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Ramatroban, a TP receptor antagonist, improves vascular responses to acetylcholine in hypercholesterolemic rabbits in vivo

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Abstract

Recent studies show that 8-*iso*-prostaglandin $F_{2\alpha}$, a member of F_2-isoprostane family, acts as a vasoconstrictor via TP receptor activation; and its local release may contribute to an abnormal vasomotor tone associated with hypercholesterolemia. The purpose of this study was to examine whether ramatroban, a TP receptor antagonist, improves abnormal vascular reactivity in vivo in hypercholesterolemic rabbits. The plasma 8-*iso*-prostaglandin $F_{2\alpha}$ levels in hypercholesterolemic groups were significantly higher than those in normal groups. The treatment by ramatroban reversed the attenuation of the vascular response to acetylcholine in hypercholesterolemic groups. However, L-N^G-nitroarginine methyl ester, a nitric oxide synthase inhibitor, did not inhibit the protective effects of ramatroban. Attenuation of the vascular response to acetylcholine in hypercholesterolemic rabbits was significantly enhanced by 8-*iso*-prostaglandin $F_{2\alpha}$.

Attenuation of the vascular response to acetylcholine by a cholesterol-rich diet and 8-*iso*-prostaglandin $F_{2\alpha}$ was canceled by ramatroban. These findings suggest that ramatroban improves the vascular response in vivo to acetylcholine in hypercholesterolemic rabbits by blocking the action of 8-*iso*-prostaglandin $F_{2\alpha}$.

Keywords: Isoprostane; Vasodilatation; Hypercholesterolemia; TP receptor; Thromboxane

1. Introduction

The endothelium modulates the response of vascular smooth muscle by producing not only relaxing factors but also mediators that cause the smooth muscle cells to contract (Furchgott and Vanhoutte, 1989). Acetylcholine, a probe commonly used for testing the endothelial function in humans, causes endothelium-dependent smooth muscle vasodilatation, which, it is believed, is caused primarily by the release of nitric oxide and an endothelium-derived hyperpolarizing factor (Furchgott and Zawadzki, 1980; Feletou and Vanhoutte, 1988; Palmer et al., 1987).

Acetylcholine-induced vasodilatation is diminished by the conductance and resistance vessels of patients with atherosclerosis and in those with risk factors for atherosclerosis, such as hypercholesterolemia, hypertension, diabetes, congestive heart failure, old age, and smoking (Zeiher et al., 1991; Egashira et al., 1993; Vita et al., 1990). This abnormality is commonly attributed to the decreased activity of endothelium-derived relaxing factors; but a recent study has shown that 8-*iso*-prostaglandin $F_{2\alpha}$, a member of the F_2-isoprostane family, is a vasoconstrictor and its local release may contribute to the abnormal vasomotor tone associated with hypercholesterolemia (Wilson et al., 1999). F_2-isoprostanes, generated by free radical-induced peroxidation of arachidonic acid (Morrow et al., 1990), have been used as markers for lipid peroxidation in human diseases (Cipollone et al., 2000). The urinary concentration of F_2-isoprostanes is increased by cardiovascular risk factors such as smoking, diabetes, hypercholesterolemia, and hyper-homocysteinemia (Morrow et al., 1995; Gopaul et al., 1995; Davi et al., 1997; Voutilainen et al., 1999). 8-*iso*-prostaglandin $F_{2\alpha}$ (isoprostane $F_{2\alpha}$-III) or isoprostane $E_{2\alpha}$-III has been shown to exert a potent contractile activity of animal and human vessels caused by the activation of TP receptors (Kromer and Tippins, 1996; Cracowski et al., 2000; Audoly et al., 2000). Renal vasodilatation in response to acetylcholine is impaired

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in cholesterol-fed rats but the response improves markedly when TP receptors are blocked (Bank and Aynedjian, 1992). The purpose of this study was to examine whether ramatroban, a TP receptor antagonist, improves the vascular response in vivo to acetylcholine in hypercholesterolemic rabbits by blocking the actions of 8-*iso*-prostaglandin $F_{2\alpha}$.

2. Materials and methods

2.1. Reagents

8-*iso*-prostaglandin $F_{2\alpha}$ was purchased from Cayman (Ann Arbor, MI). Ramatroban was provided by Bayer Yakuhin (Osaka, Japan). Acetylcholine chloride and sodium nitroprusside were purchased from Sigma Chemical (St. Louis, MO, USA). L-N^G-nitroarginine methyl ester (L-NAME) was purchased from Biomol Research Laboratories (Plymouth Meeting, PA).

2.2. Experimental model

Forty male New Zealand white rabbits weighing 2.5–2.8 kg (mean 2.7 ± 0.2 kg) were maintained in individual cages under controlled light and temperature conditions. They were fed with either a standard laboratory diet or a diet containing 1% cholesterol (Clea Japan, Tokyo, Japan) and were randomized to receive for 6 weeks either a continuous infusion of 0.7 ng/kg/min 8-*iso*-prostaglandin $F_{2\alpha}$ ($n=19$) or a vehicle consisting of 0.9% saline ($n=21$). The infusion was delivered intravenously via an Osmotic Minipump (Esox Technology, Eagan, MI) that was implanted in the groin. They were anesthetized by an intramuscular injection of 3.5 mg/kg of xylazine (Bayer, Leverkusen, Germany) and 20 mg/kg of ketamine (Parke-Davis, Morris Plains, NJ). Under sterile conditions, the right femoral vein was exposed through a groin incision and isolated. A distal vein was ligated and the catheter of the pump was inserted retrogradely to the level of the inferior vena cava and secured. The incision was then closed. Administration of exogenous 8-*iso*-prostaglandin $F_{2\alpha}$ resulted in a rise in the plasma 8-*iso*-prostaglandin $F_{2\alpha}$ level to 508 ± 85 pg/ml, which was generally comparable to 488 ± 44 pg/ml, the level of the group that had been on the 1% cholesterol diet (Table 1). Some of the rabbits on a 1% cholesterol diet received ramatroban, which was combined with the drinking water at a dosage of 5 mg/kg/day. Thus, the following six experimental groups were studied: normal diet only (NL; $n=7$), normal diet with 8-*iso*-prostaglandin $F_{2\alpha}$ infusion (NL + PG; $n=6$), 1% cholesterol diet only (CH; $n=7$), 1% cholesterol diet with 8-*iso*-prostaglandin $F_{2\alpha}$ infusion (CH + PG; $n=6$), 1% cholesterol diet with ramatroban treatment (CH + RM; $n=7$), or 1% cholesterol diet with 8-*iso*-prostaglandin $F_{2\alpha}$ infusion and ramatroban treatment (CH + PG + RM; $n=7$). These animal experiments were approved by and conducted at the National Defense Medical College, Tokorozawa, Saitama, Japan, following the guidelines for animal experimentation.

2.3. Measurement of plasma lipids, nitrate and nitrite (NOx), and 8-iso-prostaglandin $F_{2\alpha}$

Blood samples were taken from the animals in each group after 6 weeks of the experiment. The plasma was stored at −80 °C until the time of the assay. Plasma cholesterol, low-density lipoprotein (LDL), and high-density lipoprotein (HDL) cholesterol and triglycerides were measured by employing standard enzymatic techniques. The total 8-*iso*-prostaglandin $F_{2\alpha}$ levels were evaluated by determining the concentrations with the aid of an appropriate enzyme-linked immunosorbent assay kits (Cayman). Prior to enzyme immunoassay, alkaline hydrolysis was conducted (Bolterman et al., 1998) and plasma samples were purified in Sep-Pak C-18 columns (Japan Waters, Tokyo, Japan) before analysis. The samples, tracer, and antiserum were added to wells that were pre-coated with a

Table 1
Mean arterial pressure, heart rate, plasma levels of total cholesterol, NOx, and 8-*iso*-prostaglandin $F_{2\alpha}$ in six experimental groups at 6 weeks

	Mean arterial pressure (mm Hg)	Heart rate (beats/min)	Total cholesterol (mg/dl)	Plasma NOx (μM)	Plasma 8-*iso*-prostaglandin $F_{2\alpha}$ (pg/ml)
Normal diet ($n=7$)	99 ± 21	179 ± 40	38 ± 7	144 ± 25	256 ± 17
Normal diet + 8-*iso*-prostaglandin $F_{2\alpha}$ ($n=6$)	96 ± 18	174 ± 37	43 ± 13	139 ± 24	508 ± 85[a]
1% Cholesterol diet ($n=7$)	90 ± 19	181 ± 24	1695 ± 135[b]	105 ± 19[b]	488 ± 44[a]
1% Cholesterol diet + 8-*iso*-prostaglanidin $F_{2\alpha}$ ($n=6$)	99 ± 14	180 ± 20	1610 ± 193	114 ± 17	858 ± 59[c]
1% Cholesterol diet + ramatroban ($n=7$)	98 ± 24	174 ± 28	1694 ± 137	117 ± 19	498 ± 14
1% Cholesterol diet + ramatroban + 8-*iso*-prostaglandin $F_{2\alpha}$ ($n=7$)	96 ± 19	175 ± 10	1675 ± 121	103 ± 28	838 ± 17[d]

Data are means ± S.D. in the six experimental groups at 6 weeks. The numbers of samples for each group of rabbits are given.
[a] $P<0.01$ as compared to those in group fed with a normal diet.
[b] $P<0.05$ as compared to those in group fed with a normal diet.
[c] $P<0.01$ as compared to those in group fed with a 1% cholesterol diet.
[d] $P<0.01$ as compared to those in group with 1% cholesterol diet feeding and ramatroban treatment.

mouse monoclonal antibody. After the plates were washed, the substrate was added to these wells. Spectrophotometric analysis was performed at 405 nm.

2.4. Quantitative angiography

After 6 weeks of the experiment, each rabbit was anesthetized by intramuscular injection of 3.5 mg/kg of xylazine and 20 mg/kg of ketamine. Under sterile conditions, the left femoral artery was exposed through a groin incision and isolated. A 2 Fr end-hole infusion catheter (Baxter, Irvine, CA), which had been inserted into the abdominal aorta, was used to infuse vasoactive drugs, directly measure intra-arterial blood pressure via a connection to a pressure transducer, and selective angiography of the aorta. Angiography was performed immediately after drug administration with the aid of 1 ml of a contrast medium. Serial images of the abdominal aorta were recorded on 105-mm spot film at a rate of two films per second for 5 s.

Acetylcholine chloride was administered intra-arterially via a constant infusion pump (0.5 ml/min) at dosages of 0.1, 1, and 10 μg/kg/min for 2 min. To reestablish the basal blood flow rate, each dose was administered at 5-min intervals. In some cases, L-NAME (1 μmol/kg), a nitric oxide synthase inhibitor, was pre-infused for 20 min before adding acetylcholine to assess the effect of endogenous nitric oxide release on vasodilatation. The rabbits were then allowed to rest for 30 min. An intra-arterial bolus dose of sodium nitroprusside (0.1, 1, and 10 μg/kg/min) was administered to assess endothelium-independent vasomotor reactivity.

The luminal diameter of the abdominal aorta at the baseline and after drug infusion was measured from cin-

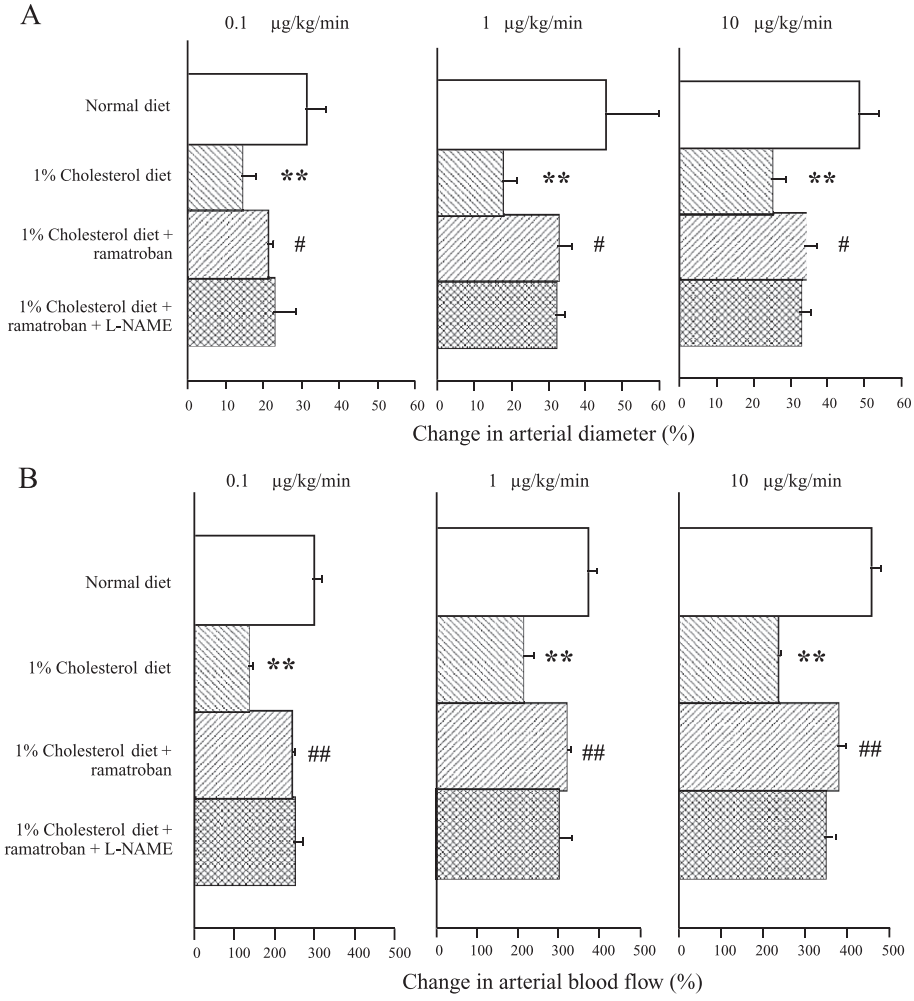

Fig. 1. Effect of ramatroban, a TP receptor antagonist, on vasodilatation (A) or the increases in arterial blood flow (B) in response to acetylcholine (0.1, 1, and 10 μg/kg/min) in the aorta from rabbits fed with a 1% cholesterol diet. Results are expressed as percentage of increase in arterial diameter (A) or arterial blood flow (B). Data are means ± S.D. of six or seven cases. $^{**}P<0.01$ as compared to those fed with a normal diet. $^{\#}P<0.05$, $^{\#\#}P<0.01$ as compared to those fed with a 1% cholesterol diet.

eangiograms by using a quantitative coronary artery angiography analysis device (Cardio 500, Kontron Elektronik, Munich, Germany) that contained a digitizing board. The vascular diameter was measured at the site where the Doppler volume was sampled (i.e., 5 mm distal to the wire tip). The cross-sectional area was calculated by assuming the lumen to be circular.

2.5. Measurement of blood flow using a Doppler guidewire

A Doppler guidewire (Cardiometrics, Mountain View, CA), a flexible 0.018-in. angioplasty guidewire with a 12-MHz piezoelectric ultrasound transducer at the tip, was inserted into the abdominal aorta and positioned 1 cm proximal to the infusion catheter. The mean blood flow velocity was measured by integrating the portion of the spectral display over the systole and diastole and dividing by the RR interval (Yamada et al., 1996; Testa et al., 1996). The results of three consecutive cycles were averaged. The mean blood flow velocity was measured at the baseline and after each administration of acetylcholine and sodium nitroprusside. Doppler-derived blood flow was calculated as follows: Doppler-derived time average flow (ml/min) = $\pi \times$ time average velocity $\times 0.125 \times$ diameter^2 (Segal et al., 1990). The Doppler-derived flow calculated in this manner has been shown to correlate with flow measurements determined by electromagnetic flowmeters, both in vitro and in vivo.

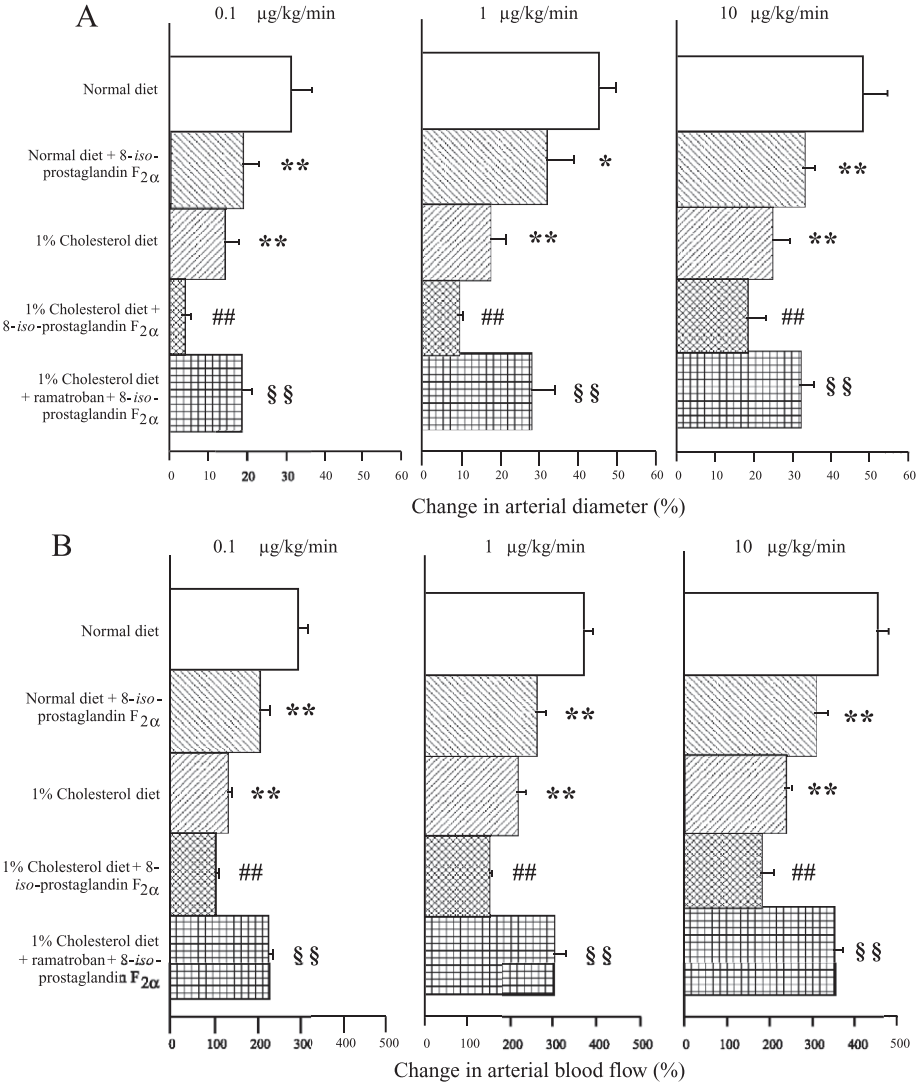

Fig. 2. Effect of 8-*iso*-prostaglandin $F_{2\alpha}$ or ramatroban on vasodilatation (A) or the increases in arterial blood flow (B) in response to acetylcholine (0.1, 1, and 10 μg/kg/min) in the aorta from rabbits either fed with a normal diet or a 1% cholesterol diet. Results are expressed as percentage of increases in arterial diameter (A) or arterial blood flow (B). Data are means ± S.D. of six or seven cases. $^{*}P<0.05$, $^{**}P<0.01$ as compared to those without 8-*iso*-prostaglandin $F_{2\alpha}$ stimulation. $^{\#\#}P<0.01$ as compared to those fed with a 1% cholesterol diet. $^{\S\S}P<0.01$ as compared to those with a 1% cholesterol diet and 8-*iso*-prostaglandin $F_{2\alpha}$.

2.6. Statistical analysis

All results were expressed as means ± S.D. Changes with respect to basal values when only two observations were made were analyzed by a paired or unpaired Student's *t*-test. Comparisons between means of multiple groups were analyzed by two-way analysis of variance (ANOVA) with the Dunnett test for post hoc comparison only when the results of the ANOVA were significant at $P < 0.05$.

3. Results

3.1. Effects of 8-iso-prostaglandin F2$_\alpha$ and ramatroban on hemodynamic variables in rabbits

There were no differences in daily oral intake or weight gain among the six groups of animals during the 6-week study period (data not shown). Neither the mean arterial pressure nor heart rate at 6 weeks was affected by the infusion of 8-*iso*-prostaglandin $F_{2\alpha}$ (Table 1). These variables were not affected by the treatment with 5 mg/kg/day of ramatroban.

3.2. Effects of 8-iso-prostaglandin F2$_\alpha$ and ramatroban on plasma lipid levels, NOx levels, or 8-iso-prostaglandin F2$_\alpha$ levels in rabbits

Plasma levels of total cholesterol in the CH + PG group did not differ from those in the CH group (Table 1). Treatment with ramatroban did not alter the plasma total cholesterol levels. There were no differences in the plasma levels of triglyceride, HDL cholesterol, and LDL cholesterol among the six groups of animals during the 6-week study period (data not shown).

There was a decrease in the plasma NOx levels in the CH group compared with the NL group ($P < 0.05$). However, neither the ramatroban treatment nor the infusion of 8-*iso*-prostaglandin $F_{2\alpha}$ affected the plasma NOx levels in the CH group.

The plasma 8-*iso*-prostaglandin $F_{2\alpha}$ levels in the CH group were significantly higher than those in the NL group ($P < 0.01$). The plasma 8-*iso*-prostaglandin $F_{2\alpha}$ levels in the NL + PG group were also significantly higher than those in the NL group ($P < 0.01$) and were about the same levels as those in the CH group. The ramatroban treatment did not affect the plasma 8-*iso*-prostaglandin $F_{2\alpha}$ levels in the CH group and the CH + PG group. The

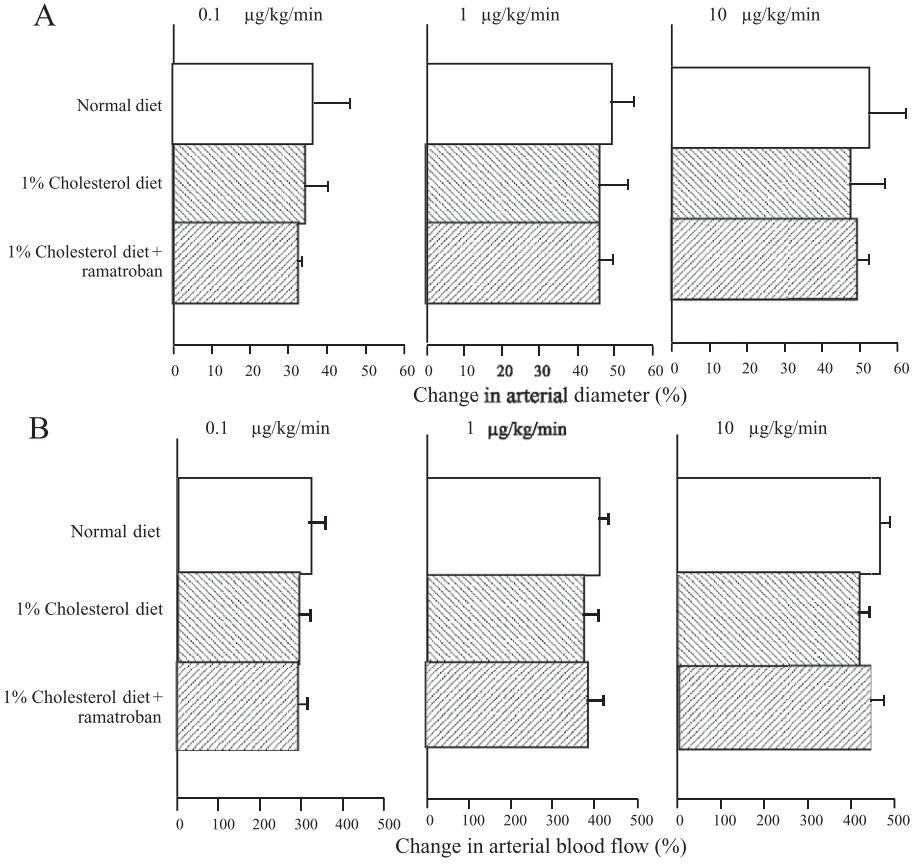

Fig. 3. Effect of ramatroban on vasodilatation (A) or the increases in arterial blood flow (B) in response to sodium nitroprusside (0.1, 1, and 10 μg/kg/min) in the aorta from rabbits fed with a normal diet or a 1% cholesterol diet. Results are expressed as percentage of increases in arterial diameter (A) or arterial blood flow (B). Data are means ± S.D. of six or seven cases.

infusion of 8-*iso*-prostaglandin $F_{2\alpha}$ significantly enhanced the plasma 8-*iso*-prostaglandin $F_{2\alpha}$ levels in the CH group ($P<0.01$).

3.3. Effects of ramatroban on vasodilatation and the increases in arterial blood flow induced by acetylcholine in hypercholesterolemic rabbits

The vasodilatation caused by acetylcholine was significantly attenuated in the CH group when compared with the NL group ($P<0.01$) (Fig. 1A). The ramatroban treatment significantly improved the vasodilatation caused by acetylcholine in the CH group ($P<0.05$). Pre-infusion with a nitric oxide synthase inhibitor, L-NAME, did not affect vasodilatation in response to acetylcholine in the CH + RM group. The acetylcholine-induced increases in arterial blood flow were significantly attenuated in the CH group in comparison with the NL group ($P<0.01$) (Fig. 1B). Ramatroban treatment significantly improved the increases in arterial blood flow in response to acetylcholine in the CH group ($P<0.01$). Pre-infusion with L-NAME did not affect the acetylcholine-induced increases in arterial blood flow in the CH + RM group.

3.4. Effects of 8-iso-prostaglandin $F2_{\alpha}$ and ramatroban on vasodilatation and the increases in arterial blood flow induced by acetylcholine in normal and hypercholesterolemic rabbits

Infusion of 8-*iso*-prostaglandin $F_{2\alpha}$ significantly attenuated vasodilatation in response to acetylcholine in the NL group (0.1 μg/kg/min; $P<0.01$, 1 μg/kg/min; $P<0.05$, 10

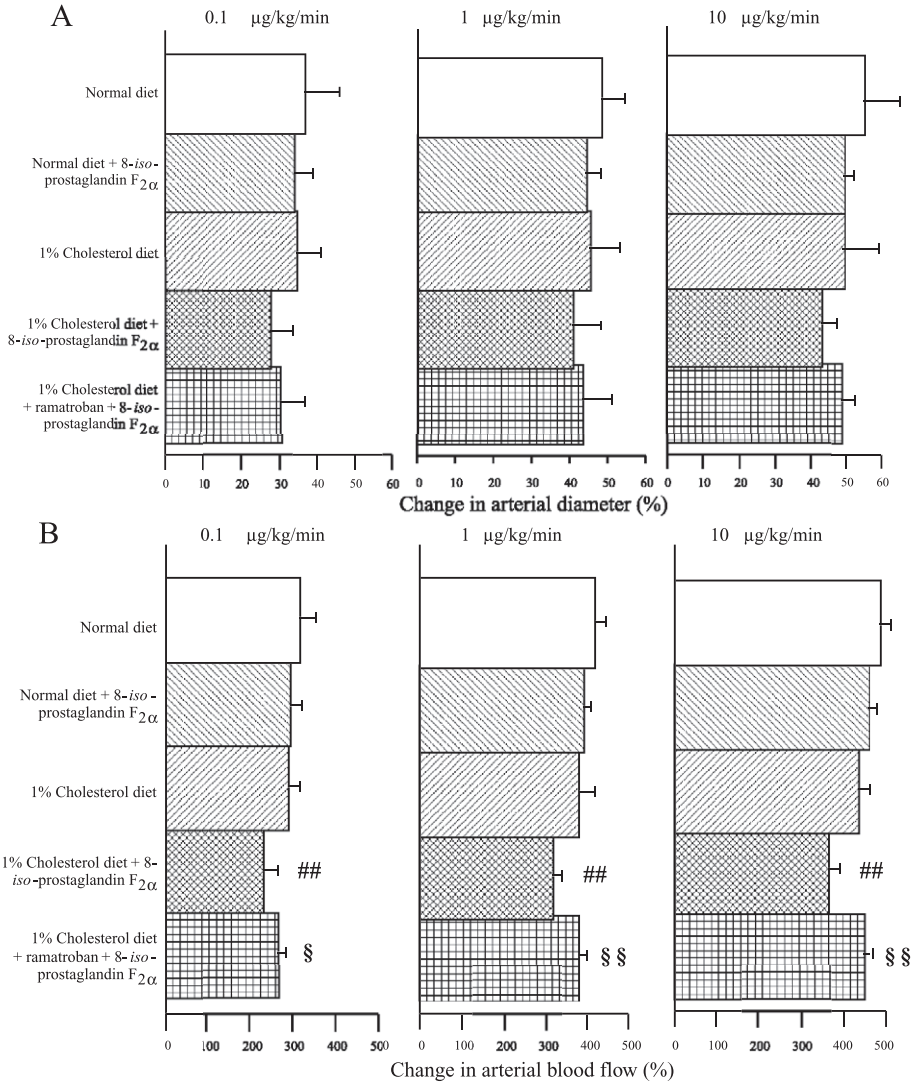

Fig. 4. Effect of 8-*iso*-prostaglandin $F_{2\alpha}$ on vasodilatation (A) or the increases in arterial blood flow (B) in response to sodium nitroprusside (0.1, 1, and 10 μg/kg/min) in the aorta from rabbits either fed with a normal diet or a 1% cholesterol diet. Results are expressed as percentage of increases in arterial diameter (A) or arterial blood flow (B). Data are means ± S.D. of six or seven cases. $^{\#\#}P<0.01$ as compared to those fed with a 1% cholesterol diet. $^{\S}P<0.05$, $^{\S\S}P<0.01$ as compared to those with a 1% cholesterol diet and 8-*iso*-prostaglandin $F_{2\alpha}$.

μg/kg/min; $P < 0.01$ versus the NL group) (Fig. 2A). Attenuation of the vasodilator response to acetylcholine in the CH group was significantly enhanced by 8-*iso*-prostaglandin $F_{2\alpha}$ ($P < 0.01$). Ramatroban treatment significantly improved vasodilatation in response to acetylcholine in the CH + PG group ($P < 0.01$). Infusion of 8-*iso*-prostaglandin $F_{2\alpha}$ significantly attenuated arterial blood flow that had been altered by acetylcholine in the NL group ($P < 0.01$, Fig. 2B). Attenuation of the increases in arterial blood flow in response to acetylcholine in the CH group was significantly enhanced by 8-*iso*-prostaglandin $F_{2\alpha}$ ($P < 0.01$). Ramatroban treatment significantly improved the increases in arterial blood flow in response to acetylcholine in the CH + PG group ($P < 0.01$).

3.5. Effects of ramatroban on vasodilatation and the increases in arterial blood flow induced by sodium nitro-

increased and heart rate decreased in hypercholesterolemic pigs (Krier et al., 2002). However, as noted in the present study, the infusion of 8-*iso*-prostaglandin $F_{2\alpha}$ did not affect mean arterial pressure or heart rate (Table 1). The concentration of 8-*iso*-prostaglandin $F_{2\alpha}$ (0.7 ng/kg/min) used in the present study was much lower than that employed by Krier et al. So it was not likely to affect the mean arterial pressure or heart rate.

In the NL group of the present study, the infusion of 8-*iso*-prostaglandin $F_{2\alpha}$ significantly attenuated the vascular response to acetylcholine but not to sodium nitroprusside in the NL group (Figs. 2 and 4). In the CH group, the effects that 8-*iso*-prostaglandin $F_{2\alpha}$ exerted to attenuate the vascular response to sodium nitroprusside was smaller than that to acetylcholine. It has been reported that cyclic GMP accumulates in the vascular smooth muscle cells and causes vasodilatation (Diamond and Blisard, 1976). Vasodilatation by sodium nitroprusside is due to direct stimulation of cyclic GMP accumulation in vascular smooth muscle cells (Schultz et al., 1977). On the other hand, acetylcholine stimulates endothelial muscaric receptors and enhances the release of nitric oxide, which causes vasodilatation via induction of the cyclic GMP levels in vascular smooth muscle cells (Ignarro and Kadowitz, 1985). In vitro and in vivo studies suggest that compared with acetylcholine in a comparable concentration, sodium nitroprusside is more potent in inducing cyclic GMP in the vascular smooth muscles, thus, it is a highly active vasodilator agent (Wang et al., 1993; Qiu et al., 1998). This appears to explain the observation that attenuation of the vasodilatation by sodium nitroprusside requires 8-*iso*-prostaglandin $F_{2\alpha}$ at a concentration higher than that when acetylcholine is used to stimulate the vascular smooth muscles. Therefore, 8-*iso*-prostaglandin $F_{2\alpha}$ synthesis in the NL, NL + PG, and CH groups may not be enough to affect the vascular response to sodium nitroprusside.

In conclusion, the present study suggests that 8-*iso*-prostaglandin $F_{2\alpha}$, through a TP receptor, plays a role in the impaired vascular response to acetylcholine associated with hypercholesterolemia; and ramatroban, a TP receptor antagonist, improves the vascular response by blocking the actions of 8-*iso*-prostaglandin $F_{2\alpha}$. Therefore, ramatroban or other TP receptor antagonists may be beneficial in the treatment of atherosclerosis, to which vascular dysfunction may contribute.

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