of VSMC, possibly through $p27^{Kip1}$ and phosphatidylinositol-3 kinase (PI-3K) (*27*). Because Ang II is known to activate PI-3K, the combination of Pra and Olm may synergistically inhibit this pathway. However, the mechanisms of the differential effect of the combined therapy on the TUNEL index and Ki-67 index is not yet clear. Further investigation is needed.

The combination of Olm and Pra was also more effective in recruiting EPCs. It was reported that statin-induced EPC mobilization requires increased endothelial nitric oxide (NO) bioavailability (*28*). Because Ang II induces production of reactive oxygen species, which quench NO, the combination of ARB and statin may increase the NO bioavailability, resulting in increased EPC recruitment. Indeed, a recent study showed that a combination treatment of Olm and Pra synergistically improved endothelium-dependent vasodilation and reduced the level of thiobarbituric acid–reactive substances in salt-loaded Dahl salt-sensitive hypertensive rats compared with treatment with either drug alone, suggesting NO has a role in the combined treatment (*29*).

However, the contribution of EPCs to the reduction of neointimal formation is not clear from this study because we did not examine whether or not these cells were incorporated into the regenerated endothelium of the injured vessel. In addition, the number of EPCs in the control BI group, which has the largest neointimal area, is the highest among all groups. Interestingly, it was reported that recruitment of vascular progenitor cells to vascular lesions is injury-dependent (*30*). Tanaka *et al*. (*30*) showed that bone marrow cells were

incorporated substantially into severely injured arteries. And many studies have suggested that EPCs are involved in the early repair of injured arteries (*31*). Because it is generally accepted that insulin resistance enhances vascular lesion formation, it may be possible that EPCs have little effect on neointimal formation under normal conditions but play an important role in the repair of injured arteries under pathological conditions such as diabetes and insulin resistance. Alternatively, these results may suggest that EPCs do not contribute to the repair of vascular injury in the acute or subacute stage of BI.

Insulin has various effects on VSMCs. Trovati *et al*. (*32*) reported that insulin increased cGMP and cAMP levels in VSMC. Because these cyclic nucleotides are reported to inhibit VSMC growth (*33*), insulin resistance may enhance VSMC proliferation after BI. In contrast, it was also reported that insulin itself has a weak growth-promoting effect on VSMC (*34*, *35*) and augments the growth-promoting effect of platelet-derived growth factor. In this case, insulin resistance may have a favorable effect on vasculature. In endothelial cells, insulin stimulates NO production (*35*). NO inhibits many of the processes associated with atherosclerosis. It is known that NO inhibits VSMC growth, suggesting that insulin resistance of endothelial cells may reduce NO production, resulting in the enhancement of VSMC growth. The reduction of NO level may enhance expression of tumor necrosis factor-α and monocyte chemoattractant protein-1 (*36*), which are believed to accelerate atherogenesis. Because of the complex nature of the direct insulin action on blood vessels as indicated above, it is difficult to determine whether or not improvement of insulin resistance by combination treatment of Olm and Pra contributed to the reduction of neointimal formation in our model. However, it is generally believed that insulin resistance and hyperinsulinemia enhance atherogenesis (*37*), suggesting that the improvement of insulin resistance by Olm and Pra treatment may play a role in the inhibition of neointimal formation.

In conclusion, we showed in the present study that the combination of Olm and Pra has beneficial effects on vascular remodeling after injury and insulin sensitivity in rats fed a fructose-rich diet. These data suggest that the combination of ARB and statin may be recommended for patients with CHD and insulin resistance. Because these drugs did not change BP or blood lipid levels, it is reasonable to assume that these beneficial effects of ARBs and statins were independent of BP- or lipid-lowering properties.

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