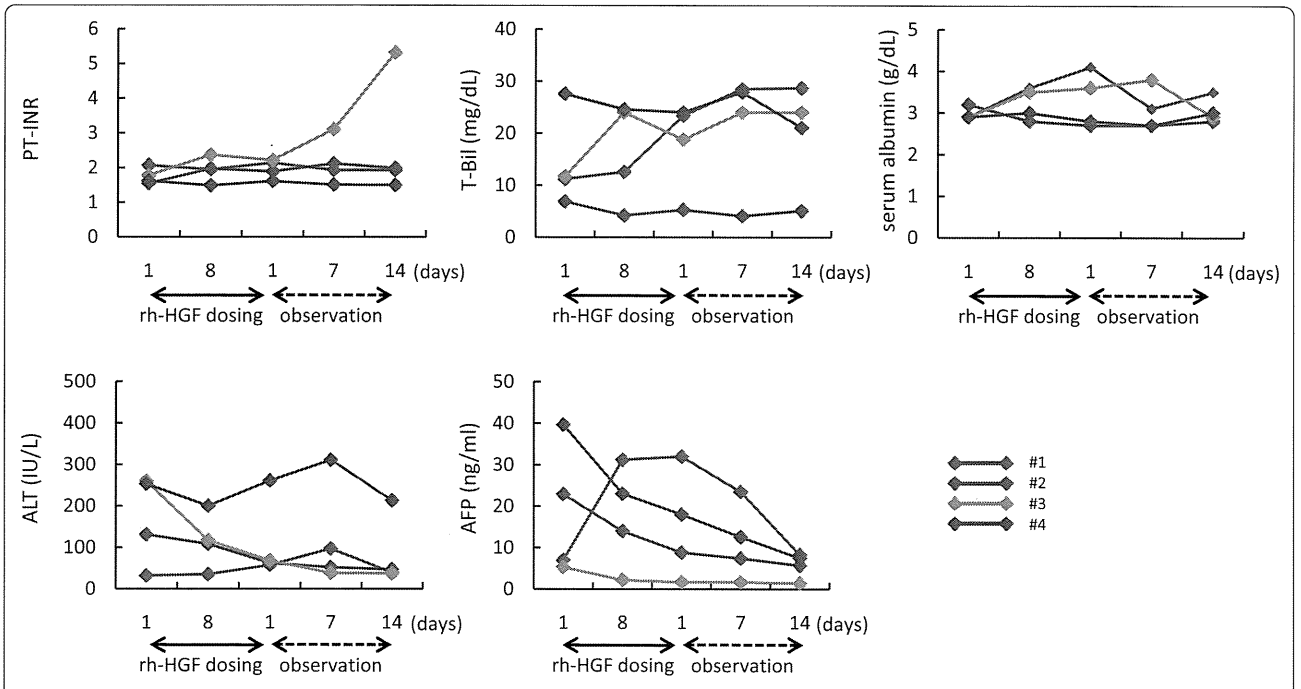

Figure 5 Changes in laboratory data results during rh-HGF dosing and observation period. PT-INR, T-Bil, serum albumin, ALT and AFP, were measured before rh-HGF administration (day 1 of rh-HGF dosing); on day 7 of the rh-HGF dosing period; and one, seven and 14 days after the protocol therapy (days 1, 7 and 14 of the observation period, respectively). Laboratory data results were not affected during or after rh-HGF administration.

complications caused by rh-HGF dosing were not observed. BP was gradually reduced during stepwise infusion of rh-HGF in three of the four patients, whereas repeated doses of rh-HGF did not affect albuminuria. In the first patient, when BP decreased during rh-HGF administration, 200-300 mL of infusion was sufficient to restore BP immediately; prior infusion ameliorated HGF-induced BP reduction, as observed in preclinical animal experiments (Figure 1C). In any event, the decrease in BP observed during HGF infusion was reversible, and did not affect patients' general condition. Although patients 2 and 3, but not 4, also exhibited BP reduction during rh-HGF infusion, their general condition was stable without additional infusion or cessation of rh-HGF. Of particular importance, patient 2, who had awakened from hepatic encephalopathy, showed no symptom or sign during rh-HGF administration. Therefore, we concluded that rh-HGF administered intravenously with a stepwise increase for up to 14 consecutive days was very well tolerated.

Table 3 Effect of rh-HGF administration on survival time

	hazard ratio	95% CI		*p value*
Survival time from:				
onset of hepatic encephalopathy	0.20	0.03	1.45	0.08
onset of disease	0.28	0.04	2.04	0.18

In this study, although two of four patients survived, there was no evidence that rh-HGF was effective in improving outcome of patients with FHSA or LOHF. There are three potential reasons for the failure of this trial to demonstrate the efficacy of rh-HGF in patients with FH or LOHF.

First, the dose of rh-HGF and/or the 14-day treatment schedule used in this study might have been too low to produce beneficial effect. The dose chosen for this study was based on a scaling of the doses used in pre-clinical animal studies, and ensured safety in several repeated dose toxicity tests. Also, this dose, corresponding to 0.1 mg/kg in rodents, has been reported to accelerate liver regeneration in normal and partially hepatectomized rats [11]. Conversely, the treatment duration was based on a nationwide survey of FH and LOHF in Japan between 1998 and 2002. In this survey, 90.4% (n = 47) of surviving patients from FHSA and LOHF (n = 52) awaked within 14 days after hepatic encephalopathy occurred, and 71% (n = 135) of non-surviving patients (n = 190) died within 28 days following the onset of hepatic encephalopathy. Therefore, rh-HGF administration for up to 14 days, followed by a 14-day observation period, was considered to be sufficient to evaluate both safety and efficacy. However, in the current study, there

was no evidence of inhibited disease progression or stimulated liver regeneration. This suggests either that the dose of rh-HGF administered in this study was insufficient to induce liver regeneration and suppress liver injury, or that the 14-day treatment regimen was too short.

Second, HGF/c-Met pathways may be impaired in patients with FH or LOHF. When rh-HGF was intravenously injected in a bolus, most rh-HGF was distributed into the liver, and development of liver injury or cirrhosis retarded clearance of rh-HGF [23,24]. In this clinical study, serum levels of HGF increased to 10-20 ng/mL (Cmax) just after a stepwise infusion of rh-HGF (0.6 mg/m^2). HGF is known to stimulate proliferation of both mature hepatocytes and hepatic progenitor cells: less than 10 ng/mL of HGF was sufficient to induce proliferation of primary cultured rat hepatocytes [12,25], and *in vivo* proliferation of rat hepatic progenitor cells was stimulated by serum levels of ~2 ng/mL human HGF [13,26]. In patients with FH, serum levels of growth and growth-inhibitory factors were elevated [27-29], and reciprocal action of these factors in FH patients results in impaired liver regeneration. In this clinical trial, the increase in serum HGF concentration did not lead to improvement of hepatic reserve; furthermore, serum levels of transforming growth factor (TGF)-β, a growth-inhibitory factor, were not affected by HGF administration (Additional file 5). However, patient 1 revealed an increase in serum AFP, a marker of liver regeneration in patients with FH, during rh-HGF dosing period, and gradually decreased after the completion of rh-HGF administration. In contrast, patients 2 and 4, who survived, showed an increase in serum AFP at enrollment, but serum AFP levels decreased during the rh-HGF dosing period. These two patients received PSL in parallel with rh-HGF (Additional files 2 and 4); AFP expression is known to be affected by a glucocorticoid responsive element (GRE) present in the 5'-flanking region of AFP gene [30]. Once serum AFP levels decreased, slowly tapered PSL did not affected serum AFP in these surviving patients. However, AFP expression at enrollment may be suppressed via the GRE, leading to a decrease in serum AFP levels. Therefore, dose escalation or prolonged exposure to rh-HGF may be able to overcome impaired liver regeneration.

Third, both FH and LOHF patients enrolled in this trial were predicted to die without liver transplantation; thus, the subjects already presented with an extremely serious condition. This life-threatening condition was influenced by the degree of impaired hepatic reserve and varying complications. Indeed, in this trial, all eligible patients with FH or LOHF developed hepatic encephalopathy, and the impaired hepatic reserve and general condition varied in severity. In these patients, even though safety could be evaluated, it may be difficult to evaluate the clinical efficacy. Therefore, it will be desirable to examine the clinical efficacy of rh-HGF in additional clinical trials involving patients with less severe conditions.

Systemic administration of potent growth factors could theoretically stimulate premalignant lesions in distant organs. Therefore, in this first clinical trial of rh-HGF, it was prudent to limit systemic therapy to life-threatening conditions. Although the two surviving patients in this study should be observed over the long term, we showed here that repeated doses of intravenous rh-HGF were well tolerated even in patients with a fatal disease. Recent investigations have indicated that HGF has the potential to improve treatment for intractable diseases of various organs, including the nervous system [31,32], lung [33], heart [34-36], intestine [26,37], kidney [38], and vessels [39]. Therefore, the safety assessment of protein-based therapy of HGF described here sheds light on the development of new therapeutic modalities aimed at treating patients with intractable diseases.

Conclusions

Despite a mild BP reduction during rh-HGF infusion, intravenous rh-HGF at a dose of 0.6 mg/m^2 was well tolerated in patients with FH or LOHF. However, there was no evidence that those dose of rh-HGF was effective for the treatment of these patients. Additional studies of rh-HGF at doses higher than 0.6 mg/m^2, for longer periods, or in treatment of patients with less severe conditions, will be valuable in determining the clinical efficacy of rh-HGF.

Additional material

Additional file 1: Clinical course of patient 1 with FHSA, the first patient receiving intravenous rh-HGF. We first administered rh-HGF to a 67-year-old Japanese man with FHSA caused by hepatitis E virus infection. On admission, he presented with hepatic encephalopathy, jaundice, ascites, edema, and microhematuria caused by bladder catheter. Although ALT had already decreased to 32 IU/L, we observed thrombocytopenia (6.1 × 10^4/μL), increased T-Bil (11.2 mg/dL), a marked decrease in serum albumin (2.9 g/dL), and prolonged PT (33%) (PT-INR 2.07), indicating severely impaired hepatic reserve. Serum HGF and AFP levels were 0.77 and 7.0 ng/mL, respectively, and liver volume measured by CT was 1055 mL. Following observation of general condition for two days, administration of rh-HGF (0.6 mg/m^2/day) was initiated. Because of an increase in serum creatinine level of 2.0 mg/dL, caused by diuretics administration to reduce massive ascites, protocol therapy was discontinued on day 14, resulting in 13-day administration of rh-HGF. Although prolonged PT was stable during rh-HGF dosing and observation period, T-Bil gradually increased and hepatic encephalopathy did not improve. Hepatic failure gradually progressed after the observation period; the patient ultimately died 68 days after the onset of hepatic encephalopathy. PE, plasma exchange; CHDF, continuous hemodiafiltration.

Additional file 2: Clinical course of patient 2 with FHSA, who survived. The second patient (patient 2) was a 71-year-old Japanese woman with FHSA of undetermined etiology. She presented with mild hepatic encephalopathy with flapping tremor, jaundice, and urinary findings, including proteinuria and microhematuria, caused by bladder catheter. Platelet count and serum albumin level decreased to 6.9×10^4/μL, and 3.2 g/dL, respectively, and PT was prolonged to 49% (PT-INR 1.55). In addition to increased T-Bil level of 6.9 mg/dL, serum ALT level increased to 131 IU/L. Serum HGF and AFP levels were 1.94 and 22.9 ng/mL, respectively, and liver volume was 595 mL. Following observation of general condition for 24 hours, treatment with rh-HGF was initiated, and the protocol therapy was continued for 14 days without any severe adverse events. Hepatic encephalopathy disappeared after plasma exchange (PE) on day 2; consciousness level was not impaired throughout the study period. Intravenous rh-HGF reduced systolic BP. The patients with lucidity, however, did not complain any symptom. Although prednisolone (PSL) was administered to reduce ALT, blood biochemical findings and patient condition were stable throughout the study period. After the completion of the study, biochemical findings were gradually improved, and, finally, the patient survived.

Additional file 3: Clinical course of patient 3, with LOHF, who died within the observation period. Sixty four-year-old Japanese woman with LOHF of undetermined etiology suffered from advanced hepatic encephalopathy (HE). She presented with platelet count of 9.2×10^4/μL, PT of 37% (PT-INR 1.78), T-Bil level of 11.7 mg/dL, ALT level of 260 IU/L, and serum albumin level of 2.9 g/dL. Serum HGF and AFP levels were 1.07 and 3.9 ng/mL, respectively, and liver volume was 640 mL. Because of oliguria (392 mL/day), protocol therapy was discontinued on day 13, resulting in 12-day rh-HGF dosing. Additionally, PSL was administered to reduce serum ALT, and plasma exchange (PE) and/or continuous hemodiafiltration (CHDF) was performed throughout the study period. Serum ALT levels reduced immediately, and hepatic encephalopathy was transiently improved during rh-HGF dosing period. However, hepatic encephalopathy, prolonged PT, and an increase in T-Bil progressed during the observation period, and the patient died during the observation period (28 days after the onset of hepatic encephalopathy).

Additional file 4: Clinical course of patient 4, with FHSA caused by a drug, who survived. Forty-year-old Japanese man with FHSA, which was caused by a supplement containing coenzyme Q-10, showed platelet count of 7.0×10^4/μL, PT of 43% (PT-INR 1.62), T-Bil level of 27.6 mf/dL, ALT level of 253 IU/L, and serum albumin level of 2.9 g/dL, but not hepatic encephalopathy (HE), which was temporarily observed before enrollment. Serum HGF and AFP levels were 1.88 and 39.7 ng/mL, respectively, and liver volume was 1110 mL. Administration of rh-HGF was continued for 14 days, and PSL was administered to reduce ALT throughout the study period. An increase in T-Bil and prolonged PT was modestly improved during rh-HGF dosing, followed by further improvement after the observation period. Ultimately, the patient survived. PE; plasma exchange.

Additional file 5: Serum levels of TGF-β were not affected by rh-HGF dosing. Serum TGF-β concentrations before and after the rh-HGF dosing period were determined by ELISA. Although patient 2 exhibited an increase in serum TGF-β after 14-day rh-HGF administration, there was no significant difference in serum levels of TGF-β (mean ± SE: 230.4 ± 21.0 vs 266.4 ± 68.1 pg/ml, $p = 0.52$).

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Authors' contributions
AI, AM, MN, and IDK conducted preclinical studies. AI, AM, MN, IDK, TM, ST, SH, MY, MF, AS, and HT participated in research design. AI, SH, AS, and HT contributed to preparation of rh-HGF at GMP grade. AI, AM, MN, TM, HM, NY, HS, IDK, TC, and MY provided medical care. ST and MF performed data analysis. AI, AM, MN, ST, AS, and HT wrote or contributed to the writing of the manuscript.

Competing interests
The authors declare no competing interests. Mitsubishi Tanabe Pharma Corporation had no role in the design of the study, in data accrual or analysis, or in preparation of the manuscript.

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Atherosclerotic plaques induced by marble-burying behavior are stabilized by exercise training in experimental atherosclerosis

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ABSTRACT

Background: We assessed the hypothesis whether behavioral stress may affect the development of atherosclerosis and whether regular exercise training may influence the composition of atherosclerotic plaques in apolipoprotein (apo) E-deficient mice.
Methods: Atherosclerosis was induced in apo E-deficient mice fed a high fat diet. Exercise training (45 min swimming, 3 times/week) was conducted, and behavioral stress was provoked by glass marble-burying procedure. Mice were treated with marble-burying, marble-burying behavior plus swimming training, and swimming alone over 8 weeks.
Results: Exercise training decreased the atherosclerotic lesions, but marble-burying behavior increased the lesions. The plaques containing macrophage accumulation with intercellular adhesion molecule-1 (ICAM-1) expression associated with reduced collagen contents were induced in the mice treated with marble-burying. However, ICAM-1 expression was suppressed and collagen contents were reversed in the mice that received marble-burying behavior plus exercise training. In addition, exercise alone and concomitant exercise training reduced the superoxide production in aortic walls, shown by dihydroethidium staining, compared with that in mice with marble-burying behavior alone. There were no significant differences in the serum lipids profiles among the groups.
Conclusions: Behavioral stress increased the atherosclerotic lesions and induced the adhesion molecule expression with superoxide production on the lesions in apo E-deficient mice. Exercise training may stabilize plaque lesions induced by marble-burying behavior in this animal model.

1. Introduction

Various stresses, inducing psychological stress, are now recognized to be an important contributor to cardiovascular diseases. Both clinical and experimental evidence supports the hypothesis that behavioral stress is linked to hypertension, cardiac arrhythmias, and sudden, unexpected death [1–5]. However, the specific sites of action and mechanisms involved in the development of atherosclerosis are not well known. Inflammation, cytokine overproduction, oxidative stress, and free radicals may be considered to be key mechanisms for the development of atherosclerosis [6]. On the other hand, exercise is a deterrent of cardiovascular disease, and its antiatherogenetic effects have been described [7–9].

Defensive burying is a behavior that can be elicited in rodents in response to aversive stimuli. The term is used to describe the behavior when rats or mice bury a shock prod, noxious food or dead conspecifics under a layer of bedding material [10,11]. In recent years, many investigators have used the marble-burying assay as a tool for assessing anxiety-like behaviors in mice [12].

In the present study, using apolipoprotein E-deficient mice, we have provided evidence for the induction of intercellular adhesion molecule-1 expression on atherosclerotic plaques by defensive burying behavior and for the suppression of the expression by exercise with the analysis of tissue superoxide production.

2. Materials and methods

2.1. Experimental atherosclerosis

The apolipoprotein E (apo E)-deficient 129ola×C57BL/6 hybrid mice were generous gifts of Dr. Edward M. Rubin (University of California, Berkeley, CA). These mice were mated with C57BL/6 mice to produce F_1 hybrids. The F_1 apo $E^{+/-}$ mice were then backcrossed to C57BL/6 mice for 10 generations. Mice homogeneous for the apo E-null allele on a C57BL/6 background were subsequently generated. Male mice were subjected to the subsequent experiments. The mice were kept in a temperature-controlled facility on a 14:10-hour light–dark cycle with free access to food and water.

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After being weaned at 4 weeks of age, mice were fed a normal chow diet (Oriental Yeast) until 6 weeks of age, when the animals were switched to a high fat diet containing 20% fat and 0.3% cholesterol as previously described [13,14].

The experimental protocols were approved by the institutional ethics committee for animal experiments of Kyoto University.

2.2. Procedure of marble-burying (Fig. 1)

The groups of 3 to 6 mice were placed in 23×17×14 cm cages for 30 min. Thereafter, mice were placed individually in a 23×17×14 cm cage with 20 glass marbles, 1.5 cm in diameter. The glass marbles were placed in close contact in the middle of the cage on a 5 cm layer of sawdust. The ceiling was smooth with a few-holes so that the mice could not cling to the cage ceiling. The mice were left in the cage with marbles for 30 min. After this, the test was terminated by removing the mice and counting the number of marbles that were more than two thirds covered by sawdust. The test was done 24 times during 8 weeks. The average value was calculated (Fig. 1).

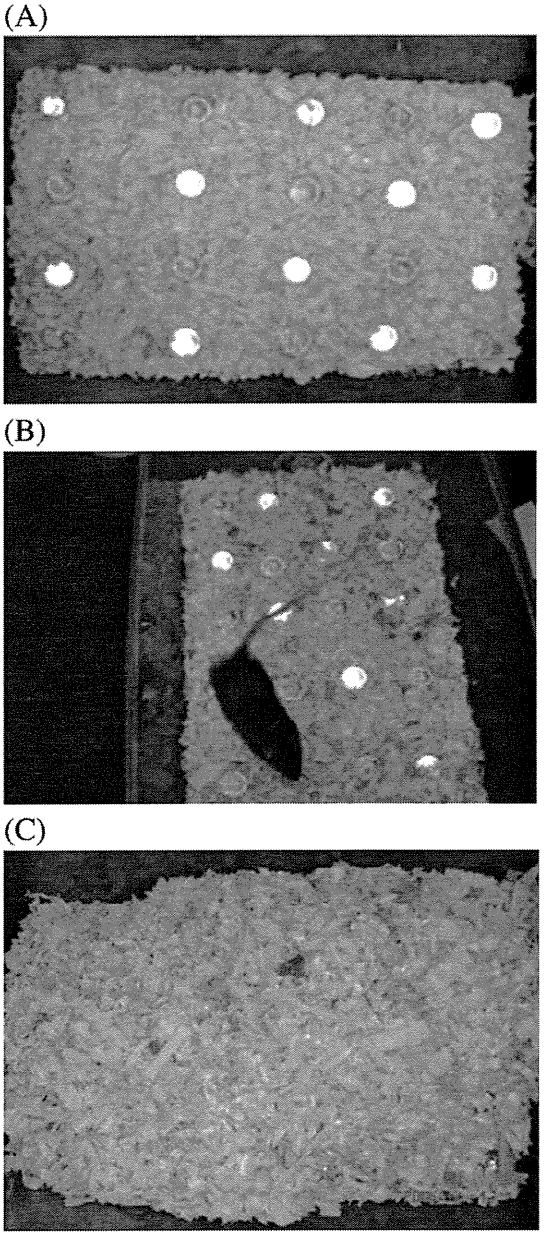

Fig. 1. Marble-burying behavior. (A)The 20 glass marbles were placed in a cage. (B) Mice were placed individually in a cage with for 30 min. After this, the test was terminated by removing the mice and counting the number of marbles that were covered by sawdust more than two thirds. (C) Number of marbles buried was calculated.

2.3. Experimental protocol

At 6 weeks of age, mice were divided into 4 groups and subjected to the study protocol. They were forced to marble-burying as mentioned before and to swim a hot water bath at 37 °C for 45 min per day 3 times on alternate days per week during 8 weeks as previously described [9]; control group ($n=14$), marble-burying group ($n=20$), marble-burying plus exercise group ($n=15$), and exercise group ($n=7$). It was already reported by us that this protocol of forced swimming exercise was not stressful for rodents [9]. The number of burying marbles was recorded at each time. The measurement was repeated three times at each time. At 14 weeks the mice were killed by puncture of the ventricle under ether anesthesia. The organs were weighed, and the ratios of organ weight to body weight were calculated.

2.4. Tissue processing

Mice were killed by bleeding with puncture of the ventricle. The vasculature was perfused with sterile phosphate buffered saline and 6.8% sucrose. The root of the aorta was dissected under a microscope and frozen in OCT embedding medium for serial cryosectioning covering 1.0 mm of the root. The first section was harvested when the first cusp became visible in the lumen of the aorta. Four sections of 6 μm thickness were harvested per slide, and thus 8 slides per mouse were prepared. All sections were immersed for 15 s in 60% isopropanol, stained for 30 min in a saturated oil-red-O solution at room temperature, counterstained with hematoxylin, and then mounted under coverslips with glycerol gelatin [13,14]. Also, collagen contents were detected by Sirius red staining. The oil-red O and Sirius red stained sections were analyzed, blinded to group, as previously described [13–15].

2.5. Immunohistochemistry

Anti-macrophage (anti-Mϕ, M 3184, 1:400, PharMigen) and anti-intercellular adhesion molecule-1(ICAM-1) (M-19, 1:100, Santa Cruz Biotechnology) antibodies were applied to acetone-fixed cryosections of aortic roots. After being washed, the sections were then exposed to second antibodies (horseradish peroxidase-conjugated antibodies), and the horseradish peroxidase-conjugated antibody binding was visualized with diaminobenzidine. Sections were counterstained with methyl green or Mayer's hemotoxylin. Macrophages in the lesions were quantitatively evaluated as previously described [15,16]. Data were obtained by dividing the number of positively stained cells by all counterstained cells inside the internal elastic lamina. Three to five random microscopic fields were analyzed at ×200. ICAM-1 expression was qualitatively evaluated.

2.6. In situ defection of superoxide production

To evaluate in situ superoxide production from aorta, unfixed frozen cross sections of the specimens were stained with dihydroethidium (DHE; Molecular Probe, OR) according to the previously validated method [17–19]. In the presence of superoxide, DHE is converted to the fluorescent molecule ethidium, which can then label nuclei by intercalating with DNA. Briefly, the unfixed frozen tissues were cut into 10-μm thick sections, and incubated with 10 μM DHE at 37 °C for 30 min in a light-protected humidified chamber. The images were obtained with a laser scanning confocal microscope. Superoxide production was demonstrated by red fluorescence labeling. For quantification of ethidium fluorescence from aortas, fluorescence (intensity × area) was measured using a high-power image. The validity of DHE staining for the evaluation of superoxide production from the atherosclerotic lesions was already reported by us [19].

2.7. Lipid measurement

Serum was separated by centrifugation and stored at −80 °C. Serum total cholesterol (TC) and triglyceride (TG) levels were measured with assay kits (Wako) according to the manufacturer's instructions.

2.8. Statistical analysis

Values were expressed as means ± SD. One-way ANOVA with subsequent Fisher protected least-significant difference tests was performed. A value of $P<0.05$ was considered statistically significant.

3. Results

3.1. Organ weights (Table 1)

There were no significant differences in body weight, heart weight, or heart weight to body weight among the groups (Table 1).

Table 1
Organ weights and lipid profiles.

	(*n*)	BW (g)	HW (g)	HW/BW (mg/g)	(*n*)	TC (mg/dl)	TG (mg/dl)
Control	14	31.5 ± 5.5	0.19 ± 0.04	6.03 ± 0.65	6	1307 ± 273	54.5 ± 28.4
Marble-burying	20	30.7 ± 6.2	0.21 ± 0.04	6.84 ± 0.63	6	1187 ± 283	68.0 ± 48.3
Marble-burying + Exercise	15	31.7 ± 4.9	0.20 ± 0.04	6.31 ± 0.60	6	1586 ± 304	52.5 ± 23.1
Exercise	7	31.7 ± 6.0	0.19 ± 0.05	5.99 ± 0.71	5	1233 ± 249	60.4 ± 34.4

(Mn ± SD).
BW = body weight, HW = heart weight, TC = total cholesterol, TG = triglyceride.

3.2. Atherosclerotic lesions (Figs. 2 and 3)

The surface areas of aortic roots covered by fatty streak lesions were quantified in oil red-O-stained samples, and specimens among the groups were compared. Controls ($105.30 \pm 39.0 \times 10^3$ μm^2, $n = 14$) and the marble-burying behavior group ($138.06 \pm 25.74 \times 10^3$ μm^2, $n = 20$) developed extensive lesions in the root of the aorta (Fig. 2). Exercise group ($61.62 \pm 15.60 \times 10^3$ μm^2, $n = 7$) showed the minimal lesions among the groups. The marble-burying behavior increased the severity of atherosclerosis significantly ($P<0.05$) compared with controls. In mice treated with marble-burying plus exercise ($69.42 \pm 19.50 \times 10^3$ μm^2, $n = 15$), the fractional area of lesions was reduced ($P<0.01$) compared with the marble-burying group as shown in Fig. 2.

Compared with the control ($38.22 \pm 10.14 \times 10^3$ μm^2), collagen contents were decreased ($P<0.05$) by marble-burying behavior ($21.06 \pm 7.8 \times 10^3$ μm^2) and were returned to the control levels by the treatment of marble-burying plus exercise ($39.78 \pm 17.94 \times 10^3$ μm^2) (Fig. 3). In exercise group ($46.80 \pm 10.92 \times 10^3$ μm^2), collagen contents were highest among the groups.

3.3. Inflammatory cell surface markers (Figs. 2 and 4)

The degree of macrophage-positive cells was decreased in the exercise-treated group and the marble-burying behavior-treated plus concomitant exercise-treated group compared with the other two groups (Fig. 2). The expression of ICAM-1 was increased by marble-burying behavior and was decreased by the concomitant exercise. The expression of ICAM-1 in exercise group was minimal (Fig. 4).

3.4. In situ superoxide production (Fig. 5)

To analyze the in situ superoxide production in the aortic wall, DHE staining was performed. Ethidium fluorescence was detected in the plaques. The intensity of DHE staining was increased by the marble-burying behavior, and was suppressed by the marble-burying plus swimming training group. The intensity of DHE staining in exercise group was lowest among the groups (Fig. 5).

3.5. Lipid profiles (Table 1)

There were no significant differences in the serum lipids profiles among the groups.

3.6. Numbers of marbles buried (Fig. 6)

Fig. 4 shows the numbers of marbles buried and the effects of exercise upon marble burying. The numbers in mice treated with exercise alone and concomitant exercise were significantly less

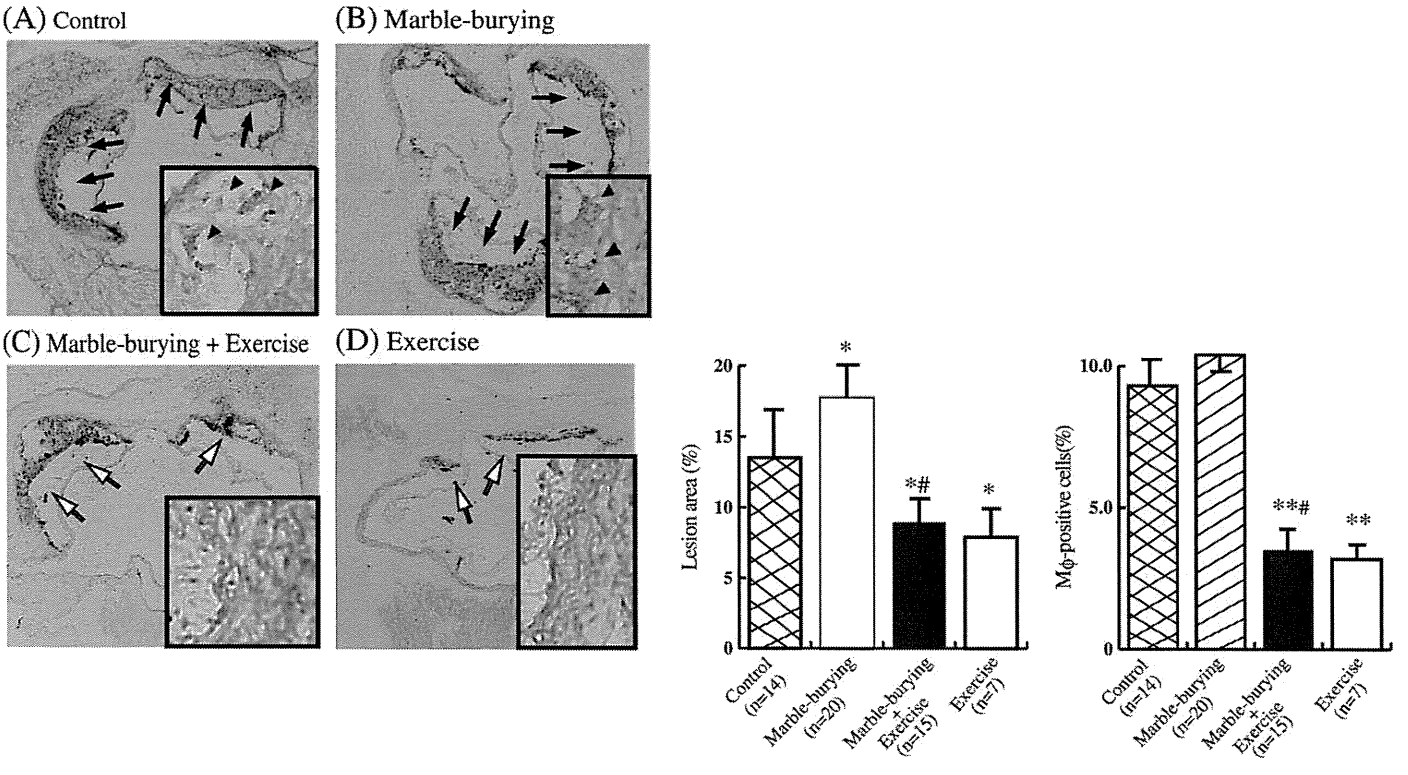

Fig. 2. Atherosclerotic lesions and, macrophage infiltration. The lesions (white arrows) in the marble-burying procedure plus exercise-treated mouse (C) and the exercise-treated mouse (D) were smaller and covered less of the inner circumference of the aortic root than those (black arrows) of the control mouse (A) and marble-burying procedure-treated mouse (B). Insets boxes show magnified samples for macrophages (Mϕ) (arrow-heads). Oil-red-O stain (A, B, C, D × 60). *$P<0.05$, **$P<0.01$ vs control group. #$P<0.01$ vs marble-burying group.

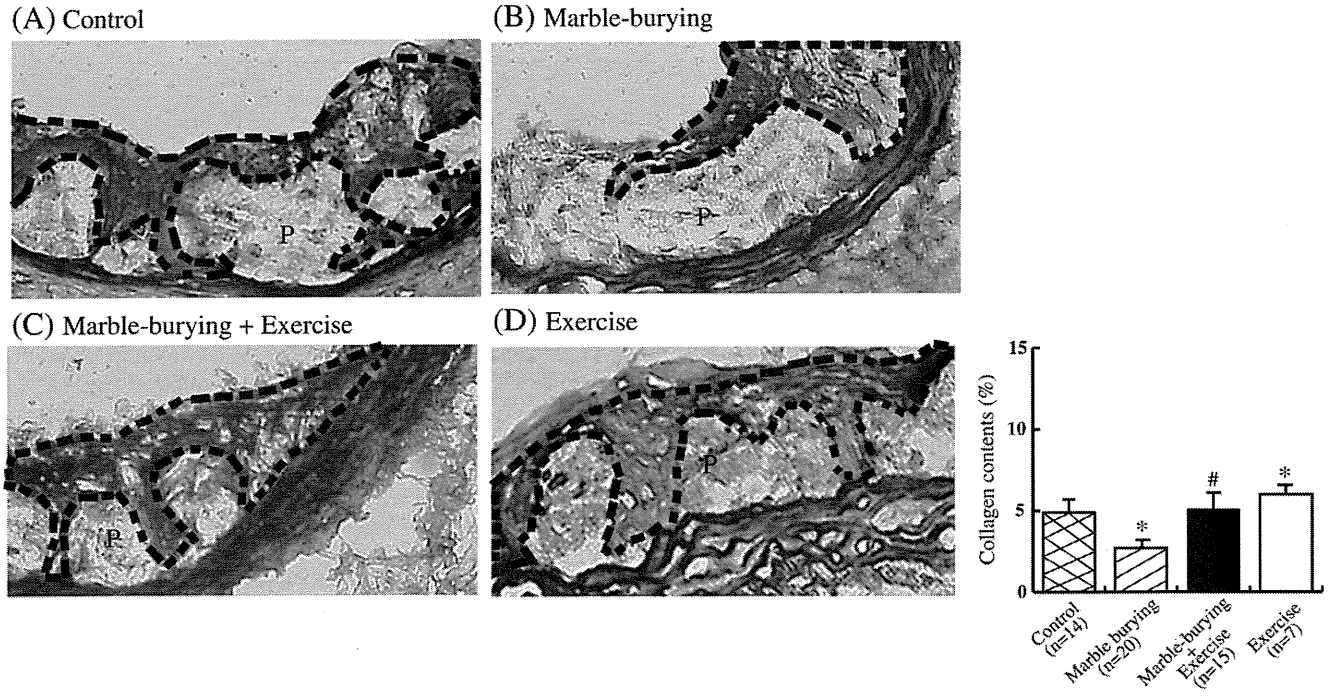

Fig. 3. Collagen contents. Compared with the control (A), collagen contents (red area, surrounded by dashed line) were decreased by marble-burying behavior (B), and were returned to the control levels by the treatment of marble-burying plus exercise (C). In exercise group (D), collagen contents were highest. P indicates plaques. Sirius red stain (A, B, C, D × 120). *P<0.05, vs control group. #P<0.01 vs marble-burying group.

compared with those in mice treated with marble-burying alone, which may reflect the less psychological stress-state in the exercise-treated group and the concomitant exercise-treated group (Fig. 6).

4. Discussion

In the current study, it was shown that (i) apo E-deficient mice fed a high fat diet over 8 weeks developed severe fatty streak lesions of aortic roots, (ii) behavioral stress increased the severity of atherosclerosis, and induced the atherosclerotic plaques with the increased ICAM-1 expression and superoxide production in aortic walls, and (iii) the propensity of the plaque unstability was reduced by the concomitant exercise treatment.

Chronic inflammation is thought to be of central importance in atherosclerosis [6,20]. It was shown that regular and chronic exercise could suppress overt and subclinical inflammation [21,22], based on the fact that atherosclerosis can be considered as generalized manifestations of an inflammatory disease [6]. We and other investigators had already reported that experimental atherosclerosis in apo E-deficient mice was markedly suppressed by Fcγ portion of immunoglobulin administration, possibly by an antiinflammatory action via inhibitory Fcγ receptor IIB [15,16,23].

As mentioned previously, the so-called negative emotions, such as depression and anxiety, have been associated with the development of atherosclerosis and coronary artery disease [1–5]. Also, psychosocial distress and anxiety disorders are a significant risk factor for atherosclerosis [24–26]. Although the exact mechanisms are not yet clear, burying behavior in the mouse is provoked by glass marble, which is considered to be a kind of defensive burying [10–12].

In the present study, using the animal model of behavior stress induced by glass-burying procedure, we clearly demonstrated that behavioral stress increased the expression of adhesion molecule and superoxide production on atherosclerotic plaques, and that chronic and regular swimming training suppressed the expression of ICAM-1 and the overload of superoxide in aortic walls. It has already been established that the intensity of exercise training used in this study ameliorated the development of atherosclerosis in apo E-deficient mice [9], and that the degree of tissue DHE staining correlates with the

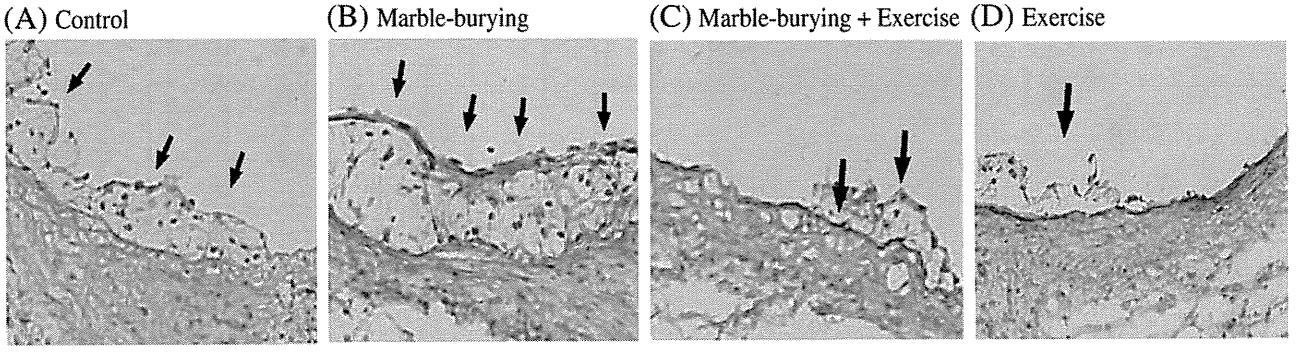

Fig. 4. Intercellular adhesion molecule-1 (ICAM-1) expression. The expression of ICAM-1 in the lesion (arrows) of the marble-burying plus exercise-treated mouse (C) were decreased compared with those of the control mouse (A) and marble-burying procedure-treated mouse (B). The expression of ICAM-1 in exercise group was minimal (D). ICAM-1 stain (A, B, C, D × 100).

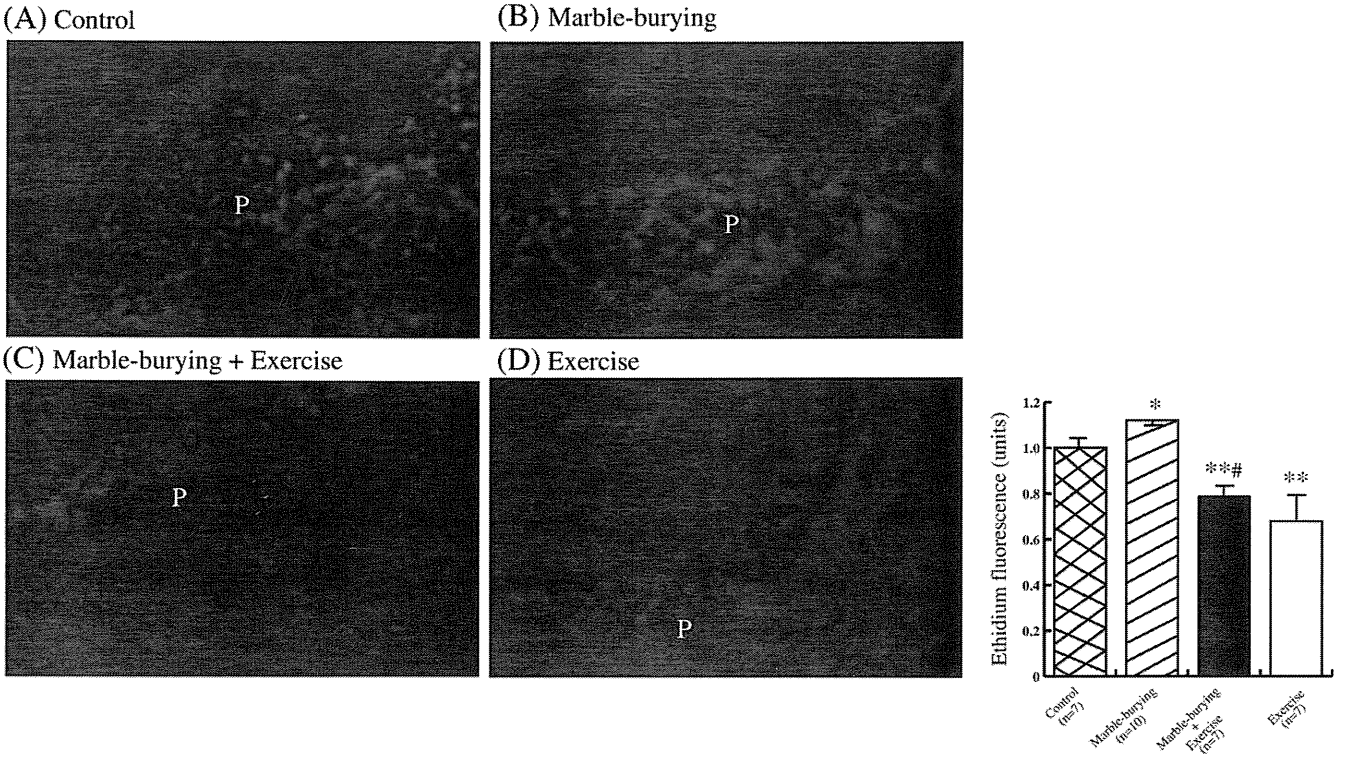

Fig. 5. Dihydroethidium (DHE) staining. Ethidium fluorescence was detected in the plaques (A). The intensity of DHE staining was increased by the marble-burying behavior (B), and was suppressed by the marble-burying plus swimming training group (C). The intensity of DHE staining in exercise group (D) was lowest among the group. P indicates plaques. DHE stain (A, B, C, D×120). *$P<0.05$, **$P<0.01$ vs control group. #$P<0.01$ vs marble-burying group.

superoxide production [17–19]. Thus, the decrease of the intensity of ethidium fluorescence expression in aortic walls may reflect the decrease of superoxide by exercise treatment.

The mechanisms by which exercise might benefit cardiovascular diseases are still unknown [7,8]. It is reported that exercise suppresses inflammation, hypertension, and atherosclerosis [7,8], and may reverse some cardiomyopathic conditions [27,28]. We already reported that chronic and regular exercise reduced the experimental atherosclerosis by the antioxidant effects [9]. Indeed, in the current study, exercise reduced the lesions and might stabilize atherosclerotic plaques by reducing the expression of macrophages and ICAM-1 as well as decreased intensity of DHE. Accordingly, regular exercise might be recommended for the clinical therapy for atherosclerotic patients.

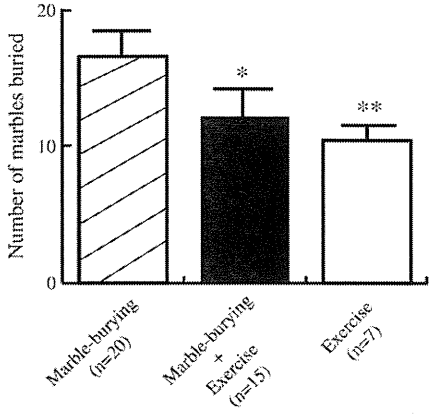

Fig. 6. Numbers of marbles buried. The numbers in mice treated with exercise alone and concomitant exercise were significantly less compared with those in mice treated with marble-burying alone. *$P<0.05$, **$P<0.01$ vs marble burying group.

The present study also demonstrated that exposure to behavioral stress induces the overexpression of ICAM-1 and the overproduction of superoxide in the aortic walls with relatively less amount of collagen contents in apo E-deficient mice, although it did not increase the severity of atherosclerosis significantly. This phenomenon may be of significant clinical relevance to humans. However, it should be noted that behavioral stress has been shown to have different effects in other strains of mice [10,11].

Behavioral stress is now recognized to be an important contributor to cardiovascular diseases, as mentioned before [1–5]. From the current study, however, appropriate and regular exercise may be recommended for the secondary prevention of atherosclerotic patients, especially having sustained psychological stress.

Several limitations of the present study should be mentioned. First, the present study lacked the precise evaluation of hemodynamic study. Recently, it was reported that high heart rate and vigorous shear stress may affect the development of atherosclerosis [29]. However, it was already reported that the severity of atherosclerosis was reduced by the appropriate exercise protocol [9,30]. Second, the present study did not demonstrate the precise and molecular mechanisms why exercise might reduce the severity of experimental atherosclerosis. Third, the present experimental findings cannot be applied to the clinical settings directly because of the species differences of sensitivity for stress or anxiety.

In conclusion, behavioral stress may induce the overexpression of ICAM-1 and superoxide overproduction in the lesions in apo E-deficient mice, and concomitant exercise training may downregulate ICAM-1 expression and superoxide production, and reversed the collagen contents, resulting in the reduction of atherosclerosis. In view of the propensity of atherosclerotic plaques having adhesion molecule overexpression and superoxide overproduction, it may be concluded that exercise training may be useful for stabilizing the atherosclerotic plaques in clinical settings. Exploration of clinical usefulness of the results might be warranted.

Acknowledgement

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Nutrition, Metabolism & Cardiovascular Diseases

Olmesartan, a novel angiotensin II type 1 receptor antagonist, reduces severity of atherosclerosis in apolipoprotein E deficient mice associated with reducing superoxide production☆

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KEYWORDS
Atherosclerosis;
Angiotensin II type 1 receptor antagonist;
Oxidative stress;
Cytokines

Abstract *Background and aim:* Oxidative stress may play an important role in the development of atherosclerosis. Some angiotensin II type 1 (AT_1) receptor antagonists have the capacity of reducing oxidative stress in addition to the hemodynamic actions. Accordingly, we assessed the hypothesis that olmesartan, a novel AT_1 receptor antagonist, reduced the severity of atherosclerosis in apolipoprotein (apo) E-deficient mice associated with reducing oxidative stress.

Methods and results: Atherosclerosis was induced in apo E-deficient mice fed a high fat diet. Mice were intraperitoneally treated with an injection of olmesartan (1 mg/kg/day) daily over 8 weeks, and were compared with the untreated controls. Blood pressure was not changed significantly by the olmesartan treatment. Fatty streak plaque developed in apo E-deficient mice, and was suppressed in mice that received olmesartan. In addition, olmesartan reduced not only superoxide production but the overload of oxidative stress in aortic walls. There were no significant differences in serum lipid levels between olmesartan-treated and -untreated groups. In vitro study showed that both olmesartan and its active metabolite RNH-6270, an enantiomer of olmesartan, suppressed interferon-γ, macrophage inflammatory protein-2, and thioredoxin (a marker of oxidative stress) concentrations in cultured cells.

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Conclusion: Olmesartan may suppress atherosclerosis via reducing not only superoxide production but also the overload of oxidative stress in this animal model.

Introduction

Inflammation, cytokine overproduction, oxidative stress, and free radicals may be considered to be key mechanisms for the development of atherosclerosis [1]. In addition, the significance of renin-angiotensin system in the development of atherosclerosis is now well known [2]. Angiotensin II is a major mediator of oxidative stress by activating NADH/NAD(P)H oxidase via the type 1 receptor, which results in the production of the superoxide anion. Accordingly, angiotensin II has deleterious effects on vessel walls.

Recent reports indicate that some angiotensin type 1 (AT_1) receptor antagonists inhibit inflammatory reactions in macrophages and myocardium [3—5]. AT_1 receptor antagonists have also been reported to inhibit interleukin (IL)-1 production as well as free radical production [6]. These results imply that olmesartan, a novel AT_1 receptor antagonist, may be an effective agent in countering inflammatory reactions and oxidative stress of vessel walls [5—7]. In addition, it was recently reported that olmesartan suppressed the development of atherosclerosis by various effects, such as prevention of endothelial disruption, decreasing effects of macrophage accumulation in the lesions, and so on [8—13]. However, the exact mechanism of olmesartan on atherosclerosis are still unknown.

In the present study, using apolipoprotein E-deficient mice which is a well-known animal model of experimental atherosclerosis [14—16], we have provided evidence for the lowering effects of atherosclerotic lesions by olmesartan, focusing upon inhibitory effects for free radical and oxidative stress production in vivo and in vitro.

Methods

Experimental atherosclerosis

The apolipoprotein E (apo E)-deficient 129ola × C57BL/6 hybrid mice were generous gifts of Dr. Edward M. Rubin (University of California, Berkeley, CA). These mice were mated with C57BL/6 mice to produce F_1 hybrids. The F_1 apo $E^{+/-}$ mice were then backcrossed to C57BL/6 mice for 10 generations. Mice homogeneous for the apo E-null allele on a C57BL/6 background were subsequently generated. Male mice were subjected to the subsequent experiments. The mice were kept in a temperature-controlled facility on a 14:10-h light-dark cycle with free access to food and water.

After being weaned at 4 weeks of age, mice were fed a normal chow diet (Oriental Yeast) until 6 weeks of age, when the animals were switched to a high fat diet containing 20% fat and 0.3% cholesterol as previously described [17,18].

The experimental protocols were approved by the institutional ethics committee for animal experiments of Kyoto University.

Treatment protocol

At 6 weeks of age, mice were treated daily with an intraperitoneal injection of either saline (olmesartan-untreated group, $n = 9$) or 1 mg/kg/day of olmesartan (olmesartan-treated group, $n = 9$) for 8 weeks. The dosage of the drug without affecting the blood pressure significantly was determined from the previous report [5]. Blood pressure and heart rate were periodically determined by the tail-cuff method using a photoelectric tail-cuff detection system (model BP-98A, Softron, Tokyo, Japan). The measurement was repeated three times at each time. At 14 weeks the mice were sacrificed by puncture of the ventricle under ether anesthesia. The organs were weighed, and the ratios of organ weight to body weight were calculated. Olmesartan and its active metabolite RNH-6270 (an enantiomer of olmesartan) [19] were kindly provided by Daiichi-Sankyo Company (Tokyo, Japan).

Tissue processing

Mice were sacrificed by bleeding with the puncture of the ventricle. The vasculature was perfused with sterile phosphate buffered saline (PBS) and 6.8% sucrose. The root of the aorta was dissected under a microscope and frozen in OCT embedding medium for serial cryosectioning covering 1.0 mm of the root. The first section was harvested when the first cusp became visible in the lumen of the aorta. Four sections of 6 μm thickness were harvested per slide, and thus eight slides per mouse were prepared. All sections were immersed for 15 s in 60% isopropanol, stained for 30 min in a saturated oil-red-O solution at room temperature, counterstained with hematoxylin, and then mounted under coverslips with glycerol gelatin [18].

Quantitation of atherosclerotic lesions

The oil-red-O-stained sections were analyzed at a magnification of ×10, as previously described [17,18]. The image was captured directly from the RGB camera attached to a light microscope and displayed on a microcomputer to quantify the cross-sectional surface area of the lesion. The fractional area of the lesion was calculated by dividing the whole vessel area including the lumen, intima, media, and adventitia, as previously described [17,18]. For each animal, 20 sections, i.e., every fourth section, were examined, and the mean of the fraction area was calculated and expressed as a percentage.

In situ defection of superoxide production

To evaluate in situ superoxide production from vessel walls, unfixed frozen cross sections of the specimens were stained with dihydroethidium (DHE; Molecular Probe, OR) according to the previously validated method [7,20,21]. In the

Table 1 Organ weights and hemodynamics.

	(*n*)	BW (g)	HW (g)	HW/BW (mg/g)	HR (/min)	SBP (mm Hg)	DBP (mm Hg)
Untreated	9	28.1 ± 5.1	0.17 ± 0.02	6.12 ± 1.12	563 ± 71	94 ± 9	57 ± 6
Olmesartan	9	28.4 ± 4.6	0.14 ± 0.02*	5.17 ± 0.83*	576 ± 34	87 ± 9	53 ± 5
							(Mn ± SD)

BW, body weight, HW, heart weight, HR, heart rate, SBP, systolic blood pressure, DBP, diastolic blood pressure. *$P < 0.05$ vs untreated group.

presence of superoxide, DHE is converted to the fluorescent molecule ethidium, which can then label nuclei by intercalating with DNA. Briefly, the unfixed frozen tissues were cut into 10 μm thick sections, and incubated with 10 μM DHE at 37 °C for 30 min in a light-protected humidified chamber. The images were obtained with a laser scanning confocal microscope. Superoxide production was demonstrated by red fluorescence labeling.

For the quantification of ethidium fluorescence from the lesions, fluorescence (intensity × area) was measured, using the Image J in high-power (×100) images. For each vessel, total fluorescence was calculated from three to four separate fields taken in each section of the vessel to produce $n = 1$. The validity of DHE staining for the evaluation of superoxide production from the atherosclerotic lesions was already reported by us [21].

Oxidative stress evaluation

To analyze the oxidative stress overload in the aortic wall, immunohistochemistry for thioredoxin (TRX), which is a redox-active protein and considered a marker of oxidative stress, was performed as previously described [22,23]. The validity of TRX staining for the evaluation of oxidative stress in the atherosclerotic lesions was already reported by us [18].

Lipid measurement

Serum was separated by centrifugation, and stored at −80 °C. Serum total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and triglyceride (TG) levels were measured with assay kits (Wako) according to the manufacturer's instructions. Low-density lipoprotein cholesterol (LDL-C) was calculated by the Friedewald formula.

Cytokine and TRX assays

U-937 human macrophages and thioglycolate-elicited peritoneal macrophages from apo E-deficient mice were cultured as previously described [5], and were stimulated with 10 μg/ml lipopolysaccharide (LPS). Olmesartan or its active metabolite RNH-6270 [5,19] was added to the cultured medium 30 min before LPS stimulation. Forty-eight hours later, interferon-γ (IFN-γ), macrophage inflammatory protein-2 (MIP-2) and TRX were assayed by enzyme-linked immunosorbent assay (ELISA) [24]. MIP-2 is thought to play an essential role in inflammatory cardiovascular diseases [25].

Statistical analysis

Values were expressed as means ± SD. Unpaired *t*-test was performed. A value of $P < 0.05$ was considered statistically significant.

Results

Effects of olmesartan on organ weights and hemodynamics (Table 1)

Heart weight and heart weight to body weight ratio were significantly decreased in olmesartan group than in untreated group. Although there is a tendency for low levels of blood pressure in olmesartan group, systolic and diastolic blood pressure, and heart rate did not differ significantly between the two groups.

Effects of olmesartan on atherosclerotic lesions (Table 2, Fig. 1)

Apo E-deficient mice were kept on a cholesterol-rich diet for 8 weeks to induce fatty streak formation. The surface areas covered by fatty streak lesions were quantified in oil-red-O-stained samples, and specimens from untreated group were compared with those from olmesartan group. Untreated mice developed extensive lesions in the root of the aorta (Fig. 1). In mice treated with olmesartan, the

Table 2 Lesion area, superoxide production and oxidative stress.

	(*n*)	Lesion area, μm² (%)	Ethidium fluorescence, units	TRX expression (+1−+4)[b]
Untreated	9	59.28 ± 21.68 × 10³(7.64 ± 2.78)	1.00 ± 0.05	+++
Olmesartan	8[a]	39.38 ± 21.69 × 10³(5.05 ± 2.74)*	0.84 ± 0.07*	+
				(Mn ± SD)

*$P < 0.05$ vs untreated group.
[a] The result of one mouse was missed due to the technical problem.
[b] The degree of TRX expression was determined semi-quantitatively as previously described [22,23].

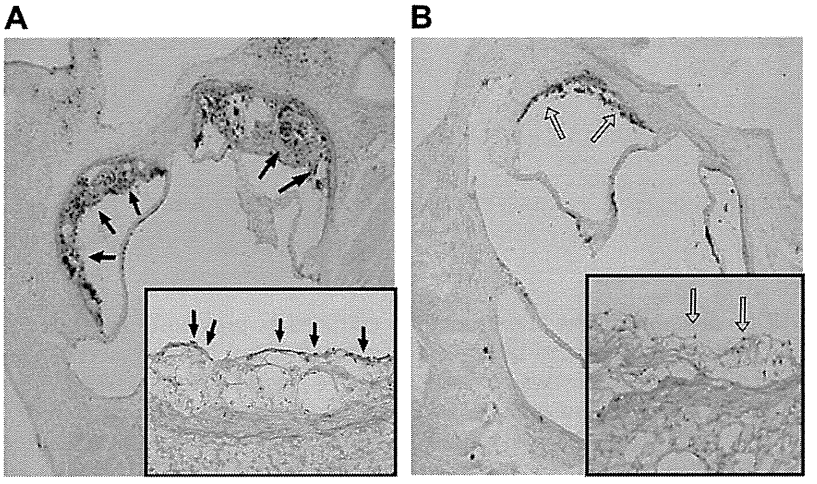

Figure 1 Effects of olmesartan on atherosclerotic lesions and oxidative stress. The lesions (white arrows) in the olmesartan-treated mouse (B) were smaller and covered less of the inner circumference of the aortic root than those (black arrows) of the olmesartan-untreated mouse (A). Insets are the expressions of thioredoxin (TRX). The expression of TRX in the lesion (white arrows) of the olmesartan-treated mouse (B) was decreased compared with that (black arrows) of the olmesartan-untreated mouse (A). Brown staining shows the positive area for TRX expression in the atherosclerotic plaques. Oil-red-O stain (×50). TRX staining (×100).

fractional area of lesions was reduced compared with the untreated mice as shown in Fig. 1.

In situ superoxide production (Table 2, Fig. 2)

In situ superoxide production was measured using DHE oxidative fluorescent microphotography. Ethidium fluorescence was detected throughout all layers of the vessel walls. Olmesartan significantly suppressed the staining of atherosclerotic plaques (Fig. 2). Quantification of the effects of olmesartan on superoxide production was listed in Table 2.

Oxidative stress (Table 2, Fig. 1)

As shown in Fig. 1, TRX expression in the aortic wall was suppressed by olmesartan treatment compared with untreated group (Table 2, Fig. 1).

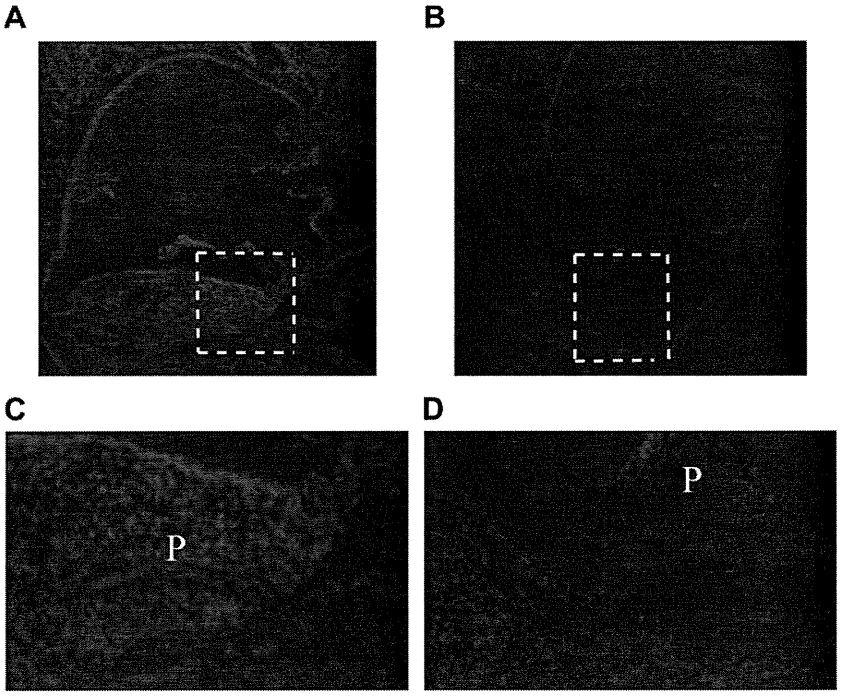

Figure 2. Representative photographs of in situ superoxide production in aortic vessel wall using dihydroethidium (DHE) staining. Treatment of olmesartan suppressed the brightness of DHE staining in vessel walls and plaques (P) in the treated mouse (B, D) compared with the untreated mouse (A, C). (C and D) are magnified pictures for dotted areas of (A and B), respectively. DHE staining (A, B ×10; C, D ×80).

Table 3 Lipid profiles.

	(*n*)	TC (mg/dl)	HDL-C (mg/dl)	LDL-C (mg/dl)[a]	TG (mg/dl)
Untreated	9	1539 ± 598	864 ± 175	669 ± 96	30 ± 53
Olmesartan	9	1449 ± 164	850 ± 104	591 ± 85	38 ± 42
					(Mn ± SD)

TC, total cholesterol, HDL-C, high-density lipoprotein cholesterol, LDL-C, low-density lipoprotein cholesterol, TG, triglyceride.
[a] The values were obtained by the Friedewald formula.

Lipid profiles (Table 3)

Olmesartan treatment did not significantly modify the serum lipids profiles.

Effects of the drug on cytokine and TRX production (Table 4)

INF-γ, MIP-2 and TRX concentrations were markedly increased by LPS stimulation (Table 4). Both olmesartan and RNH-6270 suppressed LPS-induced increased cytokine production on U-937 cells and thioglycolate-elicited peritoneal macrophages in a dose-dependent manner (Table 4).

Discussion

In the current study, it was shown that olmesartan treatment suppressed the development of experimental atherosclerosis in apo E-deficient mice associated with reducing the overload of oxidative stress and the superoxide production of aortic walls without significant hemodynamic changes. The in vitro study actually showed that both olmesartan and its active metabolite RNH-6270, an enantiomer of olmesartan, suppressed IFN-γ, MIP-2 and TRX production in cultured cells. Accordingly, the in vitro data may support the anti-atherosclerotic effects of the drugs in vivo.

Chronic inflammation is thought to be of central importance in atherosclerosis [1,26]. It was shown that regular and chronic exercise could suppress overt and subclinical inflammation [27,28]. We and other investigators had already reported that experimental atherosclerosis in apo E-deficient mice was markedly suppressed by Fcγ portion of immunoglobulin administration possibly by an anti-inflammatory action via inhibitory Fcγ receptor IIB [29—31].

There is also increasing evidence to support the critical role of both free radicals and oxidative stress in the

Table 4 Effects of olmesartan and RHN-6270 on cytokine production in vitro.

LPS (10 μg/ml)	Conditions		Cytokines concentrations		
	Olmesartan (pg)	RNH-6270 (pg)	IFN-γ (pg/ml)	MIP-2 (ng/ml)	TRX (ng/ml)
U-937 cells					
−	−		1.4 ± 0.5	8 ± 3	10 ± 4
+	−		3.9 ± 1.1	45 ± 15	34 ± 5
+	1		3.4 ± 0.5	32 ± 5	30 ± 8
+	10		2.3 ± 0.6*	21 ± 10*	20 ± 8*
+	100		2.0 ± 0.4*	10 ± 7**	12 ± 5**
−		−	1.5 ± 0.5	5 ± 4	13 ± 5
+		−	4.5 ± 0.8	52 ± 15	29 ± 6
+		1	4.0 ± 1.0	45 ± 11	25 ± 5
+		10	3.2 ± 0.8	34 ± 8	15 ± 5*
+		100	2.2 ± 0.4**	16 ± 7**	15 ± 6*
Peritoneal macrophages					
−	−		3.6 ± 1.5	10 ± 5	- - -
+	−		6.0 ± 0.5	98 ± 21	- - -
+	1		5.2 ± 1.0	74 ± 15	- - -
+	10		4.1 ± 0.7*	56 ± 20*	- - -
+	100		3.5 ± 1.0*	52 ± 24*	- - -
−		−	3.0 ± 2.1	12 ± 6	- - -
+		−	7.1 ± 1.4	104 ± 15	- - -
+		1	5.0 ± 1.5	77 ± 25	- - -
+		10	4.7 ± 0.9*	68 ± 30	- - -
+		100	4.5 ± 1.2*	55 ± 20*	- - -
					(Mn ± SD)

IFN-γ, interferon-γ, MIP-2, macrophage inflammatory protein-2, TRX, thioredoxin. Each value was derived from 4 to 6 trials. Cell viability in each experiment was more than 93% by trypan blue exclusion test. *$P < 0.05$; **$P < 0.01$ vs olmesartan (−) or RNH-6270 (−) conditions.

development of atherosclerosis [20,32—34]. We had already demonstrated that MCI-186, a free radical scavenger, suppressed the severity of experimental atherosclerosis [18]. The AT_1 receptor antagonist such as olmesartan is reported to suppress not only cytokine production but also free radical production in addition to its hemodynamic effects [5—7]. Indeed, angiotensin stimulation has been reported to produce free radicals from various cells [35—37]. Free radicals from vessel walls are thought to play critical roles in atherogenesis. Free radicals induce the expression of adhesion molecules and chemokines, accelerate atherosclerotic plaque formation, increase matrix metalloprotease production, and cause vulnerable plaques [38]. Recent studies suggested that olmesartan attenuates experimental atherosclerosis via an anti-oxidative action, prevention of endothelial disruption, and the decrease of macrophage accumulation in the lesions [8—13,39].

In the present study, we clearly demonstrated that olmesartan suppressed the overload of free radicals in aortic walls assessed by DHE staining [7,20,21] and TRX expression [18,22,23]. Immunohistochemical study showed that the intensity of both DHE staining and TRX staining in the aortic wall was reduced by olmesartan treatment compared with untreated group. It has already been established that the degree of tissue TRX staining correlates with the overload of oxidative stress [18,22,23]. Thus, the decrease of the intensity of both DHE staining and TRX expression in aortic walls may reflect the decrease of oxidative stress by olmesartan treatment.

In addition, in vitro study showed that both olmesartan and its active metabolite RNH-6270 suppressed IFN-γ, MIP-2 and TRX production. Although there were no significant differences in serum cytokines (interleukin-1β and IFN-γ) between olmesartan-treated and -untreated mice in vivo (data not shown), these in vitro results may suggest that beneficial effects of olmesartan in apo E-deficient mice is partly due to the suppression of inflammatory events in the vessel walls. The in vitro data may well explain the anti-atherosclerotic effects of olmesartan in vivo.

It is now well recognized that vascular AT_1 receptor stimulated superoxide production is NAD(P)H oxidase-dependent. However, in the current study, the aortic NAD(P)H oxidase activity with and without olmesartan treatment was not examined.

We used this dosage of the drug to avoid the effect of the drug-induced hypotension. Indeed, the drug did not exhibit the lowering effects of systolic blood pressure significantly. At present time, we have no answers whether the suppressive effects of olmesartan for oxidative stress is dependent of blood pressure lowering effect or not, because angiotensin II-mediated hypertension is associated with the increased oxidative stress [32—34].

Novel findings of the current study compared with the previous work [8—13,16,39,40] are that olmesartan inhibits experimental atherosclerosis in apo E-deficient mice without significant hemodynamic changes, that is, the drug has the potential to reduce atherosclerosis not by lowering blood pressure, and that the effects were associated with reduced superoxide production and oxidative stress even with hyperlipidemic state. It was also shown in the current study that the capability of the active metabolite RNH-6270 to reduce inflammation was demonstrated. Accordingly, olmesartan may be beneficial for atherosclerotic patients associated with reducing oxidative stress. In conclusion, inhibition of free radical production and oxidative stress may be one mechanism by which olmesartan decreases the severity of experimental atherosclerosis in apo E-deficient mice. Our results suggest that more widespread clinical use of the drug for atherosclerotic patients might be warranted.

Conflict of interest

The authors have no conflict of interest.

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Differential effect of statins on diabetic nephropathy in db/db mice

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Abstract. Recent studies suggest a potential benefit of the lipid-lowering medication in the treatment of chronic kidney disease (CKD) such as diabetic nephropathy. Although statins have been widely used to lower serum cholesterol levels, the effect of these drugs on diabetic nephropathy has not been fully elucidated. In the present study, therefore, we addressed the role of different kinds of statins on diabetic nephropathy in db/db mice. Mice were fed with a standard diet with 0.005% (w/w) of pitavastatin, rosuvastatin, and pravastatin for 8 weeks starting from 8 weeks of age. The treatment with statins did not affect the food intake, body weight gain, adiposity, or blood pressure in db/db mice. Treatment with statins also had no effect on plasma lipid levels. In terms of the effect on albuminuria, pitavastatin and rosuvastatin reduced the urinary excretion of albumin by 60 and 40%, respectively, but not pravastatin, suggesting the effect of these two drugs on diabetic nephropathy. Furthermore, pitavastatin and rosuvastatin improved glomerular hypertrophy. All statins treatment improved insulin resistance. In addition, rosuvastatin and pravastatin treatment reduced oxidative stress measured by urinary 8-OHdG level, whereas the statins had no effect on the inflammatory response in the kidney of db/db mice. These results are not consistent with the renoprotective effect of statins. In conclusion, our data suggest that pitavastatin and rosuvastatin can improve diabetic nephropathy through the suppression of glomerular hypertrophy, independent of lipid-lowering or anti-oxidative effects.

Introduction

Diabetic nephropathy is one of the most common forms of chronic kidney disease (CKD) and the most frequent cause of mortality in patients with diabetes (1,2). The number of people affected by diabetic nephropathy or who need renal replacement is steadily increasing (3). Therefore, the establishment of therapeutic strategies for diabetic nephropathy is needed. Diabetic nephropathy results from complex interactions between genetic, metabolic, and hemodynamic factors, and can be characterized by mesangial expansion followed by glomerulosclerosis and a decline in renal function. The development of glomerulosclerosis in diabetes mellitus is always preceded by persistent albuminuria and glomerular hypertrophy (2). Therefore, these two manifestations could be promising therapeutic targets for the treatment of diabetic nephropathy.

3-Hydroxy-3-methylglutaryl (HMG)-coenzyme A (CoA) reductase inhibitors (statins) are widely used for diabetic patients to reduce their cardiovascular risk (4). Statins also have renoprotective actions and have been shown to reduce albuminuria in both experimental and clinical diabetic renal disease (5-8). Some of these benefits may be due to lipid lowering, since lipid levels are strongly associated with the development and progression of diabetic kidney disease (9,10). On the other hand, statins have a range of lipid-independent actions on cell proliferation, inflammation, and oxidative stress (11,12), which may impact the development and progression of renal damage in diabetes. These pleiotropic effects have been suggested to contribute to the renoprotective effect of statins. However, the precise mechanisms of the renoprotective effects are not fully understood. In addition, whether different statins have the same effect on diabetic nephropathy is not well known.

In this study, we addressed the role of various statins, such as pitavastatin, rosuvastatin, and pravastatin on the development of diabetic nephropathy in db/db mice.

Materials and methods

Materials. Pravastatin and rosuvastatin were provided by Daiichi Sankyo Co., Ltd. and pitavastatin was provided by Kowa Pharmaceutical Co., Ltd.

Animal procedure and experimental design. Male db/db mice (n=24) and their lean control db/m (n=6) mice were obtained from Charles River at 6 weeks of age. The mice were fed with normal chow without additional supplementation (non-treated group) or with chow supplemented with 0.005% (w/w) pravastatin, pitavastatin or rosuvastatin for 8 weeks starting from 8

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Key words: statin, diabetic nephropathy, albuminuria, pleiotropic action

Table I. Characteristics of db/m and db/db mice treated with or without statins.

	db/m	db/db			
		Con	Pra	Pit	Ros
Body weight (g)	32.5±0.40	53.1±3.90	51.2±5.1	50.3±3.80	51.3±5.70
Liver weight (g)	1.15±0.29	2.99±0.41	2.78±0.54	2.55±0.36	3.19±0.85
eWAT weight (g)	0.37±0.05	3.23±0.25	3.08±0.59	3.01±0.41	3.10±0.62
Kidney weight (g)	0.31±0.07	0.50±0.02	0.51±0.06	0.42±0.01[a]	0.41±0.03[a]
Food intake (g/day)	3.82±0.33	7.45±2.43	7.15±0.72	7.70±1.65	7.04±1.58
SBP (mmHg)	NA	113.2±11.6	113.5±3.00	109.5±9.50	114.6±5.60

Con, control; Pra, pravastatin; Pit, pitavastatin; Ros, rosuvastatin; eWAT, epididymal white adipose tissue; SBP, systolic blood pressure. Results are expressed as mean ± SD (n=6 in each group). [a]P<0.05 vs. Con.

weeks of age. Animals had access to food and water *ad libitum* and were maintained on a 12-h light/dark cycle. All animal experiments were conducted according to the Guidelines for Animal Experiments at Kyoto University.

Analysis of metabolic parameter. Plasma glucose concentration was measured with a Glutest Ace (Sanwa Kagaku Kenkyusho Co., Ltd.). Plasma insulin concentration was measured with an insulin assay kit (Morinaga Institute of Biological Science). Plasma cholesterol and triglyceride levels were respectively measured with the Cholesterol E and Triglyceride E tests (Wako Pure Chemical Industries, Ltd.).

Measurement of urinary albumin and creatinine. Urinary albumin and creatinine were measured at 16 weeks of age from 24-h collection samples from mice housed in individual metabolic cages. During the urine collection, the mice were allowed free access to food and water. Albumin concentration in the urine was measured by Albuwell (Exocell). Urinary creatinine was measured with a Hitachi Mode 736 analyzer (Hitachi). The urinary albumin concentration was adjusted by the urinary creatinine concentration.

Measurement of urinary oxidative stress. Urinary 8-OHdG concentrations were measured at 16 weeks of age using a competitive enzyme-linked immunosorbent assay kit (8-OHdG Check, Japan Institute for the Control of Aging). Urinary 8-OHdG excretion was expressed as the total amount excreted in 24 h.

Quantitative real-time PCR. Total-RNA was extracted from frozen kidney tissue (50 mg) at 16 weeks of age using an RNeasy mini kit (Qiagen). The cDNA was synthesized from total-RNA using SuperScript III (Invitrogen). Real-time PCR was performed on an ABI PRISM 7900 using the SYBR-Green PCR Master Mix (Applied Biosystems). Primer sets were as follows: tumor necrosis factor (TNF)-α forward, 5'-CCCAGA CCCTCACACTCAGATC-3' and reverse, 5'-GCCACTCCAG CTGCTCCTC-3'; β-actin forward, 5'-TACCACAGGCATTG TGATGG-3' and reverse, 5'-TTTGATGTCACGCACGAT TT-3'. The mRNA levels were normalized relative to the amount of β-actin mRNA and expressed in arbitrary units.

Measurement of glomerular size. The mice were euthanized at 16 weeks of age. The kidneys were rapidly fixed in 10% formaldehyde, and embedded in paraffin. Paraffin sections were cut at 3 μm. For measurement of the glomerular size, paraffin sections were stained with hematoxylin and eosin. The size of the glomerular surface area was measured using the Image-Pro Plus software version 3.0.1 (Media Cybernetics, Inc.).

Statistical analysis. Data are expressed as the mean ± SD. Multiple comparisons among the groups were conducted by one-way analysis of variance with Fisher's PLSD test for post hoc analysis. P-values of <0.05 were considered significant.

Results

Effect of statin treatment on body weight, adiposity and systolic blood pressure. In db/db mice fed with a standard diet for 8 weeks starting at 8 weeks of age, body weight, epididymal white adipose tissue (eWAT) weight, liver weight were increased compared to those of db/m mice. Treatment with statins had no effect on body weight, food intake, liver weight and eWAT weight in db/db mice (Table I). In addition, there was no difference in systolic blood pressure between statin-treated and non-treated db/db mice.

Effect of statin treatment on renal function in db/db mice. Because albuminuria reflects renal function (13), we measured the urinary excretion of albumin in normal chow-fed db/db mice at 16 weeks of age. Urinary excretion of albumin was markedly increased in db/db mice compared with db/m mice (Fig. 1). Pitavastatin, rosuvastatin, but not pravastatin improved albuminuria in db/db mice. Kidney weights in pitavastatin- and rosuvastatin-treated db/db mice were reduced compared with non-treated db/db mice (Table I). These data suggest that pitavastatin and rosuvastatin treatment improves renal function in db/db mice.

Effect of statin treatment on plasma lipid level in db/db mice. To clarify the mechanism by which statins ameliorated renal function, we first examined the effect of statin treatment on lipid metabolism in db/db mice. Plasma triglyceride and total cholesterol level were increased in non-treated db/db mice compared with db/m mice (Fig. 2A and B). On the other hand,

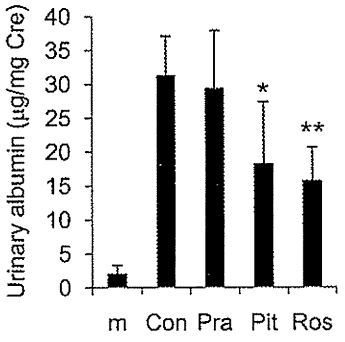

Figure 1. Effect of statins on renal function in db/db mice. The graph shows the urinary excretion of albumin in db/m mice (m), non-treated (Con), pravastatin-treated (Pra), pitavastatin-treated (Pit) and rosuvastatin-treated (Ros) db/db mice. Results are expressed as mean ± SD. *P<0.05, **P<0.01 vs. non-treated db/db mice (n=6 in each group).

statin treatment had no effect on plasma lipid levels in db/db mice (Fig. 2A and B), suggesting that the renoprotective effect of statins is independent of their lipid-lowering action.

Effect of statin treatment on insulin resistance in db/db mice. It has been reported that the development of insulin resistance contributes to renal dysfunction (14). Therefore, we next examined the effect of statin treatment on glucose metabolism in db/db mice. Blood glucose level, plasma insulin level, and HOMA-IR were markedly increased in db/db mice compared with db/m mice, indicating an increase in insulin resistance (Fig. 2C-E). Although statin treatment had no effect on plasma glucose, all statins reduced plasma insulin levels, resulting in a decrease in HOMA-IR (Fig. 2C-E). The data suggest that statin treatment improves insulin resistance.

Because hypoadiponectinemia is associated with the development of insulin resistance and kidney disease (15), we examined the effect of statin treatment on plasma adiponectin levels in db/db mice. In non-treated db/db mice, plasma adiponectin levels were decreased compared with db/m mice. Meanwhile, statin treatment had no effect on plasma adiponectin level in db/db mice (Fig. 2F).

Effect of statin treatment on the renal inflammation in db/db mice. Accumulating evidence now indicates that inflammatory mechanisms play a significant role in the development and progression of diabetic nephropathy. Especially, TNF-α is a pleiotropic inflammatory cytokine and has been shown to cause enhanced albumin permeability (16). Therefore, we next examined the effect of statin treatment on inflammation in the kidney of db/db mice. The expression of TNF-α mRNA was increased in the kidney of db/db mice compared with that of db/m mice, whereas statin treatment had no effect on its expression in db/db mice (Fig. 3A). These data suggest that statins had no effect on the inflammatory response in the kidneys of db/db mice.

Effect of statin treatment on the oxidative stress in db/db mice. To examine the effect of statin treatment on oxidative stress, we measured urinary 8-OHdG concentrations in db/db mice. Urinary 8-OHdG levels in non-treated db/db mice were significantly higher than those in db/m mice. Pravastatin and rosuvastatin reduced urinary 8-OHdG levels in db/db mice, whereas pitavastatin had no effect on oxidative stress despite detecting the amelioration of albuminuria (Fig. 3B).

Effect of statin treatment on glomerular hypertrophy in db/db mice. Glomerular hypertrophy is a hallmark in diabetic

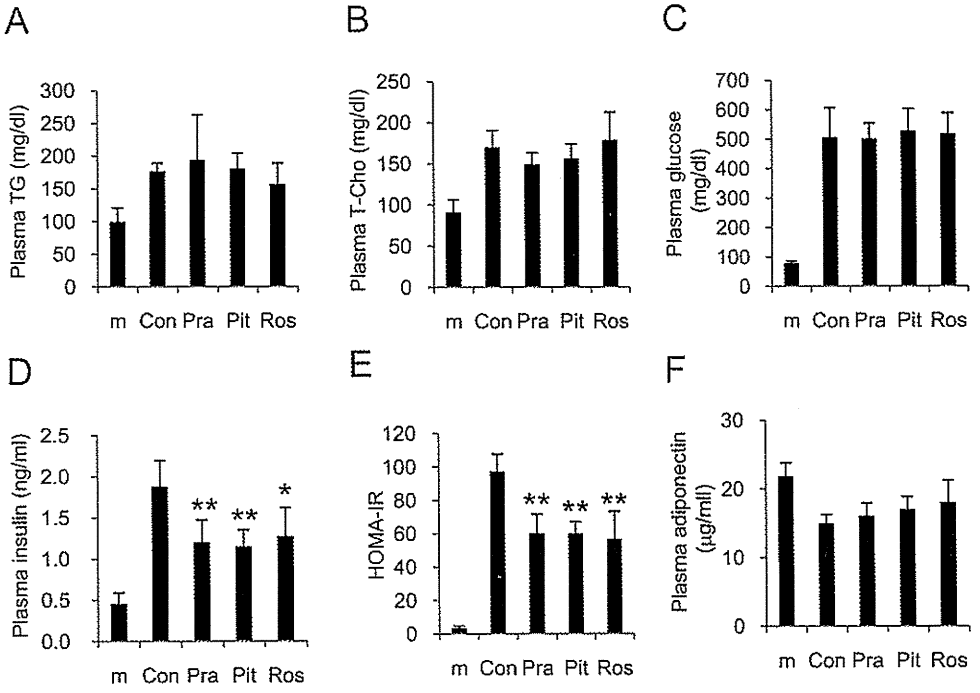

Figure 2. Effect of statins on lipid and glucose metabolism in db/db mice. (A) Plasma triglyceride (TG), (B) total cholesterol (T-Cho), (C) glucose, (D) insulin, (E) HOMA-IR and (F) adiponectin levels in db/m mice (m), non-treated (Con), pravastatin-treated (Pra), pitavastatin-treated (Pit) and rosuvastatin-treated (Ros) db/db mice. Results are expressed as mean ± SD. *P<0.05, **P<0.01 vs. non-treated db/db mice (n=6 in each group).