roles. Endothelial NOS plays an important role in vascular endothelium functions by producing NO, an important anti-atherosclerotic agent. Recent studies also showed that eNOS has a dual effect on atherosclerosis (Robert et al., 1998). eNOS itself could be an important source of endothelial superoxide production in hypercholesterolemia (Kirkwood et al., 1995). In diabetic vessels of human, the endothelium was found to be an additional net source of superoxide production because of eNOS dysfunction (Tomas et al., 2002). On the other hands, study of insulin-resistant rat aorta revealed a decreased aortic BH_4 contents as well as increased BH2 (7,8-dihydrobiopterin) levels, when compared with normal and non-insulin-resistant diabetic groups (Shinozaki et al., 1999). They reported that insulin resistance is the pathogenic factor of eNOS dysfunction and BH_4 deficiency. Other study showed that the balance between reduced and oxidized BH_4 is a key redox switch controlling superoxide formation from eNOS (Vasquez-Vivar et al., 2002). Exogenous administration of BH_4 leads to an acute amelioration of endothelium-dependant relaxation in DM rats (Pieper, 1997). Intravenous administration of sepiaptrin, which is an ancestor of BH_4, could improve the endothelial-dependent vasodilatation of diabetic patients clinically (Heitzer et al., 2000). There is little evidence that shows the relationship among high glucose, eNOS dysfunction and BH_4. Hyperglycemia is an independent risk factor for ischemic heart disease proved by clinical studies such as UKPDS. The current study is aimed to reveal the mechanisms of eNOS dysfunction leading by high glucose in an in vivo model.

Materials and methods

Cells

BAECs were isolated from fetal calf as described previously (Hayashi et al., 1995a) and cultured in DMEM with 10% (v/v) of CS, 100 u/ml of penicillin, 100 μg/ml of streptomycin, 2 mM glutamine. Cells were allowed to the confluent of 80%, and then stimulated with different concentration of D-glucose (5.5, 12.5, 25 and 50 mM) as well as other reagents in DMEM with 2% CS and phenol red free for 24 hours. Mannitol was used as control to rule out the effect of osmotic pressure.

Measurement of NOx (nitrite and nitrate)

Measurement of NOx (nitrite and nitrate) in supernatant was performed as described in previous study (Yamada and Nabeshima, 1997). Briefly, the supernatant were taken for the measurement of NOx by HPLC (ENO10, Eicom Co, Kyoto, Japan), where nitrate was converted to nitrite in an in-line copper coated cadmium reduction column (NO–RED), and then nitrite was detected based on Griess reaction.

Western blot analysis of eNOS protein

Determination of eNOS protein expression were performed as described in our previous study (Hayashi et al., 1995b). Protein concentration was determined by Dc protein assay kit (Bio-Rad, CA). 15 μg protein was loaded. Primary anti-eNOS monoclonal antibody (Anti-mouse IgG1

monoclonal antibody, Transduction Laboratories, CA) was incubated in the ratio of 1:2000, overnight. HRP-linked anti-mouse IgG antibody (Cell signaling) was used as second a antibody. Bands of eNOS protein were developed in dark on the film (Fuji Medical X-ray Film, Japan). Band densities were analyzed densitometrically by the National Institutes of Health IMAGE program.

RT–PCR analysis of eNOS and GTPCH1 mRNA

Total RNA was isolated from BAECs with TRIZOL reagent according to the manufacture's protocol (GIBCO BRL, Life Technologies). eNOS mRNA were analyzed by reactions with RNA PCR kit (One step RNA PCR Kit, Takara, Japan) as described in our previous study (Kano et al., 1999). The programmed cycles for eNOS RT PCR were as follows: 1 cycle of 50 °C × 30 minutes and 94 °C × 2 minutes; 30 cycles of 94 °C × 30 seconds, 60 °C × 30 seconds, and 72 °C × 30 seconds. Bands were visualized on dual intensity transluminator. RT–PCR of GTPCH1 mRNA were carried on such a programmed cycles: 1 cycle of 50 °C × 30 minutes and 94 °C × 2 minutes; 30 cycles of 94 °C × 30 seconds, 60 °C × 30 seconds, and 72 °C × 1 minutes. Sequence of bovine GTPCH1 primer is as follows: sense: 5′ CCGCCTACTCGTCCATCCTGA 3′, antisense: 3′ACCTCGCATTACCATACACAT 5′.

Measurement of intracellular superoxide by FACS

At the end of treatment period, cells were washed with PBS, 2 μl of 5 mM DCFH-DA was added and then incubated in 37 °C for 30 minutes. Cells were detached with trypsin, and centrifuged at 4 °C, 15 000 rpm for 5 minutes. Cell suspensions in PBS were transferred into 5 ml polystyrene round-bottom tubes with cell-strainer caps (Becton Dickinson lab ware, Becton Dickinson and company, France). And they were kept on ice for immediate measurement by FACS (Fluorescence-activated cell sorter, BD Biosciences).

Determination of intracellular BH4 level and GTPCH1 activity

Cells were harvested with trypsin and pelleted by centrifugation and frozen at −80 °C. BH_4 measurements were performed by HPLC procedure described by Fukushima and Nixon (Consrtino et al., 1997). Intracellular BH_4 levels were expressed in terms of pmoles per mg protein of the cell pellet. GTPCH1 activity was assayed based on the quantification of D-erythro-neopterin by HPLC after conversion of enzymatically formed D-erythro-7,8-dihydroneopterin triphosphate into D-erythroneopterin by sequential reaction of iodine oxidation and dephosphorylation.

Statistics

Data were reported as mean ± SD, and represent three independent experiments. Comparisons between the two groups were made based on the nonparametric Mann-Whitney *U* test. Significant differences were accepted when $P < 0.05$.

Results

Effects of high glucose on eNOS protein and mRNA expression

After exposure to high glucose for 24 hours, eNOS proteins were increased significantly, and in accordance with it, expression of eNOS mRNA were also enhanced (Fig. 1A, B). As mannitol treatment did not affect the expression of eNOS protein or eNOS mRNA, these results attributed to high glucose itself, not to osmolality.

Effects of high glucose on NOx produced by eNOS

After stimulated by high glucose (12.5 mM, 25 mM) for 24 hours, NOx production was significantly decreased compared with control (5.5 mM), but there were no significant different between the two high glucose groups (12.5 mM, 25 mM) (Fig. 2A). As mannitol treatment did not affect the concentration of NOx, the effect attributed to high glucose itself, not to osmotic pressure.

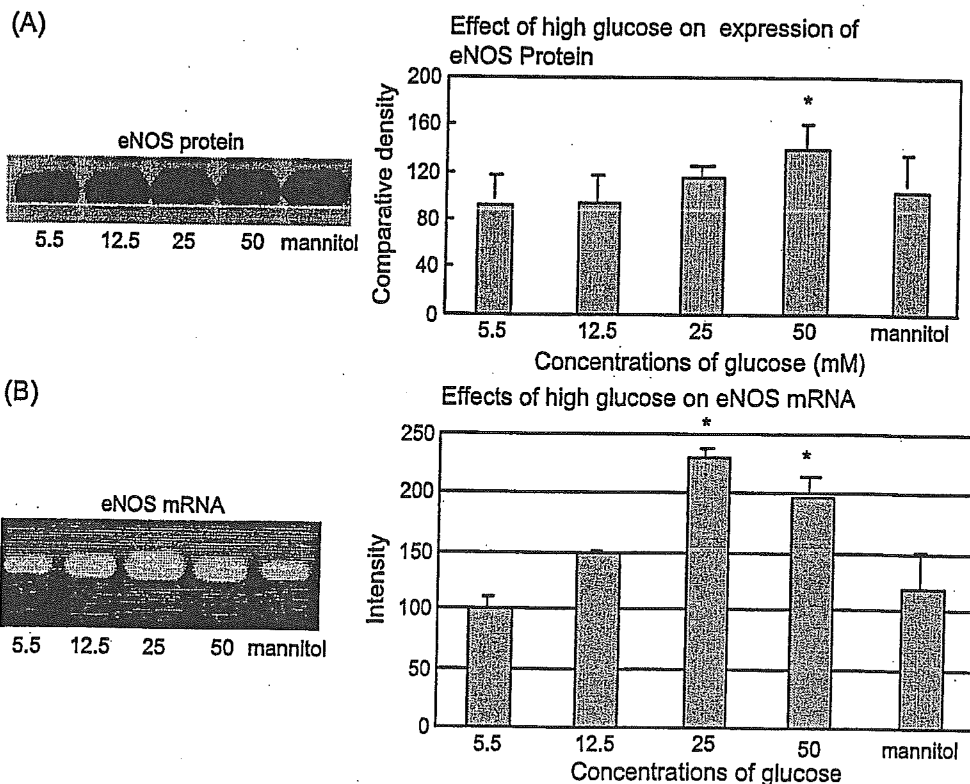

Fig. 1. The effects of high glucose on eNOS expression. Western blot and RT–PCR analysis of eNOS protein (A) and mRNA expression (B) after 24 hours exposure to different concentrations of glucose. Data represents the mean ± SEM of three separated experiments. The effects of high glucose on NOx production (A) *P < 0.05 vs control.

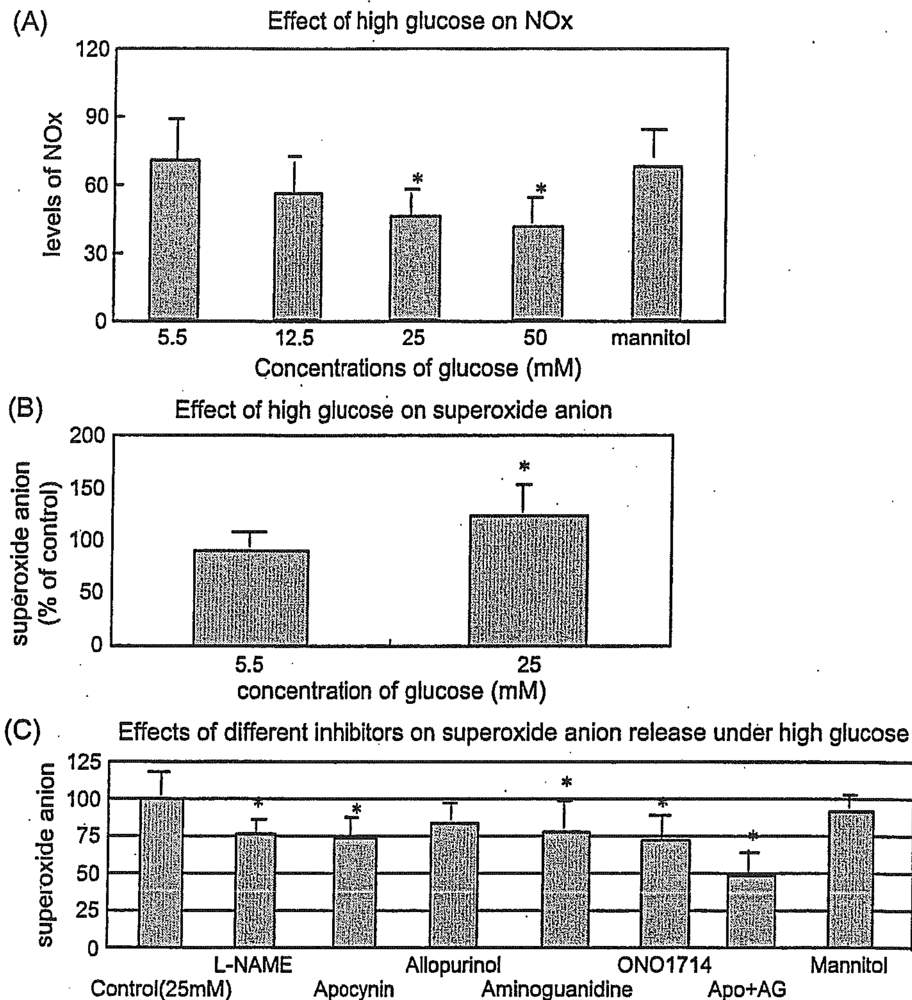

Fig. 2. The effects of high glucose on NOx production (A), superoxide anion (B). Effects of different inhibitors on superoxide anion under the stimulation of high glucose (C). Data represents the mean ± SEM. *P < 0.05 vs control.

Effects of high glucose on intracellular superoxide anion and possible route of superoxide production

The intracellular superoxide anion was largely increased by the stimulation of high glucose (25 mM), compared with control (5.5 mM), after 24 hours exposure (Fig. 2B). And the stimulatory effects of high glucose could be abolished by L-NAME(100 μM) and apocynin(10 μM), respectively (Fig. 2C). However, the effect of allopurinol (10 μM), aminoguanidine(10 μM), or ONO 1714 was relatively limited, and mannitol did not affected superoxide production (Fig. 2C).

Effects of high glucose on intracellular BH4 levels and GTPCH1 activities

As showed in Fig. 3A and B, both of intracellular BH_4 levels and GTPCH1 activities were decreased significantly by high glucose exposure.

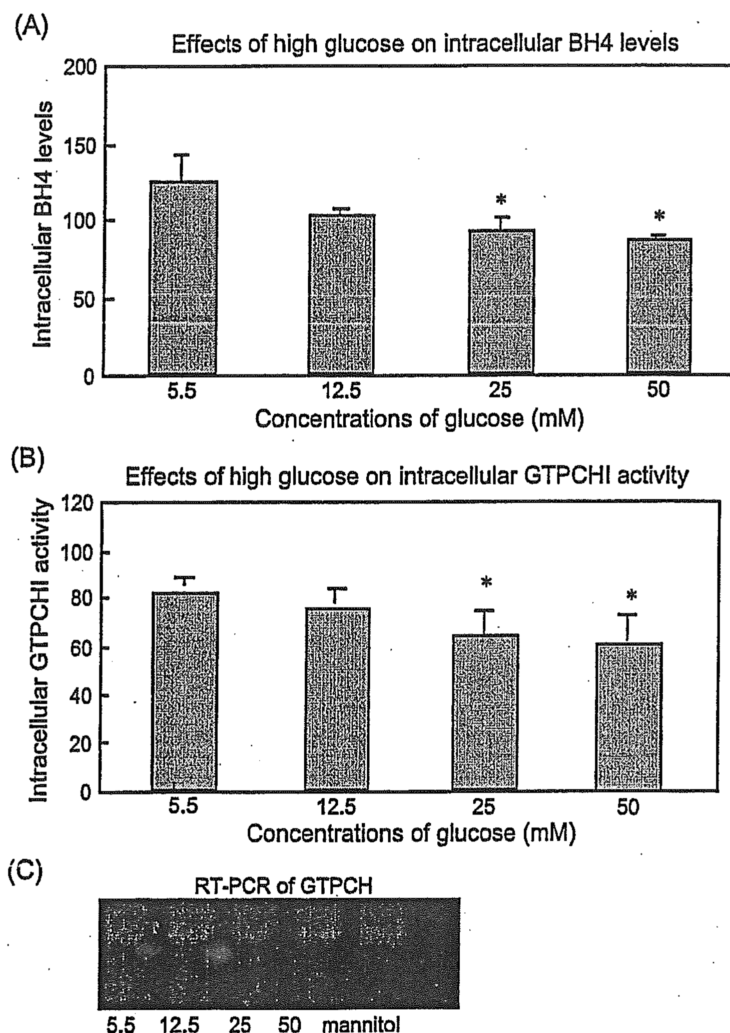

Fig. 3. The effects of high glucose on intracellular BH_4 levels (A), GTPCH1 activities (B) and GTPCH1 mRNA expression (C).

Effects of high glucose on expression of GTPCH1 mRNA

As revealed in Fig. 3C, in accordance with the inhibition of intracellular GTPCH1 activities, the expression of GTPCH1 mRNA abundance was also decreased by exposure to high glucose. It tended to correlate with GTPCH1 protein and activities (data not shown).

Effect of HMG-CoA reductase inhibitor on intracellular GTPCH1 activity and BH4 level

Atrovastatin exhibited a stimulatory effect on intracellular BH_4 accumulation (Fig. 4A) and GTPCH1 activities (Fig. 4C) and it was shown in a time- and concentration–dependent manner (part of data not shown).

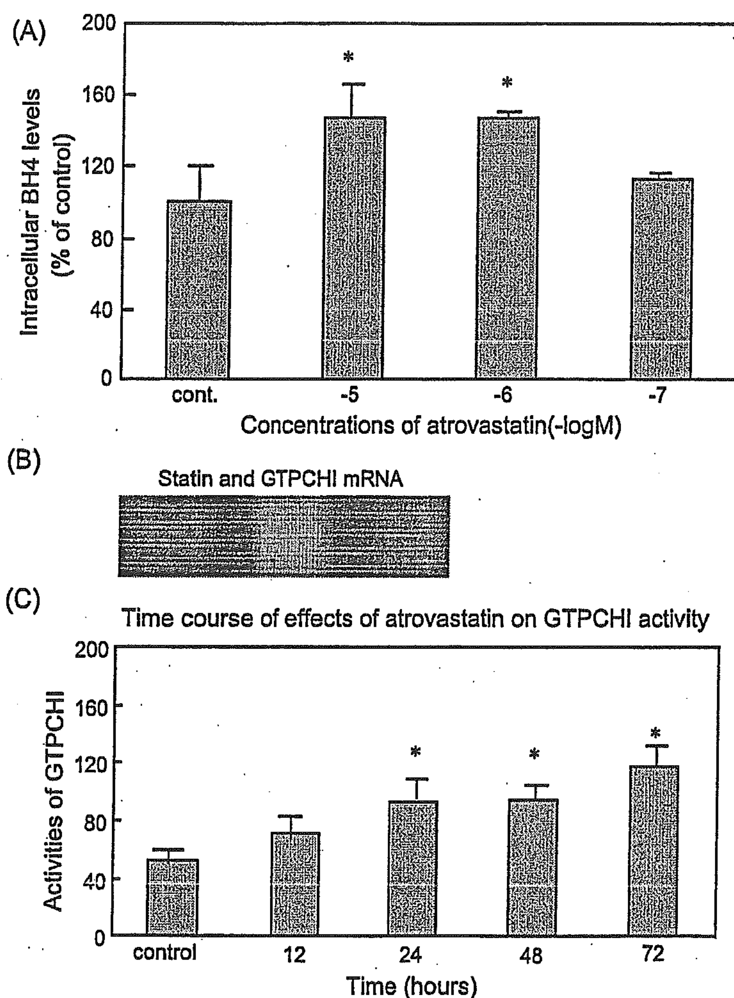

Fig. 4. Effects of different concentrations on atrovastatin on intracellular BH_4 levels (A) and GTPCH1 mRNA (B). Time-course of effects of atrovastatin on intracellular GTPCH1 activities (C). Data represents the mean ± SEM of three independent experiments. $^*P < 0.05$ vs control.

HMG-CoA reductase inhibiters and expression of GTPCH1 mRNA

In accordance with the stimulatory increase of intracellular GTPCH1 activities and BH_4 accumulations, expression of GTPCH1 mRNA was also augmented by atrovastatin (Fig. 4B).

Discussions

This study demonstrated that in bovine aortic endothelial cells, the expression of eNOS was increased by exposure to high glucose (Fig. 1A). This may be the result of augmentation of eNOS mRNA expression by high glucose (Fig. 1B). Although, the effect of high glucose on eNOS protein

was dose dependent, the maximum level of eNOS mRNA expression was maximum between 25 and 50 mM glucose. We cannot elucidate the mechanism in the difference of effective concentration between protein and mRNA levels. In the preliminary experiment of 72 hours exposure to high glucose, eNOS expression was maximum between 25 and 50 mM glucose. We speculate that the difference of effective glucose concentration between eNOS protein and mRNA is due to the difference of time course. It exhibited conflictions to the reasonable hypothesis that, the eNOS abundance should be decreased by high glucose, which is based upon the clues of impaired endothelium-dependent relaxation in diabetic vessels of both human and animal experiments (Consrtino et al., 1997; Ding et al., 2000; Johnstone et al., 1993; Makimattila et al., 1996; Noyman et al., 2001; Steinberg et al., 1996). Further, measurement of NOx revealed a marked decrease when cells were allow to grow in high glucose (Fig. 2). Two possible explanations could be applied: one comes from the rapid reaction between NO and superoxide, while another one means the possibility if virtually decreased capacity of NO production by eNOS. One possible clue for eNOS dysfunction caused by high glucose gives rise to the hypothesis that increased abundance of eNOS caused by high glucose could not produce NO in proportion to that under normal glucose.

It is now generally agreed that oxidative stress plays a crucial role in the formation and deterioration of atherosclerosis (Tomas et al., 2002; Chen et al., 1995). In order to clarify the dysfunction of eNOS, we also studied the production of superoxide anion after exposure of high glucose by FACS. As shown in Fig. 2, high glucose increased intracellular superoxide anion significantly. In order to identify the sources of superoxide, different kinds of inhibitors which is related to possible pathways of superoxide were applied. It is amazingly to find that L–NAME, which is the specific inhibitor of NOS, exhibited a strong inhibitory effect on superoxide production and restored superoxide anion to almost the same level as control. It means that eNOS becomes an important source of superoxide anion in high glucose. From this point of view, high glucose could lead to dysfunction of eNOS.

Since increased superoxide anion could also be inhibited partially by apocynin, but not by allopurinol and aminogunidine independently, it confirmed that in case of high glucose, NADPH oxidase, but neither xanthine oxidase nor iNOS is not the possible source of superoxide production as well as eNOS. The mechanisms underline these phenomena are still unknown. Evidences from diabetic animal models and human studies showed cofactor of eNOS, tetrahydrobiopterin (BH_4), may be the redox of NO or superoxide production of eNOS (Heitzer et al., 2000; Pieper, 1997). We further focused on the effects of high glucose on intracellular BH_4 level and activity of GTPCH1-the rate-limiting enzyme in the de novo biosynthesis of BH_4, which is the most important pathway under physiological conditions. BH_4 is absolutely required for eNOS activity (Chen et al., 1995; Hattori et al., 2003). By acting as a cofactor of eNOS, evidences showed that it is involved in: 1) stabilization eNOS in its dimeric form, which is pivotal for eNOS to function normally; 2) electron transfer from the reductase domain to oxidase domain; 3) active site integrity. And in some pathological situations, it could even help overcome 'paradoxical deficiency' of L-arginine. As it is showed in Fig. 3, both intracellular BH_4 levels and activities of GTPCH1 were markedly decreased by the exposure to high glucose comparing with control. It has been revealed that BH_4 react with superoxide rapidly, thus decreases BH_4 accumulation in cells. Results of the present study show that in case of high glucose, the deceased activity of GTPCH1 could also be an important reason for the decreased BH_4 levels. So it is reasonable to think that it is the combination of the two possibilities lead to an absolute shortage of intracellular BH_4, and accordingly, the dysfunction of eNOS arises. But it is still difficult to identify which one plays a more important role.

We speculated that the transcriptional regulation of GTPCH1 mRNA is responsible for the decreased GTPCH1 mRNA by high glucose treatment. Preliminary experiment showed that the decreased activity of GTPCH1 associated decreased protein level. Gesierich et al. reported the importance of the complex formation of GTPCH1 with GTPCH1 feedback regulatory protein (GERP) in negative feedback regulation by end product BH_4, and phenylalanine upregulated GTPCH1 mRNA without changing GERP (Gesierich et al., 2003; Hattori et al., 2003). They speculated that the substrate level and transcription of the interacting protein regulation of BH_4 biosynthesis. In the present study, the amount of BH_4 was decreased by high glucose treatment, and the protein also decreased.

HMG-CoA reductase inhibitors are now generally convinced to be a potent antiatherosclerotic agent. Its pleiotropic effects include a direct stimulatory effect on eNOS or iNOS as reported (Gorren et al., 2002; Hayashi et al., 1995a; List et al., 1997). And we have for the first time revealed that HMA-CoA reductase inhibitors could upregulate GTPCH1 mRNA expression, thus stimulate the activity of GTPCH1 as well as intracellular BH_4 levels in cultured endothelial cells, directly. Statin was reported to enhance cytokine-mediated inducible nitric oxide synthesis in smooth muscle cells (Hattori et al., 2002). It has been reported that the effect of statin was abolished by exogenous mevalonate or GTPCH1 inhibitor, GGTI-298. Our data further, give a richer meaning to the pleiority of antiatherosclerotic effects of HMG–OA reductase inhibitor (Laufs et al., 1998; Tsunekawa et al., 2001). Finally, mannitol concentration was adjusted to the osmotic pressure in 50 mM high glucose. Preliminarily, we examined the effect of mannitol on high glucose treatment, and we made sure that it did not significant effect on eNOS protein and mRNA and GTPCH1 mRNA, and BH_4 concentration.

Conclusively, high glucose could lead to the dysfunction of eNOS by inhibiting the synthesis of BH_4 and activating NADPH oxidase. Statin could enhance eNOS activity through stimulating GTPCH1, thus increases BH_4 levels, directly.

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A new HMG-CoA reductase inhibitor, pitavastatin remarkably retards the progression of high cholesterol induced atherosclerosis in rabbits

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Abstract

Background: The remarkable anti-atherosclerotic effects of 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitor have not been demonstrated in diet induced severe hyperlipidemia in rabbit model. *Objective:* We have investigated the effect of pitavastatin, a newly developed statin, on atherosclerosis in rabbits. *Methods and results:* Oophorectomized female NZW rabbits were fed 0.3% cholesterol chow for 12 weeks with or without pitavastatin (0.1 mg/kg per day) (Gp.NK and HCD). The level of serum cholesterol was decreased in Gp.NK compared with Gp.HCD (772.8 ± 70.2 versus 1056.9 ± 108.3 mg/dl), whereas no significant alterations were observed in triglyceride and HDL-cholesterol. NO dependent response stimulated by acetylcholine and calcium ionophore A23187 and tone related basal NO response induced by N^G-monomethyl-L-arginine acetate were all improved by pitavastatin treatment. Pitavastatin treatment increased the level of cyclic GMP in the aorta of cholesterol fed rabbits. In the aorta, the expression of eNOS mRNA was significantly up regulated and O_2^- production was slightly reduced in Gp.NK animals. Atherosclerotic area was significantly decreased in aortic arch and thoracic aorta from Gp.NK compared with those from Gp.HCD (15.1 ± 5.3 versus 41.9 ± 10.2%, 3.1 ± 1.1 versus 7.9 ± 1.2% in Gp.NK and Gp.HCD aortic arch and thoracic aorta). Anti-macrophage staining area, the MMP1 or 2 and the nitrotyrosine positive area were decreased in Gp.NK. *Conclusion:* Pitavastatin retards the progression of atherosclerosis formation and it improves NO bioavailability by eNOS up-regulation and decrease of O_2^-.
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Keywords: Nitric oxide; Endothelial nitric oxide synthase; Superoxide anion; HMG-CoA reductase inhibitor; Atherosclerosis

1. Introduction

3-Hydroxymethyl-3-glutaryl-CoA (HMG-CoA) reductase inhibitors (statins) are potent inhibitors of cholesterol biosynthesis in the liver by blocking the conversion of HMG-CoA to mevalonate [1]. They were widely used for the treatment of hyperlipidemia and used for the prevention of coronary artery disease. Landmark clinical trials with pravastatin (WOSCOPS) and simvastatin (4S) demonstrate that these statins decrease the serum cholesterol level and reduce the incidence of myocardial infarction and also cardiovascular mortality [2,3]. Additionally, several large statin trials such as AFCAPS/TEXCAPS and LIPID showed the beneficial effect of other statins [4,5].

Pitavastatin is a newly developed statin whose cholesterol reducing effect is stronger than the other new statins such as atorvastatin or lovastatin and its side effects such as liver dysfunction, were reported less when compared to the other statins [6]. However, the anti-atherosclerotic effect of pitavastatin on high cholesterol diet induced atherosclerosis was unknown in the rabbit model. High cholesterol diet itself inhibits HMG-CoA reductase activity of cells throughout the body, especially in the liver. In addition, application of statin inhibits HMG-CoA reductase absolutely, and the complete suppression of HMG-CoA reductase inhibitor may result in it being impossible of produce cell membrane composed of cholesterol, a life-threatening condition. It is thus very important to evaluate the anti-atherosclerotic effect of

Abbreviations: NO, nitric oxide; NOS, nitric oxide synthase; e-NOS, endothelial nitric oxide synthase; $ONOO^-$, peroxynitrite

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strong statin administration using high cholesterol diet induced atherosclerosis animal models.

HMG-CoA reductase inhibitors were shown to improve the endothelial function in a short time period [7]. Superoxide anion (O_2^-) production was increased in vessels of hyperlipidemic rabbits, and the release of peroxynitrite; $ONOO^-$ (formed from the reaction of NO and O_2^-) release was also increased in atherosclerosis [8]. These studies demonstrated that atherosclerosis was closely related to the level of NO production and reactive oxygen species (ROS). Hence, the present study was decided to determine whether the anti-atherosclerotic effects of pitavastatin is observed, and whether it is mediated by its lipid lowering effect and/or nitric oxide or superoxide mediated system in high cholesterol diet induced atherosclerosis in oophorectomized female rabbits. We used rabbits because they are herbivorous and easy to make atherosclerotic and it is also easy to damage liver function by high cholesterol diet or statins. Further, as sex steroids are known to affect on atherosclerosis formation via NO and antioxidant action, we used oophorectomized female rabbits in this study [9].

2. Materials and methods

2.1. Chemicals and solutions

Acetylcholine chloride (ACh), prostaglandin F2α (PGF2α), indomethacin and L-monomethyl-arginine (L-NMMA) were purchased from Sigma Chemical Co. (St. Louis, MO). Nitroglycerin (NTG) was from Nihon Kayaku Co. (Tokyo, Japan). Krebs'–Henseleit solution (118 mM NaCl, 4.7 mM KCl, 1.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 11 mM glucose, and 0.002 mM EDTA; disodium ethylendiamine-tetraacetic acid, pH7.4) was saturated with 95% O_2/5% CO_2. All concentrations are those in the final bath.

2.2. Animals

A total of 28 female New Zealand white rabbits, 3–4 months aged, weighing about 2.0 to 2.4 kg were obtained from Kitayama Rabbis (Ina, Japan). The rabbits were housed individually at 20 ± 3 °C with free access to water. Twenty rabbits were bilaterally oophorectomized and 8 were left non-oophorectomized. Four weeks after oophorectomy, the rabbits were divided into two groups (n—each = 10) and treated for 12 weeks. Gp.HCD was fed HCD (regular diet plus 0.3% cholesterol; Gp.NK was fed HCD with pitavastatin (0.1 mg/kg per day). Separately, 10 oophorectomized female rabbits were fed with regular diet with or without pitavastatin (0.1 mg/kg per day)(Gp.R, Gp.R-NK). Feeding was restricted to 120 g per day. Blood was withdrawn 24 h after the last feeding. All the rabbits were appeared to be healthy throughout the course of study. All the experiments were conducted in accordance with institutional guidelines for animal research.

2.3. Determination of plasma lipids

Plasma lipids levels were measured by enzymatic assays as described previously [10].

2.4. Isometric tension measurements

After twelve weeks of treatment, the rabbits were sacrificed by exanguination after being anesthetized with pentobarbital (50 mg/kg i.v.). The thoracic aorta was carefully taken from the portion of the orifice of left first costal artery down to the portion enclosed by the diaphragm, and cut into 2-mm wide transverse rings. Isometric tension measurement was performed as described before [11]. The rings were stretched to their optimal force, which was predetermined as the contractile response to 122 mM KCl, mounted in organ chambers and bathed in Krebs' Henseleit solution at 37 °C. Prostaglandin F2α induced sub-maximal force (2.6×10^{-6} M). Endothelium-dependent relaxation induced by ACh and endothelium-independent relaxation by NTG were determined. To investigate tone-related basal NO release assessed by responses to L-NMMA from aortic rings, moderate vascular tone (35–50% of the contraction obtained with 122 mM KCl) was induced by low prostaglandin F2α concentrations (0.8×10^{-6} M). In some experiments, indomethacin (5×10^{-6} M) was added for 60 min before the experiment to rule out contribution of prostanoids.

2.5. Histological evaluation of atherosclerosis and assays for tissue cholesterol content

Cross-sections of the aorta adjacent to segments of vascular responses were examined [12]. Briefly, the contours of the lumen and the internal elastic lamina (IEL) were traced. The mean surface involvement by atherosclerotic lesion per vessel (extent) was calculated after dividing the lesion circumference by the circumference of the internal elastic lamina. The circumferences of the lesion and the healthy region were defined as the circumferences of the respective parts of the internal elastic lamina. The area occupied by atherosclerotic lesions (total lesion burden: size/thickness) was defined as the % area bounded by the lumen and internal elastic lamina for luminal area ($n = 6$ for one vessel). The intima/media ratio was also measured. A 0.8 cm-long segment was homogenized and lipids were extracted and resuspended, then cholesterol levels were measured [13].

2.6. Immunocytochemical analyses

Cross-sections of the thoracic aorta were analyzed as described previously [14]. They were incubated with primary monoclonal antibody [for anti-macrophages (RAM11), smooth muscle cells (HHF35), MMP-1 and -2, nitrotyrosine or iNOS] for 60 min at room temperature. Negative controls

included substitution of primary antiserum for either PBS or irrelevant antibodies. Each field was scored for number of each antibody positive cells on slides and analyzed statistically as described by previous report [14]. Five samples were prepared from each rabbit.

2.7. Determination of cyclic GMP (cGMP)

The aortic cGMP concentration was determined by a specific radioimmunoassay (RPN226, Amersham, Buckinghamshire, England) [15]. Four aortic rings (each wet weight is 10 ± 1 mg) per rabbit were investigated.

2.8. Measurement of endothelial NO synthase (eNOS) mRNA

The expression of eNOS mRNA in the arterial wall was measured by RT-PCR methods [16]. Briefly, to make a DNA competitor, we designed and synthesized two primers [5′-ATTTAGGTGAC-ACTATAGAATACCAGTGTCCAA-CATGCTGCTGGAAATTGGTACGGTCATCATC-TGAC-AC-3′ (sense primer), 5′-TAAAGGTCTTCTTCCTGGTGA-TGCCAATACATC-AAACGCCGCGAC-3′ (anti-sense primer)] based on the sequences of human eNOS cDNA. We used a competitive RNA transcription kit (Takara Shuzo, Otsu, Japan). eNOS cDNA primers amplify a product with a predicted length of 486 bp, and the competitor was produced at a length of 558 bp. The same amount of mRNA was corrected using a β-actin.

2.9. Detection of aortic superoxide anion (O_2^-) generation

Formation of O_2^- from vessel was assayed by measuring the intensity of chemiluminescence probes in the presence of one of the Cypridina luciferin analogs, 2-methyl-6-(p-methoxyphenyl)-3,7-dihydroimidazo[1,2-a] pyrazine-3-one (MCLA) [17]. In brief, the O_2^- generation signal from the 2 mm length of vessel with or without endothelium was detected by a luminescence reader (BLR-201, Aloka Co., Tokyo). To ensure the specificity of MCLA to detect O_2^- increasing concentrations of SOD (1–50 U/ml) were added to the tissues.

2.10. Data analysis

Results were expressed as mean ± S.E.M. Data were compared by analysis of variance with repeated measurements. A level of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Plasma lipid concentration

Plasma lipid levels were measured before oophorectomy and after 0, 4, 8 and 12 weeks of oophorectomy. The addition of 0.3% cholesterol to the diet increased the total cholesterol level significantly compared with the baseline value. Plasma cholesterol levels were decreased in the pitavastatin group at 4, 8 and 12 weeks after oophorectomy compared with that of the HCD group. There were no significant differences in other lipid components such as HDL-C observed between the control and treated group animals (Table 1). Pitavastatin treatment in the regular diet group did not show any change of lipid profile (data not shown).

3.2. Histological examination of atherosclerosis

Histological examination of the thoracic aortae revealed more atheromatous lesions, as indicated by the mean percentage of luminal encroachment and the mean lesion area, in the hypercholesterolemic (Gp.HCD) than in NK104 treated groups (Gp.NK) (Fig. 1). Regular diet group showed no atherosclerotic lesion w/wo pitavastatin treatment.

3.3. Endothelium-dependent and -independent relaxation

In all groups, ACh produced endothelium dependent relaxations (EDRs) of the aortic rings with an intact en-

Table 1
Profile of plasma biochemistry

	BeO	0 week	4 weeks	8 weeks	12 weeks
T.Chol. (mg/dl)					
HCD	58.2±4.9	62.2±5.5	884.2±110.5	1082.2±123.3	1056.9±110.4
NK104	56.2±5.5	59.1±4.9	582.2*±95.8	752.4*±91.7	772.8*±79.1
Control	59.2±5.9	58.1±5.9	54.2±4.9	60.4±6.3	56.2±5.3
T.G. (mg/dl)					
HCD	48.5±6.3	51.2±8.3	54.2±10.1	57.4±13.7	52.9±24.0
NK104	46.2±6.5	49.1±5.1	51.2±10.3	52.1±11.7	40.8±6.8
Control	49.6±5.1	48.6±7.1	47.4±5.9	50.8±8.9	53.1±15.3
HDL-C (mg/dl)					
HCD	34.4±4.8	31.2±4.3	34.1±2.1	32.2±3.1	34.4±4.3
NK104	33.2±5.1	31.4±3.4	37.2±4.6	36.2±4.6	34.4±3.2
Control	34.6±5.1	31.5±2.1	31.4±5.2	30.8±4.9	30.1±5.1

* $P < 0.05$ vs. control.

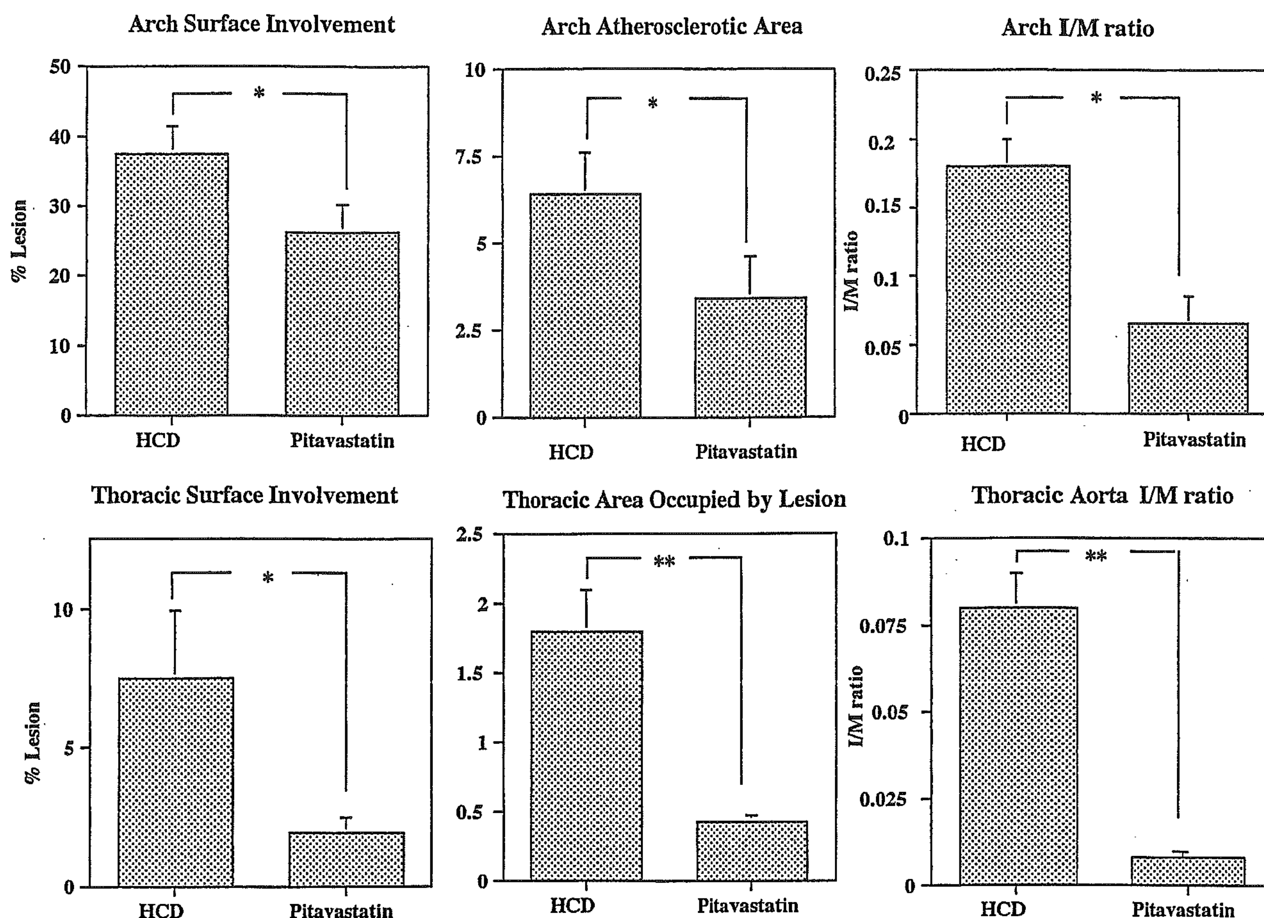

Fig. 1. Histological evaluation of atherosclerotic area (upper) of the thoracic aortae (lower). Left: The surface involvement of atherosclerotic area in the aortic arch and the thoracic aorta from of rabbits (Gp.HCD: high cholesterol diet [HCD, standard diet plus 0.3% cholesterol], Gp.NK, pitavastatin: HCD plus pitavastatin (1 mg/kg per day) $^{*}P < 0.05$, $^{**}P < 0.01$. Center: The area occupied by atherosclerotic areas of the aortic arch and the thoracic aorta from four groups of rabbits. Right: The Intima/Media ratio of the aortic arch and the thoracic aorta from four groups of rabbits.

dothelium (Fig. 2, left). The magnitude of the relaxation of aorta from the hypercholesterolemic animals (Gp.HCD) was diminished compared to those from regular diet group w/wo pitavastatin. However, EDRs in arteries from Gp.NK was remarkably larger than that from Gp.HCD. There was no significant difference in the response of vessels from regular diet group (Gp.R) and pitavastatin treated hypercholesterolemic animals (Gp.R-NK, data not shown). The non-receptor mediated vasorelaxation by calcium ionophore, A23187 showed the same tendency as ACh induced relaxation (data not shown). The endothelium-independent vasodilator, NTG, produced concentration-dependent relaxation in the thoracic aortic rings. No significant difference in relaxation was observed in aortic rings of all groups (Fig. 2, center). The inhibition of NOS by L-NMMA led to a contractile response in the aortic rings. The L-NMMA contractile response was higher in pitavastatin treatment (Gp.NK) (Fig. 2, right). Preincubation of indomethacin did not affect EDRs (data not shown).

3.4. *Tissue cyclic GMP concentration*

NO activates soluble guanylate cyclase in smooth muscle cell and led to produce cGMP. We examined cGMP concentration in homogenate samples of rabbit aorta. Pitavastatin treatment showed a significant increase of cGMP level Gp.NK as compared with HCD group ($3.11 + 0.42$ versus $2.24 + 0.34$ pmol/wet g in Gp.NK versus Gp.HCD, $P < 0.05$).

3.5. *Detection of mRNA for endothelial NO synthase*

The ethidium bromide-stained bands were quantified by densitometry from a photograph of the gel. The signal for eNOS increased about 50% in samples from aortae of hypercholesterolemic rabbits (Gp.HCD), as compared to those from control (Gp.R)(data not shown). The amount of eNOS mRNA was increased in Gp.NK compared with that in Gp.HCD.

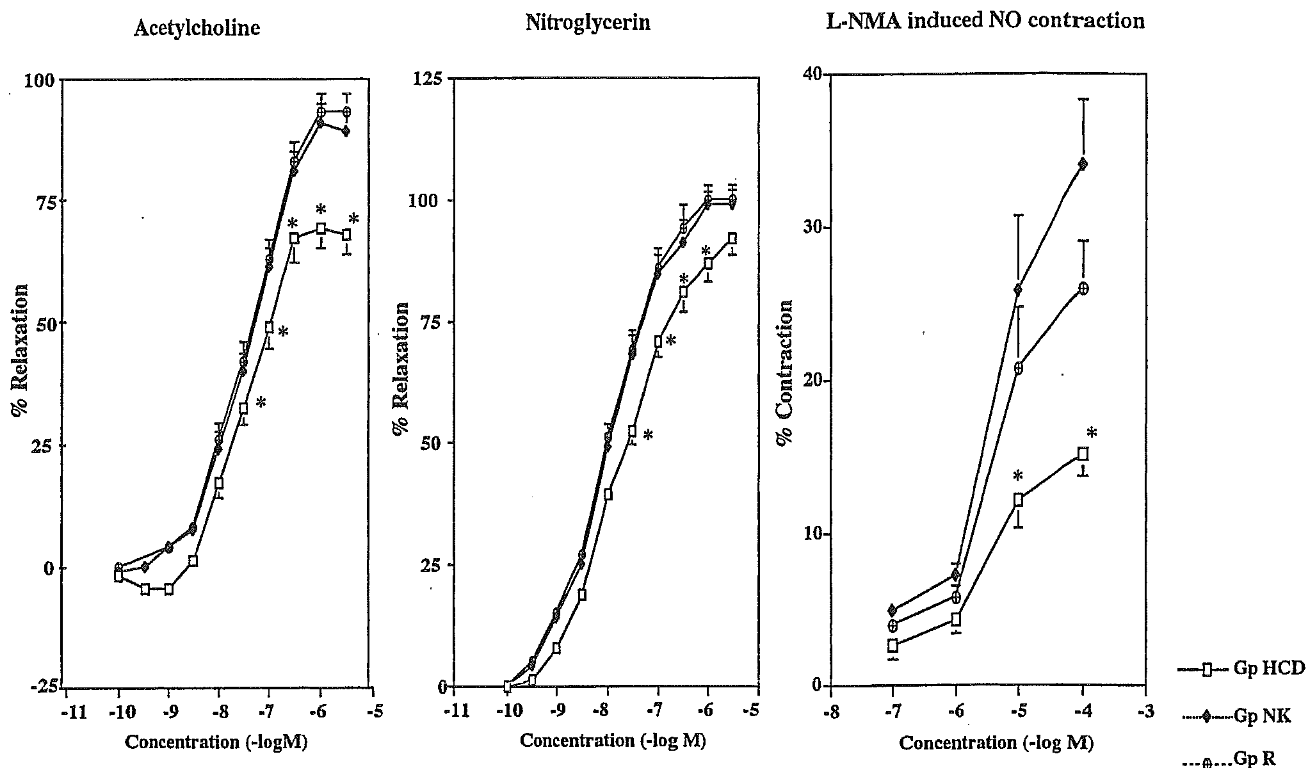

Fig. 2. Left: Cumulative concentration-response curves to acetylcholine (ACh) during contraction evoked by prostaglandin F2α (2.6×10^{-6} M) in the thoracic aortas of rabbits fed with a high-cholesterol diet (HCD), HCD plus pitavastatin (NK), or a regular rabbit chow (R). Significant difference ($^{*}P < 0.05$) vs. HCD. Data are shown as means ± S.E.M. Center: Cumulative concentration-response curves to L-NMMA during contraction evoked by prostaglandin F2α (0.8×10^{-6} M). Right: Cumulative concentration-response curves to nitroglycerin (NTG) during contraction evoked by prostaglandin F2α (2.6×10^{-6} M) in the thoracic aortas. There is no significant difference between three groups.

3.6. An aortic superoxide anion production

We measured superoxide anion production from arterial wall with lucigenin analogue (MCLA). The chemiluminescence signals (CL signals) as superoxide anion production increased in aorta from cholesterol fed rabbits (Gp.HCD) as compared with regular diet group of rabbits (Gp.R) (Fig. 3 right). CL signals from vascular tissue with endothelium showed a decrease in Gp.NK as compared with HCD group. It means that the amount of O_2^- released is greater in aorta from HCD group than in those from pitavastatin group. In aorta without endothelium, CL signals were decreased in pitavastatin treated rabbits as compared to cholesterol fed rabbits (Fig. 3 right). The endothelium dependent chemiluminescence was drastically decreased in pitavastatin treated group. In other words, the relative difference of aortic O_2^- generation between HCD group and pitavastatin group was higher in the part of endothelium, and pitavastatin treatment decreased O_2^- generation more in endothelium than that in components of vessels other than endothelium. The amount of O_2^- released decreased in Gp.NK compared with in Gp.HCD.

3.7. Immunohistochemical study

Immunohistochemical analyses demonstrated a significant decrease in the number of macrophage derived cells in the atherosclerotic lesions in pitavastatin treated rabbits as compared to those from HCD group (Fig. 4). At the same time, the number of smooth muscle derived cells in atherosclerotic lesions of pitavastatin treated rabbit aortae tended to be decreased without statistical significance (data not shown). Pitavastatin treatment not only reduced the area of atherosclerosis, but also decreased the area stained by the macrophage antibody, the area stained by the iNOS antibody, and the areas positive for $ONOO^-$ established by nitrotyrosine staining. MMP-1 (interstitial collagenase), a matrix metalloproteinase that initiates collagen degradation, was localized predominantly in macrophages. The expression of MMP-1 and MMP-2 decreased in the pitavastatin treated group compared with that of Gp.HCD.

4. Discussion

Epidemiological studies have shown that lipid lowering therapy with statins such as simvastatin leads to a significant reduction in cardiac mortality and morbidity [2–5]. Atorvastatin was also shown to reduce the progression of coronary atherosclerosis through its strong lipid lowering action. In this experiment, we tried to investigate the anti-atherosclerotic effect of pitavastatin, a newly developed

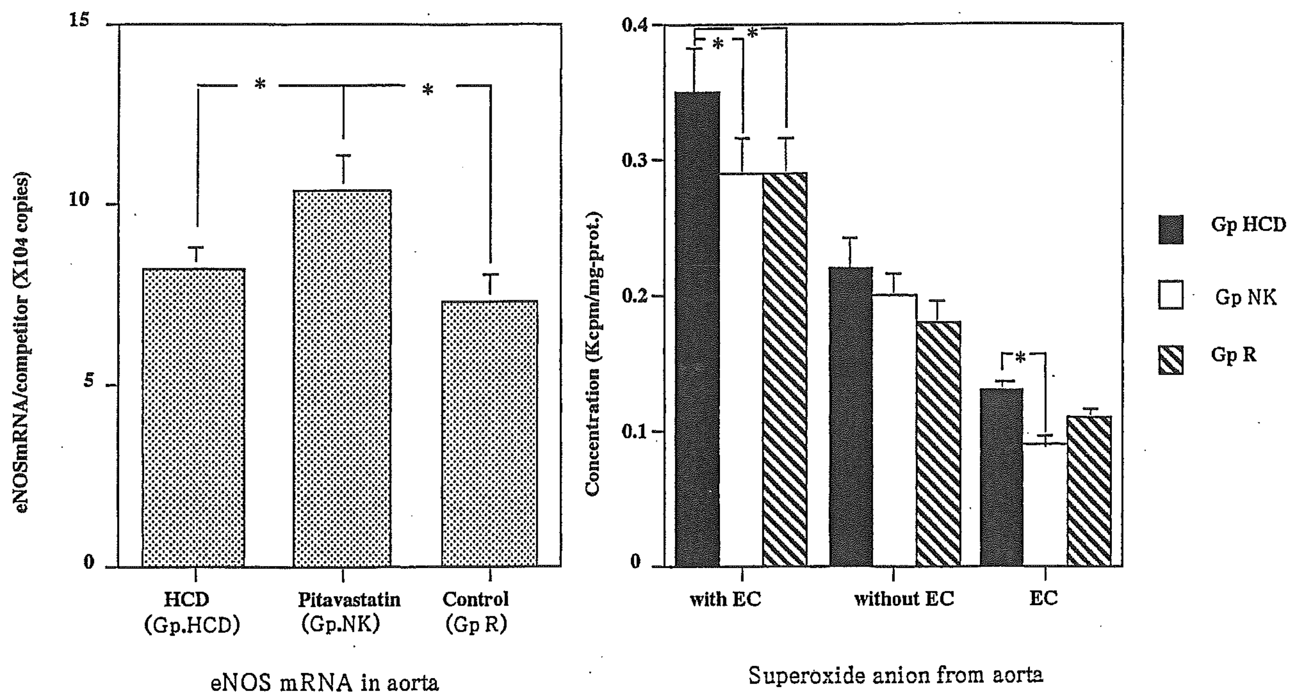

Fig. 3. Left: Quantification of eNOS mRNA in aortic arch using competitive RT-PCR. Ethidium bromide-staining gel after electrophoretic resolution of the competitor (558 bp) and eNOS target bands (486 bp) were determined. Relative amounts of eNOS mRNA to competitors using densitometry was shown. Data are shown as means ± S.E.M. Significant difference (* $P < 0.05$). Right: Effects of Pitavastatin on superoxide production from rabbit aortae. 'Endothelium+' means the data of vessel with endothelium. 'Endothelium−' means the data of vessel without endothelium.

statin, on aorta by selecting dose of pitavastatin which was reported to be comparable to the dose used in humans [6]. Attention has recently been focused on the molecular mechanisms responsible for these effects of statins, as well as their lipid lowering action. The present study therefore focuses on the status of endothelial functions, especially NO related, as determined by vascular responses. We measured nitric oxide metabolites, cGMP concentration, and eNOS mRNA expression in oophorectomized rabbits with or without pitavastatin treatment. In addition, we examined the O_2^- generation in the vessels with or without endothelium, immunohistochemistry related to peoxynitrite, matrix metalloprotease and apoptosis, and atherosclerotic lesions of hypercholesterolemic rabbits with or without pitavastatin treatment.

The HMG-CoA reductase inhibitors are potent inhibitors of cholesterol biosynthesis [1], decreasing serum cholesterol level by blocking the hepatic conversion of HMG-CoA to L-mevalonate in cholesterol biosynthetic pathway [1]. In the present study, serum cholesterol level was significantly decreased whereas no difference was observed in TG and HDL cholesterol (Table 1). This lipid lowering effect of pitavastatin in high cholesterol induced atherosclerotic rabbit model may be stronger than that of fluvastatin or simvastatin which we did previously, and the data is compatible with clinical data reported in Japan [18–20]. On the other hand, there are few reports concerning the effect of atorvastatin or lovastatin where the lipid lowering effects in rabbits were reported to be stronger than simvastatin or fluvastatin in hyperlipidemic patients. As these statins have inhibited HMG CoA reductase strongly, it may cause liver damage in rabbit. Pitavastatin was reported to have stronger LDL receptor induction in liver, however weaker HMG-CoA reductase inhibition than atorvastatin or simvastatin [21].

The EDRs were impaired in animals with experimentally induced atherosclerosis, which has been correlated to the decreased biological activity of endothelium derived NO [9,13]. The present investigation shows that endothelium dependent nitric oxide mediated relaxation in response to acetylcholine and calcium ionophore. A23187 and tone-related basal NO release evaluated by L-NMMA contraction were improved significantly by pitavastatin treatment (Fig. 2). The improvement of endothelial function by statin is often attributed to the reduction in serum cholesterol concentration. Indeed, a study demonstrated that a single treatment of LDL apheresis is sufficient to significantly improve EDRs in hypercholesterolemic humans [22]. Further, tissue cGMP concentration in aorta was also increased by pitavastatin treatment (Fig. 3). NO activates vascular smooth muscle soluble guanylate cyclase, thereby increasing cGMP in turn responsible for decreased intracellular Ca^{2+} concentration. The increased cGMP concentration clearly indicates that the increased production and bioavailabity of NO. In other words, increase of cGMP and greater contraction of aorta in response to L-NMMA shows increase of the basal

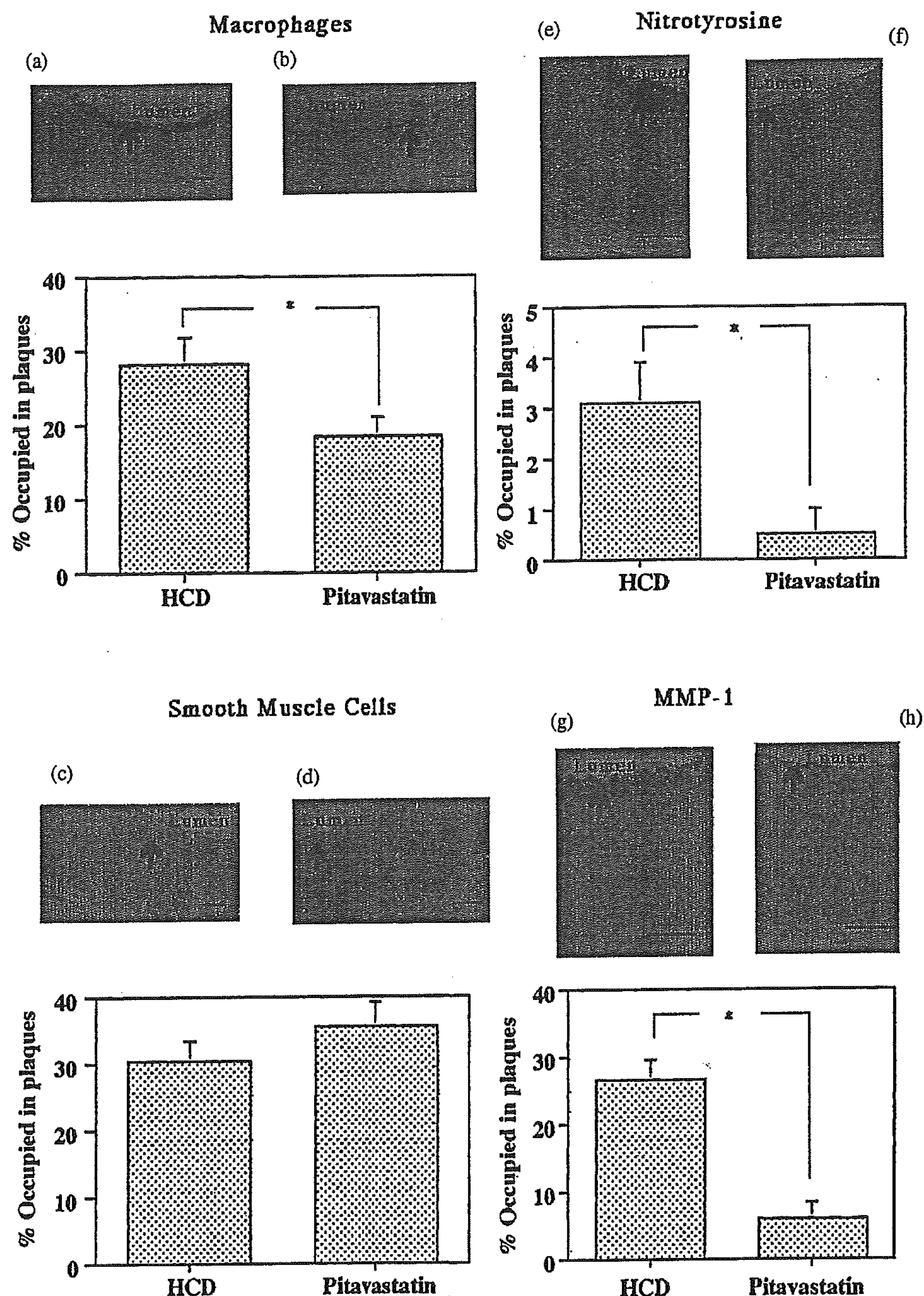

Fig. 4. Photograph: Immunohistochemical analysis of the thoracic aortae of rabbits from the HCD group and NK group (pitavastatin) using macrophages (a, b), smooth muscle cells (c, d), nitrotyrosine, a marker of peroxynitrite (e, f) and MMP-1 (g, h). Original magnification: 100× (a, b, c, d); 200× (e, f, g, h). Bar is 50 μM. Graph: % occupied area in atherosclerotic plaque by macrophages, smooth muscle cells, nitrotyrosine and MMP-1 evaluated by immuno-histochemistry.

release of NO. We hypothesized two mechanism of this improved NO bioavailability.

The eNOS mRNA expression was increased significantly in the aorta of pitavastatin treated rabbits (Fig. 3). This result is compatible with the observation that eNOS mRNA expression was increased in simvastatin treated cultured endothelial cells without changing lipid sub-fraction in the medium [23], and that the eNOS mRNA expression was increased by the stabilization of mRNA, not by the stimulation of transcription [23]. eNOS upregulation and inhibition of iNOS induction by statin were also reported [24]. We have also observed the increased expression of eNOS mRNA and protein in pitavastatin treated cultured bovine aortic endothelial cells and that it was also mediated by the stabilization of eNOS mRNA (data not shown). The increased expression of eNOS mRNA attributes increased NO synthesis. In endothelial cells, eNOS protein is translocated to the caveolae for myristoylation and palmitoylation. Our preliminary experi-

ment based on immunohistochemical study showed that the majority of eNOS protein exists in cytoplasm of endothelial cells in atherosclerotic lesions of cholesterol diet fed rabbits whereas almost all of eNOS exist in membranous part of aortic endothelial cells of regular diet fed rabbits (data not shown). The eNOS mRNA was increased by cholesterol diet in this study and recent other studies [16]. Taken together, we speculated the possibility that eNOS activity was regulated by both mRNA level and location of protein in cells.

The other mechanism of improved NO bioavailability is the decrease of O_2^- production. The oxidative inactivation of NO is regarded as an important cause of its decreased biological activity. O_2^- reacts with NO faster than SOD and forms peroxynitrite anion [25]. The peroxynitrite anion oxidizes sulfhydryls groups and yields products indicative for hydroxyl radical reaction with deoxyribose and dimethyl sulfoxide. These reactions induce membrane lipid peroxidation, to stimulate progression of vascular atherosclerosis. The presence of peroxynitrite-derived nitrotyrosines in atherosclerotic lesions has been demonstrated in our previous study in rabbit models [26]. The vascular release of superoxide was increased significantly in hypercholesterolemia and atherosclerosis [9]. This study shows that O_2^- production was decreased in arteries by pitavastatin treatment, especially in endothelial cells. Among several oxidases, as O_2^- producing enzymes, three are possible candidates in the release of O_2^- from endothelial cells. In the hypercholesterolemic rabbit, increasing serum activity of xanthine oxidase release increased amounts of O_2^- [27]. Recently, NO was reported to inhibit, in vitro [28], xanthine oxidase and xanthine dehydrogenase, which are present in endothelial cells. NADPH oxidase exists in culture endothelial cells and smooth muscle cells activated by TNF-α, and its activity is increased in hypercholesterolemia [29]. eNOS was also one of the candidates, because it was reported to release O_2^- in diabetic vessels [30]. Preliminarily, our data have shown that pitavastatin decreases O_2^- from NADPH oxidase in endothelial cells, and eNOS did not release O_2^- in high-cholesterol diet induced atherosclerosis (data not shown). However, we have to consider that statins may have a potential effect on superoxide production by mitochondria, considering the potential effects of statins on the metabolism of CoQ10 [31]. Coenzyme Q can undergo oxidation/reduction reactions in other cell membranes such as lysosomes, plasma membranes; deficiency of coenzyme Q has been described based on failure of biosynthesis by statins [31].

Nitroglycerin mediated endothelium independent relaxation is also improved by pitavastatin treatment. We speculated that it was due to the retardation of atherosclerosis formation by pitavastatin. However it is possible that pitavastatin has some effect on smooth muscle cell sensitivity to NO. It maybe necessary to elucidate more to understand the underlying mechanism.

Accumulation of macrophages in the vascular wall might be responsible for a variety of pathological events, such as generation of superoxide radicals, oxidation of LDL, subsequent foam cell formation, and release of cytokines, resulting in smooth muscle cell proliferation, and migration. The present investigation depicts the decreased number of macrophages in the intima following pitavastatin treatment. It may be due to the prevention of macrophages adhesions and migration by increasing NO bioavailability.

To determine other mechanisms of the anti-atherosclerotic effect of pitavastatin, we investigated the proportion of MMP-1 and 2 positive areas. The interstitial collagenase (matrix metalloproteinase-1, MMP-1) and MMP-2 expression in the lesion were measured by quantitative image analysis [32]. MMP-1 is localized predominantly in the macrophages and that plays a key role in initiating collagen degradation. The baseline lesions in the HCD group expressed high levels of MMP-1. Macrophage-related proteolysis might contribute to weakness of the protective fibrous cap of the plaque (Fig. 4). A reduction of both the macrophage content and the expression of immunoreactive MMP-1 were observed in aortae from pitavastatin treated rabbits. Immunoreactive MMP-2 showed the same tendency. This suggests that pitavastatin treatment plays a major role in plaque stabilization. Conclusively, the present study demonstrates that pitavastatin safely reduces plasma cholesterol level in high-cholesterol diet induced atherosclerosis, and that the anti-atherosclerotic effect of pitavastatin is mediated at least partly by increasing endothelium dependent vascular responses, eNOS mRNA expression, cGMP level and decreasing superoxide anion production. The antiatherosclerotic property of pitavastatin is due to two major pathways: one is due to its pleiotropic effect, such as improvement of endothelial function; the second stems from its lipid lowering effect. Although suggest the experiment was carried out in rabbits, the results suggest new possibilities of the usefulness of pitavastatin in cases of atherosclerosis, due to its NO bioavailability.

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Gene transfer of endothelial NO synthase, but not eNOS plus inducible NOS, regressed atherosclerosis in rabbits

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Abstract

The effects of in vivo gene transfer of endothelial nitric oxide synthase (eNOS) and inducible NOS (iNOS) genes on severe atherosclerosis were investigated in rabbits. The recombinant adenoviruses, Ad.eNOS and Ad.iNOS, which respectively express eNOS and iNOS, were constructed. Atherosclerosis was induced by a balloon injury followed by a high cholesterol diet for 12 weeks. The rabbits were divided into six groups: Gp cont (no treatment); Gp null (adenovirus sham-infected); Gp eNOS (Ad.eNOS); Gp iNOS (Ad.iNOS); Gp e+i (Ad.eNOS plus Ad.iNOS); and Gp heNOS (a high dose of Ad.eNOS). Examinations were carried out 7 days after gene transfer. Plasma lipid levels were not significantly changed, but transfection with Ad.eNOS (Gp eNOS and Gp heNOS) decreased the tissue cholesterol concentration and regressed atherosclerotic lesions. Vessels treated with Ad.iNOS (Gp iNOS and Gp e+i) showed iNOS staining in the atheroma, and slight staining at other parts of the vessels; those treated with Ad.eNOS showed eNOS staining in the endothelium and subintima, and slight staining at other parts. Ad.eNOS transfection, but not Ad.iNOS or Ad.eNOS+Ad.iNOS transfection, improved the impaired aortic endothelium-dependent relaxation (EDR) and basal NO-dependent response, increased tissue cyclic GMP (cGMP), and decreased the release of O_2^- from vessels. eNOS treatment showed a decreasing tendency in regions with peroxynitrite staining, MMP1 staining, and suspected apoptosis. In conclusion, in vivo gene transfer of eNOS, but not iNOS or eNOS plus iNOS, regressed atherosclerosis. The relations among NO, O_2^-, and peroxynitrite may be critical, and lipid resorption from the lesions may be responsible for the regression.
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Keywords: Atherosclerosis; Nitric oxide; Gene theraphy

1. Introduction

The atherogenic process is characterized by an early deficit in nitric oxide (NO) and related biomolecules [1,2]. Chronic inhibition of NO in addition to a high cholesterol diet has been shown to induce severe atherosclerosis [3]. These data suggest that NO has direct effects on the progression of atherosclerosis. In fact, NO has anti-atherosclerotic effects, because it can inhibit monocyte adhesion to endothelium, smooth muscle cell chemotaxis, and proliferation [4]. It has been speculated that improving NO bioavailability, which in turn helps to restore the impaired endothelium-dependent relaxation (EDR) in atherosclerotic vessels, is important to stabilize atheroma and regress atherosclerotic lesions—two important goals in the treatment of atherosclerosis. On the other hand, removing dietary cholesterol has not been consistently shown to restore EDR [5,6]. In addition, clinical and experimental trials have failed to show sufficient regression of atheroma by dietary cholesterol reduction [5,7]. Even if such a regression occurs, it is

Abbreviations: NO, Nitric oxide; NOS, Nitric oxide synthase; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; NTG, nitroglycerin; EDR, endothelium-dependent relaxation; L-NMA, N^G-monomethyl-L-arginine acetate, inhibitor of NO synthase; ACh, acetylcholine chloride; SOD, superoxide dismutase; PGF2α, prostaglandin F2α.

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expected to take years to obtain a partial regression of atherosclerosis in humans, although such a regression occurs quickly in animals [5,7]. We speculated that vascular gene transfer of nitric oxide synthase (NOS) isoforms via adenoviruses might be effective to hasten the process of NO-induced regression of the atheroma and lesions associated with atherosclerosis. Nabel et al. [8] successfully transfected genes in vivo by using a double-balloon catheter in a pig ileofemoral artery. Ooboshi et al. [9] showed that ex vivo endothelial nitric oxide synthase (eNOS) gene transfer improved the impaired EDR, and Leyen et al. [10] reported that in vivo eNOS gene transfer inhibited neointimal vascular lesions in rabbits. However, the effects of inducible NOS (iNOS) on the vascular function and the regression of atherosclerosis remain a subject of controversy [11,12]. In this study, we first ensured the successful regression of severe atherosclerosis by eNOS gene transfer. To speculate on the dose-dependency of the transferred NOS, we prepared two doses of Ad.eNOS vectors. To determine whether or not iNOS gene transfer is beneficial, we compared the effects of in vivo gene transfer of eNOS and iNOS in a model of advanced atherosclerosis with vascular dysfunction. We investigated the exact role played by iNOS when combined with eNOS gene transfer to confirm its synergistic effect with eNOS gene transfer.

2. Materials and methods

2.1. Chemicals

Acetylcholine chloride (ACh), prostaglandinF2α (PGF2α), indomethacin, and N^G-monomethyl-L-arginine acetate (L-NMA) were purchased from Sigma (St. Louis, MO). Nitroglycerin (NTG) was obtained from Nihon Kayaku (Tokyo, Japan). Monoclonal antibodies against eNOS and iNOS (Transduction Laboratories, Lexington, KY), rabbit macrophages (RAM11), muscle actin-specific one (HHF35) (DAKO, Carpinteria, CA), nitrotyrosine (Upstate Biotechnology, Lake Placid, NY), MMP-1 (Fuji Yakuhin, Takaoka, Japan) and β galactosidase (Chemicon International, Lexington, NY) were also used [6,13].

2.2. Construction and purification of recombinant adenovirus

Recombinant adenoviruses containing eNOS cDNA and iNOS cDNA were constructed using an ADENO-QUEST Kit (Quantum, Quebec, Canada) [14]. Briefly, bovine eNOS cDNA (provided by Dr. T. Michel, Harvard University) and rat iNOS cDNA (provided by Drs. H. Esumi and Y. Ogura, National Cancer Center, Tokyo) were cloned into the AdBM5pAG vector. The resulting plasmid was co-transfected with viral DNA into 293 cells. For all of the studies, the viral titers were adjusted to 2×10^9 pfu/ml. Adenoviruses carrying an *Escherichia coli* Lac Z gene encoding a nucleus-localized variant of β galactosidase (Ad. β gal) or no cDNA (Ad.null) were also used. To investigate the expression of eNOS and iNOS in normal and atherosclerotic aorta, we separately prepared four rabbits (two animals were fed with a regular diet, and other two animals were fed with 0.5% cholesterol and regular diet for 12 weeks). Gene transfer of eNOS and iNOS was performed using dispatch catheter as described in Section 2.3 into abdominal aorta in regular diet and high cholesterol diet groups of rabbits. Animals were sacrificed at day 4 for the detection of the transgene [14].

2.3. Animals

Male New Zealand white rabbits (age, 3–4 months; weight, 2.0–2.4 kg) were obtained from Kitayama Rabbis (Ina, Japan) and housed with free access to water. The abdominal aortae were injured by means of a balloon catheter as described previously [15]. In brief, a 3Fr Fogarty catheter was inserted from right femoral artery and advanced as far as the just below diaphragm. The balloon was inflated with 0.6 ml of saline and the catheter pulled three times until reaching a portion of the bifurcation of iliac arteries. Finally, the balloon was deflated and the catheter withdrawn. After aortic injury, atherosclerosis was induced by feeding regular chow plus 0.5% cholesterol for 12 weeks. The animals were divided into six groups ($n = 8$ each): Gp cont (no treatment); Gp null (treated with Ad.null); Gp eNOS (Ad.eNOS); Gp iNOS (Ad.iNOS); Gp e+i (Ad.eNOS plus Ad.iNOS); and Gp heNOS (high dose of Ad.eNOS). In an additional experiment, the rabbits were treated with Ad. β gal, Ad.eNOS plus Ad.null, or Ad.iNOS plus Ad.null after aortic injury and feeding with regular chow plus 0.5% cholesterol for 12 weeks ($n = 6$, each). As an additional control, six rabbits were fed regular chow for 12 weeks, with neither aortic injury nor cholesterol supplementation (Gp regular control). In all experimental trials, transfection of genes into the arteries was done using a 3.2 Fr. dispatch catheter as follows (Boston Scientific, Boston, MA). Under anesthesia with pentobarbital (20 mg/kg intravenously), a catheter was advanced into the abdominal aorta to facilitate intramural drug delivery via longitudinal strips (about 3.5 cm) of six raw-profile 0.254-mm injector plots capable of penetrating delivery. Inflation of the balloon at 6 atm was followed by injection of 0.3 ml (Gp cont, Gp null, Gp eNOS and Gp iNOS), 0.6 ml (Gp e+i) or 3 ml (Gp heNOS) of viral solution over a period of 3 min. In an additional experiment, the viral injection volume was 0.3 ml (β gal treatment group) or 0.6 ml (Ad.null plus Ad.eNOS treatment group or Ad.null plus Ad.iNOS treatment group). All experiments were conducted in accordance with the institutional guidelines for animal research, and conforms with the Guide for the Care and Use of