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Endothelial Constitutive Nitric Oxide Synthase Protein and mRNA Increased in Rabbit Atherosclerotic Aorta Despite Impaired Endothelium-Dependent Vascular Relaxation

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Atherosclerotic arteries are well known to exhibit impaired endothelium-dependent relaxation (EDR), but the exact mechanism of this impairment remains unclear. Recently, endothelial constitutive nitric oxide synthase (ECNOS), which generates nitric oxide and mediates EDR, was cloned, and ECNOS mRNA expression was reported to be modified by various cytokines, lipoproteins, and shear stress. To investigate the expression of ECNOS mRNA and protein in atherosclerotic arteries with impaired EDR, thoracic aortas isolated from Watanabe heritable hyperlipidemic (WHHL) rabbits were examined by using in situ hybridization and immunohistochemistry. Compared with thoracic aortas from Japanese White rabbits, WHHL aortas exhibited significantly impaired EDRs, although both in situ hybridization and immunohistochemistry exhibited enhanced expression of ECNOS mRNA and protein in WHHL aortas. There was no significant relationship between expression of ECNOS mRNA and protein of endothelial cells and age of the examined WHHL aortas. These data suggest that the mechanism of impaired EDR in atherosclerotic arteries is not due to the decrease in ECNOS mRNA and protein. (Am J Pathol 1996, 148:1949–1956)

Alterations in the endothelium-dependent relaxation (EDR)[1] play an important role in the pathophysiology of many cardiovascular disorders. Many *in vivo* and *in vitro* studies showed that atherosclerotic arteries exhibited impaired EDR,[2–6] and decreased production[7,8] or increased inactivation[9] of the endothelium-derived relaxing factor (EDRF), a substance responsible for EDR, has been suggested as its mechanism. However, the exact mechanism of the impairment is controversial. Recent investigations revealed that EDRF is either nitric oxide (NO)[10] or a nitrosylated compound[11] and that NO is synthesized from the guanidino nitrogen of L-arginine via an action of NO synthase.[12] NO synthase was purified and cloned from various tissues including brain,[13,14] activated macrophages,[15,16] activated smooth muscle cells,[17] and endothelial cells.[18,19] Previous studies revealed that the expression of endothelial constitutive NO synthase (ECNOS) mRNA was modulated by shear stress,[19] cytokines,[20] transforming growth factor-β,[21] and estrogen[22] *in vitro*. However, there are no reports about ECNOS expression in atherosclerotic arteries with impaired EDR, although the reduction of ECNOS in the endothelium of atherosclerotic vessels is speculated to explain impaired EDR. The purpose of this study is to elucidate whether ECNOS protein and mRNA are decreased in atherosclerotic arteries with impaired EDR. We employed immunohistochemistry and *in situ* hybridization to assess the expression of protein and mRNA of ECNOS in aortas from Watanabe heritable hyperlipidemic (WHHL) rabbits and isometric contraction experiments to quantify their EDR.

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Materials and Methods

Animals

As an atherosclerotic group, homozygous male WHHL rabbits, aged 11 to 25 months (n = 5; average, 16 ± 2.3 months), were used. As controls, age-matched normal male Japanese White rabbits were studied (n = 5). All animals were fed standard rabbit chow (Oriental Yeast, Tokyo, Japan).

Preparation of Tissue

The rabbits were sacrificed by an overdose of pentobarbital sodium (80 mg/kg, intravenous), and the descending thoracic aorta was isolated and cleaned of surrounding tissue. The aortic segments for isometric contraction experiments were isolated from the adjacent portions of those for pathological analysis. For *in situ* hybridization assays and pathological examinations, tissues were fixed in 0.1 mol/L phosphate buffer (pH 7.4) containing 4% paraformaldehyde, and cross sections of tissues were embedded in paraffin. Tissues were cut at 6 μm and air dried onto slides that were coated with 3-aminopropyltriethoxysilane (Shin-Etsu Chemical Co., Tokyo, Japan). For immunohistochemistry, the serial tissues were embedded in OCT compound (Ames Co., Division of Miles Laboratories, Elkhart, IN) and prepared by snap-freezing in liquid nitrogen and stored at −80°C. Serial cryostat sections (4 μm thick) were cut and air dried onto poly-L-lysine-coated slides. Additional serial paraffin and cryostat sections were stained with hematoxylin and eosin (H&E) and Verhoeff van Gieson for analysis of morphological details by light microscopy.

Isometric Contraction Experiments

Aortic rings approximately 2 mm wide were cut and opened. For isometric force measurements, transverse aortic strips were suspended in 5-ml organ baths containing Krebs' bicarbonate buffer composed of 118 mmol/L NaCl, 4.0 mmol/L KCl, 1.5 mmol/L $CaCl_2$, 1.2 mmol/L $MgSO_4$, 1.2 mmol/L NaH_2PO_4, 25 mmol/L $NaHCO_3$, and 5 mmol/L glucose and were equilibrated at 37°C with a 95% O_2/5% CO_2 gas mixture (Mizushima Oxygen, Kobe, Japan). The final pH was approximately 7.38. One end of the strip was attached to the bottom of the chamber, and the other end was attached to a Statham 4C-2 force transducer (Gould, Glen Burnie, MD), which was connected to an amplifier/recorder system (Nihon Kohden, Tokyo, Japan). An initial preload of 1.5 g was applied, and the strips were allowed to stabilize for 90 minutes. A test contraction was induced by raising KCl concentration to 40 mmol/L. When the developed tension attained its peak value, the strips were relaxed by rinsing with the buffer. Then the strips were precontracted with 0.3 μmol/L L-phenylephrine hydrochloride and subsequently relaxed by a cumulative addition of acetylcholine chloride. After washing and equilibration, the contraction-relaxation cycle was produced by a cumulative addition of nitroglycerin. Relaxation values were expressed as percent decreases of the constrictor tone induced by phenylephrine (0.3 μmol/L). For statistical analysis, the mean and standard error of the mean were calculated for all variables. Significance of difference was determined with the unpaired Student's *t*-test and $P < 0.05$ was regarded as significant. The tissues after isometric contraction experiments were fixed with 10% formalin and embedded in paraffin to confirm the preservation of endothelial cells.

In Situ Hybridization

Serial paraffin-embedded sections of rabbit aorta were hybridized with both anti-sense and sense probes. The bovine aortic ECNOS cDNA was a gift from David G. Harrison (Cardiology Division, Emory University School of Medicine).[19] To detect the ECNOS mRNA, the coding −15 to 245 area in ECNOS DNA was amplified with a primer Np-1 (5′- ATAGAATTCACCAGCACCTTTGGGAATGGCGAT) and a Cp-1 (5′- ATAGAATTCGGATTCACTGTCTGTGTTGCTGGACTCCTT), which contains the amino-terminal myristoylation site. For the synthesis of digoxigenin-labeled ECNOS-specific riboprobe, the 254-bp *Eco*RI- and *Bam*HI-digested amplified DNA was subcloned into *Eco*RI- and *Bam*HI-digested pGEM-4Z plasmid. After linearization with either *Eco*RI or *Bam*HI, both sense and anti-sense labeled RNA were generated in the presence of digoxigenin-labeled UTP as runoff transcripts with T7 and SP6 RNA polymerase, respectively. To confirm the preservation of mRNA on the studied specimen, we also examined the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. To detect GAPDH mRNA, the 124-bp *Apa*I-*Alu*I fragment of GAPDH cDNA[23] was subcloned into the *Apa*I-*Sma*I-digested pBluescriptII plasmid. This plasmid was linearized with *Bam*HI and *Kpn*I and transcribed with T7 and T3 RNA polymerase to generate anti-sense and sense riboprobes, respectively.

The slides were deparaffinized and dehydrated in graded ethanol. The sections were rehydrated in 0.1

mol/L phosphate buffer for 5 minutes and pretreated by 20 μg/ml proteinase K in phosphate buffer at 37°C for 15 minutes. After treatment with 0.2 N HCl, slides were washed in 0.1 mol/L triethanolamine buffer (pH 8.0) for 5 minutes and then in 0.1 mol/L triethanolamine buffer containing 0.25% (v/v) acetic anhydride for 10 minutes. The sections were dried and hybridized for 16 hours at 50°C with the riboprobe in a humidified chamber. After hybridization, coverslips were removed in 5X standard saline citrate (SSC) at 50°C, and slides were washed in 2X SSC containing 50% (v/v) formamide for 30 minutes at 50°C and 2X SSC for 30 minutes at 50°C. Finally, slides were washed in 0.2X SSC at 50°C for 20 minutes twice. To block nonspecific immunoglobulin-binding sites, the slides were incubated with 0.1 mol/L phosphate buffer containing 0.1% bovine serum albumin at room temperature for 60 minutes and washed in buffer containing 100 mmol/L Tris-HCl, 150 mmol/L NaCl. To detect the digoxigenin-labeled riboprobe, the sections were incubated with alkaline-phosphatase-conjugated anti-digoxigenin polyclonal antibody (Boehringer Mannheim, Penzberg, Germany) for 30 minutes and incubated with nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolylphosphate for 4 hours at 37°C. The slides were counterstained with hematoxylin.

The hybridization with the sense probe and omission of both the antisense probe and the anti-digoxigenin antibody were used as negative controls. In addition, we examined the following negative controls of *in situ* hybridization to confirm the positive signal specific to ECNOS mRNA: 1) tissues pretreated with ribonuclease A (20 μg/ml) for 2 hours at 37°C before hybridizing with the antisense probe and 2) aortas with endothelial cells that were denuded by cotton swab before fixing in 4% paraformaldehyde. Before hybridization, the specificity of the probes was confirmed by competition studies with a 100-fold excess of unlabeled probe.

Immunohistochemistry

The murine monoclonal antibody against ECNOS derived from bovine aortic endothelial cells was kindly provided by Jennifer Pollock (Vascular Biology Group, Abbott Laboratories, Abbott Park, IL).[24] The serial sections of aortic specimens were fixed in acetone at −20°C for 5 minutes. Nonspecific immunoglobulin-binding sites were blocked with 10% (v/v) bovine serum albumin diluted in phosphate-buffered saline. The sections were incubated overnight at 4°C in a humidified environment with the primary antibody against ECNOS (1/200 dilution), a rabbit macrophage-specific monoclonal antibody (RAM 11; DAKO Japan, Tokyo, Japan), α- and γ-actin-specific monoclonal antibodies (HHF-35; DAKO Japan), and a factor-VIII-related antigen-specific monoclonal antibody (vWF; DAKO Japan). To visualize the areas of staining with the first antibody, the anti-mouse IgG-horseradish peroxidase system (DAKO Japan) with diaminobenzidine was used. Murine nonimmune IgG as the primary antibody was used as negative control. Some sections were counterstained with methyl green or hematoxylin. Omission of primary antibodies in the peroxidase systems served as additional negative controls.

Evaluation of in Situ *Hybridization and Immunohistochemistry*

Area and strength of staining in *in situ* hybridization and immunohistochemistry were assessed by two observers (K. Kanazawa and S. Mikami) independently and blindly at a ×400 magnification and confirmed by the third observer (Y. Hayashi). There was no significant difference among the assessment by the three. The degrees of positivity of the *in situ* hybridization reaction and immunoreactivity were semiquantitatively graded as 0 for absence of staining and 1+ for weak, 2+ for moderate, and 3+ for strong staining.

Results

Isometric Contraction Experiments

Figure 1A shows the dose-response relationship for acetylcholine in control and WHHL rabbits. All relaxations were unaffected by pretreatment with indomethacin (10 μmol/L) and therefore not induced by the release of cyclo-oxygenase products. Acetylcholine-induced EDR was diminished in WHHL rabbit aortas, and the maximal relaxation was also decreased (control *versus* WHHL; 92 ± 3.1% *versus* 26 ± 5.5%, respectively; $P < 0.01$). There was no difference in nitroglycerin-induced endothelium-independent relaxation between control and WHHL rabbits (Figure 1B).

Histological Analysis of Aortas from Control and WHHL Rabbits

All aortas from WHHL rabbits exhibited atherosclerotic changes that consisted of raised fibro-fatty plaque. In the non-plaque lesions, subendothelial lipid accumulations were occasionally observed. In

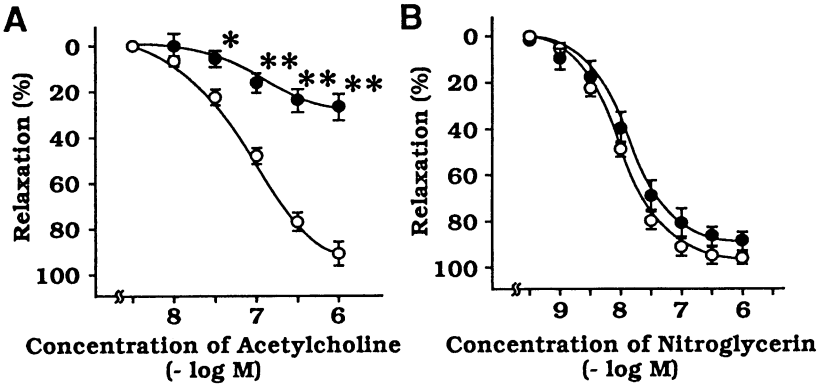

Figure 1. *Concentration-response relationships for acetylcholine* (A) *and nitroglycerin* (B) *in aortic strips from control* (○, *n* = 11) *and WHHL rabbits* (●, *n* = 11). *The 100% level represents the levels of muscle tension induced by 0.3 μmol/L phenylephrine. Concentrations of acetylcholine and nitroglycerin are expressed as a negative logarithm.* *P < *0.05;* **P < *0.01* versus *control value.*

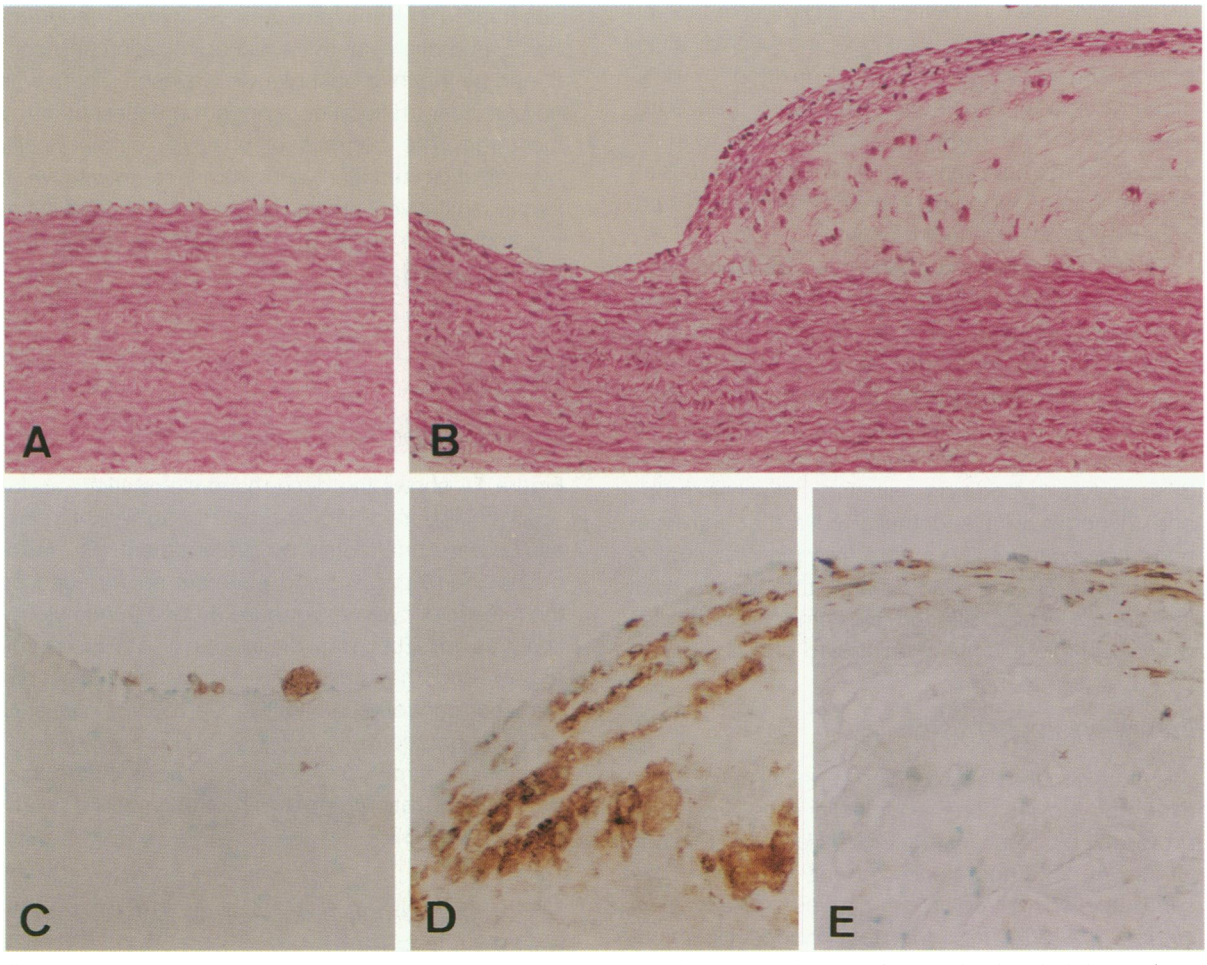

Figure 2. *Light micrographs of sections stained with H&E and immunostained with anti-macrophage-specific monoclonal antibody (RAM 11) and anti-α- and anti-γ-actin-specific monoclonal antibody (HHF 35) in aortas from control and WHHL rabbits.* A: *Aorta from a control rabbit stained with H&E. No atherosclerotic changes were observed. Magnification, ×8.8.* B: *Aorta from a WHHL rabbit stained with H&E. A fibro-fatty plaque was observed. Magnification, ×8.8.* C: *At the non-plaque lesion of WHHL rabbit aorta, staining of RAM 11 was detected only in the minimal thickened intima. Magnification, ×44.* D: *At the plaque lesion, staining of RAM 11 was observed in macrophage-form cells of fibro-fatty plaque. Magnification, ×44.* E: *Staining of HHF 35 was detected only in the thin fibrous cap of the plaque. Magnification, ×44. All immunostained specimens were counterstained with methyl green.*

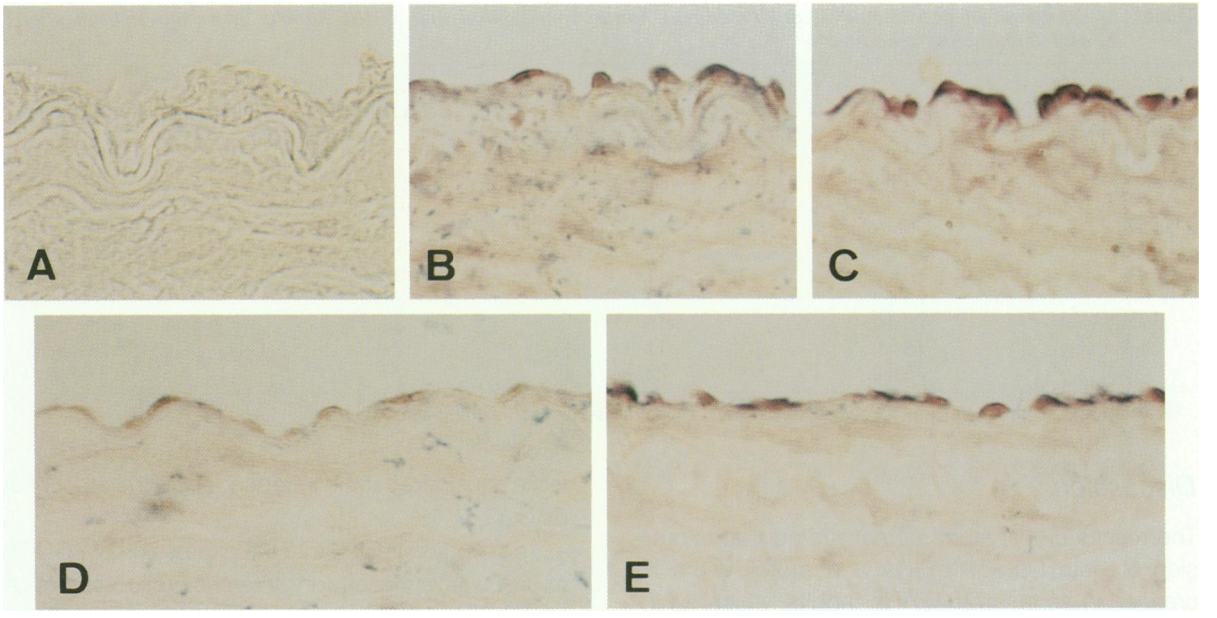

Figure 3. *Light micrographs of cells containing ECNOS mRNA and GAPDH mRNA in aortas from control and WHHL rabbits.* A: *No hybridization with the ECNOS sense probe was observed in aortic endothelial cells from WHHL rabbits.* B *and* C: *Expression of GAPDH mRNA was observed in the endothelial cells and smooth muscle cells in a control rabbit* (B) *and a WHHL rabbit* (C). D: *The positive staining with the ECNOS anti-sense probe was detected within the endothelial cell in a control rabbit aorta.* E: *The positive hybridization signal for the ECNOS anti-sense probe within endothelial cells in the WHHL rabbit aorta was significantly enhanced compared with that in a control aorta. No counterstain was used. Magnification, ×88.*

the plaque lesion, cholesterol clefts, ground substance, and a thin fibrous cap were observed (Figure 2B), but neither calcification nor intramural neovascularization was detected. The endothelial cell lining was preserved in all specimens. Aortas from control rabbits had no atherosclerotic changes (Figure 2A). In immunohistochemical analysis, no positive staining of RAM 11 was detected in the intimal lesion of aortas from control rabbits. The positive staining of RAM 11 was observed in the minimally thickened intima (Figure 2C) in non-plaque lesions as well as in atherosclerotic plaques (Figure 2D) of aortas from WHHL rabbits. Positive staining of HHF 35 was detected in the thin fibrous cap of atherosclerotic plaques and the medial layer (Figure 2E).

ECNOS mRNA Expression in Aortas from Control and WHHL Rabbits

The specificity of the *in situ* hybridization reaction with the antisense probe was analyzed by various methods. 1) The use of the sense probe resulted in a background hybridization signal (Figure 3A). 2) Ribonuclease A pretreatment before hybridization and denudation of endothelium before fixing abolished the positive hybridization signal with the antisense probes. 3) Competition experiments with 100-fold unlabeled probes led to a marked decrease in the hybridization signal. The hybridization signal of GAPDH mRNA was present in endothelial cells and subendothelial smooth muscle cells in all sections of both control and WHHL rabbits (Figure 3, B and C). In control rabbits, the positive ECNOS mRNA signal was seen only in endothelial cells (Figure 3D). Endothelial cells in WHHL rabbits expressed stronger hybridization signals with the anti-sense ECNOS probe compared with those in control rabbits (Figure 3E). There was no relationship between the strength of hybridization signals of ECNOS mRNA in the aortas and age of the examined WHHL rabbits ranging from 11 to 25 months old (Table 1).

Immunoreactive ECNOS Expression in Aortas from Control and WHHL Rabbits

Sections from control rabbits expressed weak positive staining of immunoreactive ECNOS only in the endothelial cell (Figure 4A). Serial sections incubated with nonimmune IgG were entirely negative (Figure 4C). Endothelial cells in WHHL rabbits showed stronger immunoreactivity for ECNOS than those in control sections (Figure 4B). The staining areas of the ECNOS antibody corresponded with those of the vWF antibody. There was no significant variation in the degree of immunoreactivity for ECNOS in endothelial cells among the examined WHHL rabbits (Table 1).

Table 1. *Expression of ECNOS mRNA and Protein in Aortas from Control and WHHL Rabbits by* in Situ *Hybridization and Immunohistochemistry*

Number	Rabbit	Age (months)	*In situ* hybridization	Immunohistochemistry
1	Control	8	1+	1+
2	Control	10	1+	1+
3	Control	14	2+	1+
4	Control	16	1+	1+
5	Control	17	1+	1+
6	WHHL	11	3+	2+
7	WHHL	12	3+	1+
8	WHHL	15	3+	2+
9	WHHL	17	2+	1+
10	WHHL	25	2+	2+

Staining was scored as follows: 1+, weak staining; 2+, moderate staining; 3+, strong staining.

Discussion

The results of this study demonstrated that expression of both ECNOS mRNA and protein were increased in aortas from WHHL rabbits, which exhibited impaired EDR by acetylcholine, compared with those in control rabbits.

Many investigations *in vivo* and *in vitro* demonstrated that atherosclerotic arteries had impairments of EDR,[2–4,6] and several mechanisms for the impaired EDR have been proposed.[25] Previous bioassay studies showed that there was inability to generate EDRF in atherosclerotic vessels.[26] On the other hand, other studies suggested that there was an excess generation of oxygen-derived free radicals within atherosclerotic vessels.[27] These increased radicals might augment inactivation of EDRF through their diffusion in the thickened intima and thereby cause impaired EDR in atherosclerotic arteries. However, quantitative evaluation of NO levels in tissues is difficult because of its lability and reactivity, and the conclusive mechanism of impaired EDR remained unclear. We demonstrated that ECNOS itself increased in both mRNA and protein levels rather than decreased in atherosclerotic aortas. Therefore, the mechanism of impaired EDR in atherosclerotic arteries is considered to be independent of the amount of ECNOS.

This study revealed that the expression of ECNOS mRNA was enhanced in atherosclerotic vessels. A few studies of transcriptional regulation of ECNOS mRNA have been reported. Yoshizumi et al[20] reported that tumor necrosis factor-α down-regulated an ECNOS mRNA via an increased instability of mRNA. Other studies indicated that shear stress,[19] interferon-$\alpha\beta$,[28] transforming growth factor-β,[21] and estrogen[22] potentiated the expression of ECNOS mRNA *in vitro*. The expression of these cytokines is observed and shear stress is increased in atherosclerotic arteries,[29–31] and therefore they have the potential to modulate ECNOS mRNA expression.

Recently we reported that oxidized low density lipoprotein (LDL) up-regulated the expression of ECNOS mRNA and protein *in vitro*, whereas native LDL did not change them.[32] The presence of oxidized LDL in atherosclerotic lesions has been demonstrated in the atherosclerotic rabbit aortas,[33,34] and an increase in these oxidized lipoproteins is considered to cause an accumulation of macrophages in the atherogenic process.[35] Therefore, increased oxidized LDL may serve as a key stimulus in

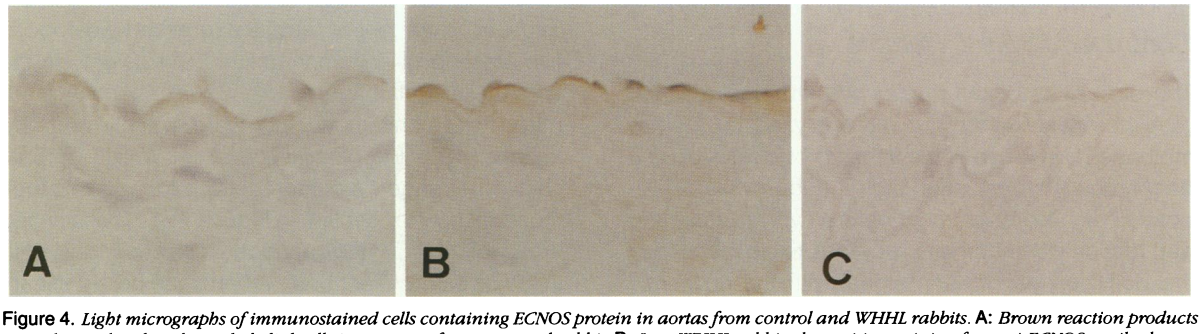

Figure 4. *Light micrographs of immunostained cells containing ECNOS protein in aortas from control and WHHL rabbits.* **A:** *Brown reaction products were detected within the endothelial cells in an aorta from a control rabbit.* **B:** *In a WHHL rabbit, the positive staining for anti-ECNOS antibody was detected only in endothelial cells. The staining in a WHHL rabbit was stronger compared with that in a control rabbit.* **C:** *There was no staining with nonimmune IgG in WHHL rabbits. All specimens were counterstained with methyl green. Magnification, ×88.*

the enhanced ECNOS expression in atherosclerotic arteries. However, conclusive mechanisms of this enhanced ECNOS expression in atherosclerotic vessels remain to be clarified.

In summary, expression of ECNOS mRNA and protein in atherosclerotic aortas was increased, accompanied by impairment of EDR. These findings suggest that the mechanism of impaired EDR is not due to a decrease of ECNOS mRNA or protein.

Acknowledgments

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