

pathway accelerates lesion progression in hypercholesterolemic rabbits (30,31) and low density lipoprotein receptor-deficient mice (32). Furthermore, the endogenous effect of vascular ETB stimulation in vivo favors vasodilatation (4,5). Indeed, systemic and selective blockade of the ETB receptor induces hypertension and increases peripheral vascular resistance (33,34).

Although ETA receptor has been shown to be involved in atherosclerosis in animals (19,20), the pathophysiological roles of the ETB receptor in atherogenesis have not been fully elucidated. In this study, we examined the effect of non-selective ETA/ETB receptor antagonist SB209670 (35-37) on atherosclerotic lesions in apoE-deficient mice, a suitable animal model of atherosclerosis (38-41). We found that chronic administration of SB209670 reduces diet-induced hypercholesterolemia and atherosclerosis in apoE-deficient mice.

## **2. Materials and methods**

### **2.1 Experimental animals and study design**

All experimental procedures were approved by the Animal Center Committee of Tsukuba University. Homozygous apoE-deficient mice were obtained from The Jackson Laboratory (Bar Harbor, ME), and animals were derived from brother-sister matings. Before the study, animals were kept on standard mouse chow (0.075% cholesterol, 4% fat, Oriental yeast, Chiba, Japan). At the age of 10 weeks, 94 male mice were randomly divided into four groups: 1. Mice fed a Western-type diet (21% fat, 0.15% cholesterol; Harlan Teklad no.88137) with placebo (0.9% saline)(n=24), 2. Mice fed the Western type diet with SB209670 treatment (n=24), 3. Mice fed the chow diet with placebo (n=23), 4. Mice fed the chow diet with SB209670 treatment (n=23). Dosing was continued until the end of the 12-week observation period. Mice were given SB209670 (10mg/kg/day) (Smithkline Beecham Pharmaceuticals, PA) or placebo by subcutaneously

implanted osmotic minipumps (Alza; model 2001, Palo Alto, CA). This dose and regimen was selected based on pharmacology studies performed in rats<sup>36-37</sup>.

Systolic blood pressure, body weight, and plasma lipid were measured at baseline (10 weeks of age), then periodically (14, 18, and 22 weeks of age) during the study.

## **2.2 Characteristics of SB209670**

SB209670 is a high affinity ligand at human cloned ETA and ETB receptors ( $K_i = 0.2$  and  $18$  nM, respectively) (36). Systemic administration of SB209670, given as a continuous intravenous infusion ( $1-100$   $\mu\text{g}/\text{kg}/\text{min}$ ), does not alter basal hemodynamic parameters in the anesthetized rat (37). Infusion of SB209670 ( $10$   $\mu\text{g}/\text{kg}/\text{min}$ ) selectively inhibits the depressor and carotid vasodilator response to exogenous ET-1 ( $0.3$  nmol/kg);  $100$   $\mu\text{g}/\text{kg}/\text{min}$  is required to inhibit significantly the biphasic hemodynamic actions of ET-1 (37). In healthy male volunteers, administration of SB209670 increases mean renal hemodynamic responses (para-aminohippurate clearance) and preserved renal sodium excretion (38).

## **2.3 Measurement of blood pressure**

Unanesthetized mice were introduced into a small holder mounted on a thermostatically controlled warming tube at  $37^\circ\text{C}$ . Systolic blood pressure was measured with a programmable sphygmomanometer (BP-98A; Softron, Tokyo) by the tail-cuff method.

## **2.4 Plasma concentration of total cholesterol and triglycerides**

After a 4 hour fast, blood samples were taken from the retroorbital plexus under anesthesia with diethylether into Eppendorf tubes containing  $1\text{mM}$   $\text{Na}_2\text{EDTA}$ . Plasma was separated by centrifugation at  $3000$  rpm for  $20$  min at  $4^\circ\text{C}$ . Total plasma cholesterol and triglycerides were assayed by enzymatic colorimetric assay kits (Wako chemicals, Osaka, Japan).

## 2.5 Lipoprotein profile in the plasma

Whole plasma was resolved by electrophoresis in 1% agarose gels (Helena Laboratories, Saitama, Japan). Then, the gels were dried and stained with Fat Red 7B to identify lipoproteins containing neutral lipids. And the plasma of 5 mice, in 200 $\mu$ l aliquots, was mixed, and lipoproteins were isolated by small-volume sequential ultracentrifugation with a Beckman TLA100.2 rotor as described (42). Total cholesterol concentrations in each density fraction were determined by the enzymatic colorimetric assay as described above.

## 2.6 Measurement of aortic ET-1 content and plasma ET-1 concentration

Whole aortic tissue was weighed, snap-frozen in liquid nitrogen, and kept at -80°C. Tissue was homogenized by using a polytron for 60sec in 1M acetic acid-20mM HCl buffer. Then homogenates were boiled for 10min and centrifuged at 10,000 rpm for 10min. Supernatant solution was used for the extraction. A 0.5ml volume of pooled plasma was diluted with 1.5ml of 10% acetic acid. The solution was subsequently pumped (1ml/min) through a Sep-pak C-18 cartridge (Waters Associates, Milford, Mass). After a wash with distilled water, the absorbed peptides were eluted with 60% acetonitrile containing 0.1% trifluoro acetic acid. The elute was dried in a centrifugal evaporator and reconstituted in working assay buffer. A sandwich-type enzyme immunoassay kit (IBL, Gumma, Japan) was used for the measurement of ET-1. Cross-reactivity characteristics of the assay were big ET-1 ( $\leq 0.1\%$ ), ET-2 (3.32%), and ET-3 ( $\leq 0.1\%$ ).

## 2.7 Measurement of plasma NOx

Plasma NOx was measured photometrically via its oxidation products nitrite and nitrate using a nitric oxide colorimetric assay kit (Boehringer Mannheim, Germany). Briefly, 200 $\mu$ l aliquots of the plasma were diluted with 800 $\mu$ l of potassium phosphate buffer (pH 7.4), and deproteinized in an ultrafilter

(Centrisart cut-off 10000, Sartorius, Germany). Samples were incubated 30min at room temperature in the presence of Aspergillus nitrate reductase, FAD and NADPH. Then, color reagent including sulfanilamide and N-(1-naphthyl)-ethylene diamine dihydrochloride was added to each sample. After a 5-min incubation at room temperature, the absorbance of each sample was determined at 550nm. The result was calculated from the calibration curve using the standard nitrate solution.

## 2.8 Analysis of atherosclerotic lesions

Mice were killed by neck sprain under anesthesia with diethylether. After blood collection via the right ventricle, phosphate buffered saline (PBS, pH7.4) was introduced into the left ventricle via a 26 gauge needle and allowed to flow out of a cut point at the right atrium. When the perfusate became clear, 4% paraformaldehyde in 0.1M phosphated buffer (PB; pH7.4) was perfused. The aorta was exposed, minor branching arteries were cut off, and the adventitial tissue was removed as far as possible. Then the aorta was opened longitudinally from the aortic root to the iliac bifurcation, and pinned out on a black wax surface. This was followed by additional fixation with 4% paraformaldehyde in 0.1M PB for 12h. The aortas were stained with oil-red O, mounted en face on slides under coverslips within glycerol gelatin, and photographed. The image data of the entire aorta were analyzed with a Mac Scope image analysis software: (Mitani Co, Chiba, Japan). The extent of atherosclerosis was expressed as the sudanophilic area as a percentage of the total aortic surface area.

Next, plaque morphology was examined with histological cross-sections of the aortic arch. Tissue was stained with hematoxylin and eosin, and Elastica van Gieson.

## 2.9 Statistical analysis

Results are expressed as the mean  $\pm$  SEM. Statistical analyses were

performed with the use of StatView J-4.5 software (Abacus concepts). Data in Table 1 and Fig.1 were analyzed by repeated measure ANOVA. If significance was detected, differences between groups were analyzed for each experimental period by one-way ANOVA followed by Scheffe's F test. Data in Fig.3 were analyzed by one-way ANOVA followed by Scheffe's F test. Aortic lesion area was analyzed by the Kruskal-Wallis test followed by Mann-Whitney's U test between two groups. A value of  $P < 0.05$  was considered significant.

### 3. Results

#### 3.1 Blood pressure.

Table 1 shows the periodical change in systolic blood pressure during the study. Systolic blood pressure did not differ between the groups, and was not affected by chronic SB209670 treatment.

#### 3.2 Plasma lipid levels and body weight.

A Western-type diet resulted in marked increases in the plasma total cholesterol, triglycerides and body weight. (Fig.1abc). In mice fed the Western-type diet, SB209670 significantly reduced the plasma total cholesterol level ( $1079.7 \pm 74.5$  vs.  $1503.1 \pm 101.4$  mg/dl,  $P < 0.001$ , at the end of the experiment, Fig.1a) and body weight ( $44.6 \pm 1.4$  vs.  $52.0 \pm 1.5$  g,  $P < 0.001$ , Fig.1c) without altering the plasma triglyceride level ( $133.2 \pm 10.7$  vs.  $130.6 \pm 12.3$  mg/dl, Fig.1b) compared with control mice. However, on a chow diet, plasma lipid levels ( $448.6 \pm 15.6$  vs.  $468.8 \pm 23.8$  mg/dl in total cholesterol level,  $81.7 \pm 7.4$  vs.  $78.5 \pm 6.0$  mg/dl in triglyceride level) and body weight ( $36.2 \pm 0.6$  vs.  $35.1 \pm 0.8$  g) were no different between the groups (Fig.1abc).

#### 3.3 Plasma lipoprotein profiles.