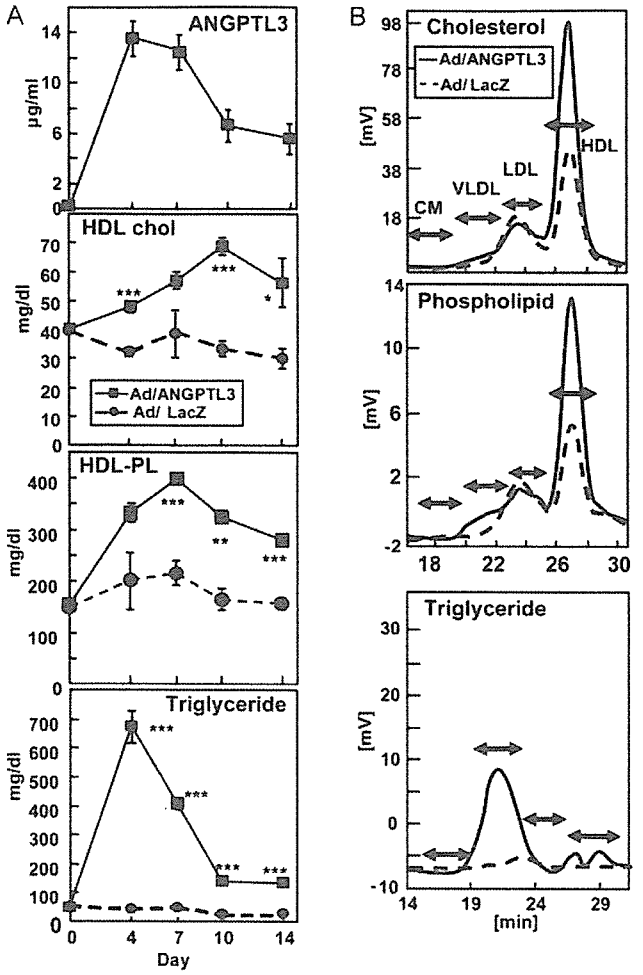

Figure 2. Alterations of plasma lipid profiles by supplementation of ANGPTL3 via adenovirus in Angptl3-deficient mice. A, Angptl3-deficient congenic C57BL/6J *Angptl3*^{hypl} mice were treated with recombinant adenoviruses carrying β-galactosidase (Ad/lacZ, circles) or human ANGPTL3 (Ad/ANGPTL3, squares). On the indicated days after the viral injection, HDL cholesterol, HDL-PL, and triglyceride concentrations in plasma were measured as described in Methods. Data are the mean±SE values of 4 mice per group. *P<0.05, **P<0.01 and ***P<0.001 vs Ad/lacZ group. B, Plasma samples were collected on day 14 from mice injected with Ad/LacZ (dotted line) or Ad/ANGPTL3 (bold line). Pooled plasma samples from each group were subjected to highly-sensitive HPLC. Cholesterol, phospholipid, and triglyceride profiles in lipoprotein fractions were determined as described in Methods. The indicated fractions are CM, chylomicron; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein.

lipase in the plasma of C57BL/6J and Angptl3-deficient mice before and after a heparin injection. Plasma phospholipase activities were slightly elevated by heparin-injection in C57BL/6 mice (100±2 versus 108±3%, Figure 4). On the other hand, in Angptl3-knockout mice, the elevation of plasma phospholipase activities by heparin-injection was marked compared with C57BL/6J mice (103±4 versus 163±19%, Figure 4). These results indicate that circulating Angptl3 should contribute to the inhibition of the phospholipase activity of EL via the heparin-binding site in vivo.

Plasma HDL Cholesterol, HDL-PL, and ANGPTL3 Levels Correlated in Humans

To date, the physiological role of Angptl3 has only been assessed in rodents. To investigate the physiological and pathological roles of ANGPTL3 in humans, we constructed an ELISA system to measure ANGPTL3 concentration in human plasma. To construct the sandwich ELISA system, mouse monoclonal antibody (45B1) and rabbit polyclonal antibody (No.1) were raised against human ANGPTL3. These antibodies specifically detected recombinant human ANGPTL3 protein (please see supplemental materials). In the sandwich ELISA system, we used the 45B1 monoclonal antibody as the first antibody and detected ANGPTL3 with HRP-conjugated No.1 polyclonal antibody. We confirmed that this sandwich ELISA system specifically detect ANGPTL3 protein in human plasma by western blotting (please see supplemental materials). Using this sandwich ELISA system, we were able to generate a linear calibration curve using serial dilutions of the recombinant human ANGPTL3 protein (please see supplemental materials).

We found that the presence of other plasma proteins in the sample hindered quantitative analysis, especially when the plasma samples were directly subjected to ELISA. This was avoided by dilution of the plasma samples by more than 1/16. Neither ethylenediaminetetraacetic acid (EDTA) nor heparin, which are anticoagulants used for collecting plasma samples, had any effect on the above measurement (data not shown). The quantifiable range of the ANGPTL3 concentration in human plasma was 50 to 800 ng/mL using our system. Furthermore, ANGPTL3 concentrations of plasma samples were stable throughout five freeze-thaw cycles (data not shown).

To investigate the significance of ANGPTL3 in lipid homeostasis in humans, we analyzed plasma lipids and ANGPTL3 concentration of Japanese healthy volunteers [n=87, mean age, 33.6±8.4 years (±SD, range, 21 to 57), male/female: 45/42] (Figure 5). This study revealed that plasma ANGPTL3 concentrations (470±122 ng/mL) correlated strongly with plasma HDL cholesterol (62±14 mg/dL; r=0.500, P<0.001) and HDL-PL levels (92±25 mg/dL; r=0.286, P=0.007), but not with plasma total cholesterol (182±33 mg/dL; r=0.169, P=0.117) or TG level (77±54 mg/dL; r=−0.125, P=0.249).

Discussion

A low level of plasma HDL has been recognized as an aspect of metabolic syndrome and is a crucial risk of cardiovascular events. Various factors have been demonstrated to influence plasma HDL-cholesterol level, including apoA1, ATP-binding cassette transporter (ABC) A1, lecithin:cholesterol acyltransferase (LCAT), PLTP, and cholesteryl ester transfer protein (CETP), etc.[20] However, to date, pathophysiological regulation of the HDL level in plasma is not completely defined. Recently, lines of study have revealed that EL is a crucial factor in determining the plasma HDL level. Overexpression of EL in mice resulted in reduced plasma HDL levels, and EL knockout mice showed significant increase of HDL levels.[5–7] In another study, injection of a neutralizing

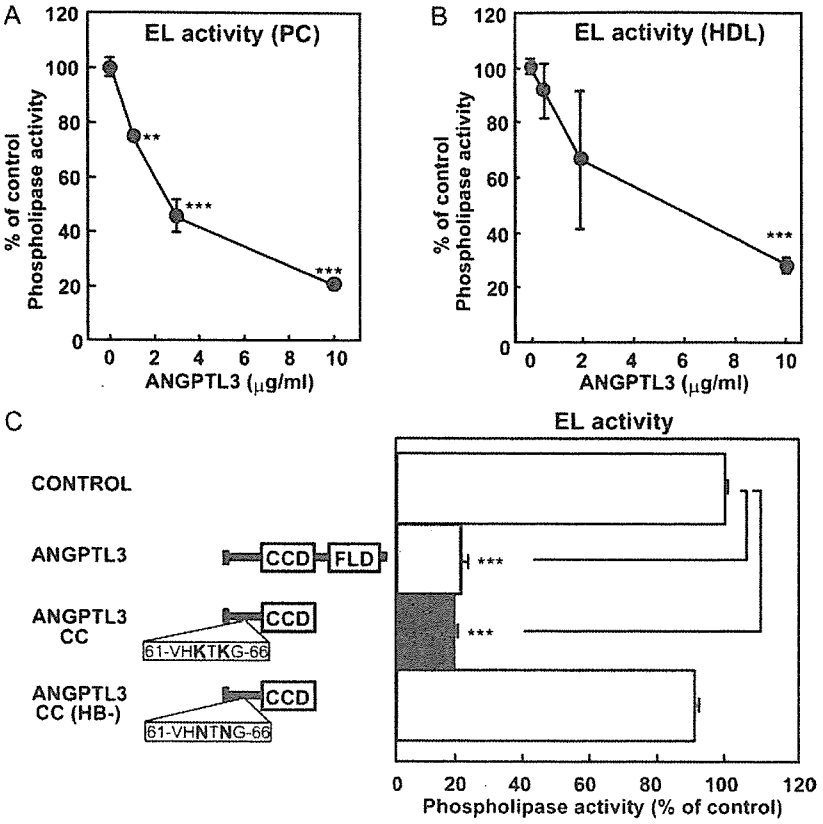

Figure 3. Inhibition of phospholipase activity of EL by ANGPTL3. A, Phospholipase activities of EL were determined using phosphatidylcholine emulsion (PC) as substrate as described in Methods, in the presence of recombinant human ANGPTL3 at the indicated doses (0, 1, 3, and 10 μg/mL). B, Similarly, they were determined using HDL particles as substrate, in the presence of the indicated dose of ANGPTL3 (0, 0.4, 2, and 10 μg/mL). Relative phospholipase activities of EL are expressed as a percentage of the value in the absence of ANGPTL3 treatment. C, The schemas indicate recombinant full-length ANGPTL3, N-terminal domain containing coiled-coil region (CCD) (ANGPTL3 CC), and the N-terminal domain containing CCD with mutation in the putative heparin-binding site [ANGPTL3 CC (HB-)]. Phospholipase activities of EL were determined in the absence of ANGPTL3 (control, open bar), in the presence of 10 μg/mL full-length ANGPTL3 (gray bar), 5 μg/mL ANGPTL3-CC (solid bar), or 5 μg/mL ANGPTL3-CC (HB-) (open bar). Relative phospholipase activities of EL are expressed as a percentage of the control. Data are the mean±SD of 3 experiments. $^{**}P<0.01$ and $^{***}P<0.001$ vs control.

antibody against EL increased plasma HDL in mice.[21] Human genetic analysis showed that a single nucleotide polymorphism (584C/T) in EL cDNA, causing one amino acid replacement (T111I), was significantly associated with plasma HDL concentrations, but not with plasma total cholesterol or TG.[6] However, the mechanism which regulates EL activity in vivo has not been clarified yet. In the present study, we showed that ANGPTL3, a hepatic secretory factor, significantly inhibited the activity of recombinant EL protein. We also found that the N-terminal domain, especially the putative heparin-binding region, is crucial for ANGPTL3-mediated suppression of EL activity. Furthermore, in Angptl3-deficiency, the phospholipase activity of post-heparin plasma was significantly elevated in vivo. Besides EL, LPL and HL also have phospholipase activity. However, McCoy et al previously demonstrated that the phospholipase activity of LPL and HL was extremely low compared with EL, whereas they had relatively high levels of triglyceride-lipase activity.[4] Moreover, the loss of EL in the homozygous knockout mice resulted in a significant decrease in the post-heparin augmentation of phospholipase activity.[7] These data clearly point to EL as a major contributor to heparin-releasable phospholipase activity in mice. Based on this previous evidence and our in vitro data, we assume that the elevation of heparin-releasable phospholipase activity in Angptl3-null mice should be explained by the lack of inhibitory effect of Angptl3 on EL. However, further analyses, eg, with double knockout mice of Angptl3 and EL, are still required to provide definitive evidence.

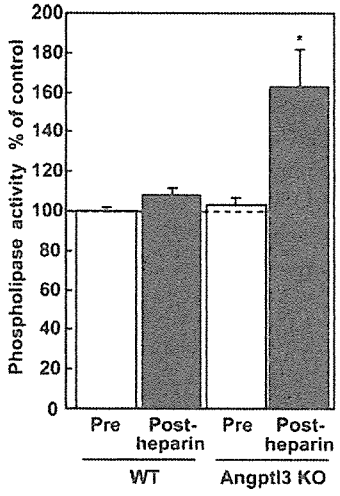

Figure 4. Phospholipase activities of pre- and post-heparin plasma in wild-type and Angptl3-knockout mice. Phospholipase activities of pre- (white bars) and post-heparin plasma (black bars) from wild-type (WT, n=5) and Angptl3-knockout mice (KO, n=5) were determined using 1,2 di[1-^{14}C] oleyl-L-3-phophatidylcholine and triolein as substrates. Relative phospholipase activities are expressed as a percentage of the values of the pre-heparin plasma in wild-type mice. Data are the mean±SEM of the values of 5 mice. $^{*}P<0.05$ vs the post-heparin plasma of wild-type mice.

Our previous and current studies demonstrated that ANGPTL3 suppressed the activities of two lipases, LPL and EL, in vitro, and Angptl3-deficiency led to a significant reduction of plasma TG and HDL levels, and supplementation of ANGPTL3 restored them in vivo. Furthermore, in the current study, we constructed an ELISA system for measuring ANGPTL3 concentrations in human plasma,

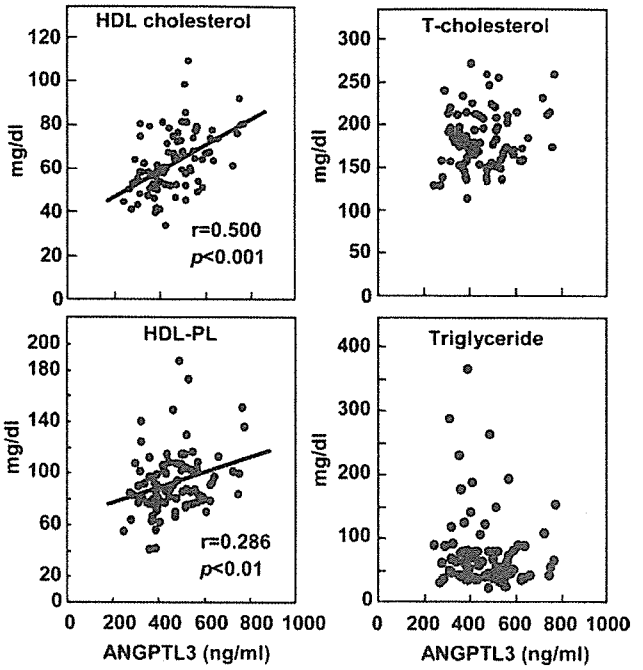

Figure 5. Plasma lipids and ANGPTL3 levels in humans. Plasma concentrations of ANGPTL3, HDL cholesterol, HDL-PL, total (T-) cholesterol and triglyceride were determined under overnight fasting conditions in healthy Japanese subjects (n=87). The values of correlation and probabilities are shown in the figures of ANGPTL3 and HDL cholesterol, and HDL-PL.

and revealed that the plasma ANGPTL3 level significantly correlated to the plasma HDL cholesterol, suggesting that ANGPTL3 should play an essential role as a regulatory factor of plasma HDL-cholesterol levels in humans, but not of plasma TG. Our previous studies showed that in mice, either the administration of ANGPTL3 protein or an injection of ANGPTL3-adenovirus promptly elevated the plasma TG level, but the elevated TG level started to decrease shortly afterward, in spite of the high level of ANGPTL3 in the plasma,[8,10] suggesting that the inhibition of LPL by ANGPTL3 does not appear to persist in vivo. In addition, plasma TG levels are easily affected by various nutritional and hormonal factors in humans. It is conceivable that these elements might be related to the finding that there was not a simple correlation between plasma ANGPTL3 and TG levels in human subjects.

Previously, we and other groups reported that insulin and leptin inhibited the production of Angptl3,[16,22] and liver X receptor (LXR) agonist upregulated the mRNA and protein expression of Angptl3 via the activation of its promoter by LXR/ retinoic X receptor (RXR).[14,23] In a recent study, downregulation of human ANGPTL3 gene by thyroid hormone was reported.[24] These previous data suggest that the expression of ANGPTL3 can be altered metabolically or nutritionally, and altered plasma levels of ANGPTL3 might be involved in the pathophysiological alterations of plasma HDL levels.

In conclusion, ANGPTL3 may be involved in the regulation of plasma HDL cholesterol levels through the inhibition of EL activity. Our findings provide new insight into understanding the regulation of EL activity and HDL metabolism via angptl3. Further epidemiological studies will provide more information for understanding the complicated HDL metabolism in humans.

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Disclosures

None.

References

1. Gordon DJ, Rifkind BM. High-density lipoprotein–the clinical implications of recent studies. *N Engl J Med.* 1989;321:1311–1316.
2. Reaven P. Metabolic syndrome. *J Insur Med.* 2004;36:132–142.
3. Krauss RM. Lipids and lipoproteins in patients with type 2 diabetes. *Diabetes Care.* 2004;27:1496–1504.
4. McCoy MG, Sun GS, Marchadier D, Maugeais C, Glick JM, Rader DJ. Characterization of the lipolytic activity of endothelial lipase. *J Lipid Res.* 2002;43:921–929.
5. Jaye M, Lynch KJ, Krawiec J, Marchadier D, Maugeais C, Doan K, South V, Amin D, Perrone M, Rader DJ. A novel endothelial-derived lipase that modulates HDL metabolism. *Nat Genet.* 1999;21:424–428.
6. Ma K, Cilingiroglu M, Otvos JD, Ballantyne CM, Marian AJ, Chan L. Endothelial lipase is a major genetic determinant for high-density lipoprotein concentration, structure, and metabolism. *Proc Natl Acad Sci U S A.* 2003;100:2748–2753.
7. Ishida T, Choi S, Kundu RK, Hirata K, Rubin EM, Cooper AD, Quertermous T. Endothelial lipase is a major determinant of HDL level. *J Clin Invest.* 2003;111:347–355.
8. Koishi R, Ando Y, Ono M, Shimamura M, Yasumo H, Fujiwara T, Horikoshi H, Furukawa H. Angptl3 regulates lipid metabolism in mice. *Nat Genet.* 2002;30:151–157.
9. Conklin D, Gilbertson D, Taft DW, Maurer MF, Whitmore TE, Smith DL, Walker KM, Chen LH, Wattler S, Nehls M, Lewis KB. Identification of a mammalian angiopoietin-related protein expressed specifically in liver. *Genomics.* 1999;62:477–482.
10. Shimizugawa T, Ono M, Shimamura M, Yoshida K, Ando Y, Koishi R, Ueda K, Inaba T, Minekura H, Kohama T, Furukawa H. ANGPTL3 decreases very low density lipoprotein triglyceride clearance by inhibition of lipoprotein lipase. *J Biol Chem.* 2002;277:33742–33748.
11. Ono M, Shimizugawa T, Shimamura M, Yoshida K, Noji-Sakikawa C, Ando Y, Koishi R, Furukawa H. Protein region important for regulation of lipid metabolism in angiopoietin-like 3 (ANGPTL3): ANGPTL3 is cleaved and activated in vivo. *J Biol Chem.* 2003;278:41804–41809.
12. Shimamura M, Matsuda M, Kobayashi S, Ando Y, Ono M, Koishi R, Furukawa H, Makishima M, Shimomura I. Angiopoietin-like protein 3, a hepatic secretory factor, activates lipolysis in adipocytes. *Biochem Biophys Res Commun.* 2003;301:604–609.
13. Hirata K, Dichek HL, Cioffi JA, Choi SY, Leeper NJ, Quintana L, Kronmal GS, Cooper AD, Quertermous T. Cloning of a unique lipase from endothelial cells extends the lipase gene family. *J Biol Chem.* 1999;274:14170–14175.
14. Inaba T, Matsuda M, Shimamura M, Takei N, Terasaka N, Ando Y, Yasumo H, Koishi R, Makishima M, Shimomura I. Angiopoietin-like protein 3 mediates hypertriglyceridemia induced by the liver X receptor. *J Biol Chem.* 2003;278:21344–21351.
15. Fujimoto K, Koishi R, Shimizugawa T, Ando Y. Angptl3-null mice show low plasma lipid concentrations by enhanced lipoprotein lipase activity. *Exp Anim.* 2006;55:27–34.
16. Shimamura M, Matsuda M, Ando Y, Koishi R, Yasumo H, Furukawa H, Shimomura I. Leptin and insulin down-regulate angiopoietin-like protein 3, a plasma triglyceride-increasing factor. *Biochem Biophys Res Commun.* 2004;322:1080–1085.

17. Burstein M, Scholnick HR, Morfin R. Rapid method for the isolation of lipoproteins from human serum by precipitation with polyanions. *J Lipid Res.* 1970;11:583–595.
18. Usui S, Hara Y, Hosaki S, Okazaki M. A new on-line dual enzymatic method for simultaneous quantification of cholesterol and triglycerides in lipoproteins by HPLC. *J Lipid Res.* 2002;43:805–814.
19. Okazaki M, Usui S, Ishigami M, Sakai N, Nakamura T, Matsuzawa Y, Yamashita S. Identification of unique lipoprotein subclasses for visceral obesity by component analysis of cholesterol profile in high-performance liquid chromatography. *Arterioscler Thromb Vasc Biol.* 2005;25:578–584.
20. Ohashi R, Mu H, Wang X, Yao Q, Chen C. Reverse cholesterol transport and cholesterol efflux in atherosclerosis. *Qjm.* 2005;98:845–856.
21. Jin W, Millar JS, Broedl U, Glick JM, Rader DJ. Inhibition of endothelial lipase causes increased HDL cholesterol levels in vivo. *J Clin Invest.* 2003;111:357–362.
22. Inukai K, Nakashima Y, Watanabe M, Kurihara S, Awata T, Katagiri H, Oka Y, Katayama S. ANGPTL3 is increased in both insulin-deficient and -resistant diabetic states. *Biochem Biophys Res Commun.* 2004;317:1075–1079.
23. Kaplan R, Zhang T, Hernandez M, Gan FX, Wright SD, Waters MG, Cai TQ. Regulation of the angiopoietin-like protein 3 gene by LXR. *J Lipid Res.* 2003;44:136–143.
24. Fugier C, Tousaint JJ, Prieur X, Plateroti M, Samarut J, Delerive P. The lipoprotein lipase inhibitor ANGPTL3 is negatively regulated by thyroid hormone. *J Biol Chem.* 2006;281:11553–11559.

Modulating role of estradiol on arginase II expression in hyperlipidemic rabbits as an atheroprotective mechanism

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We evaluated the effects of a 0.5% cholesterol-enriched diet (HCD) on nitric-oxide synthase (NOS) and arginase expression and the modulating role of 17β-estradiol (E_2) on this phenomenon. Thirty oopherectomized rabbits were divided into three groups and treated for 15 weeks. Group I received normal chow; group II, HCD; and group III, HCD plus E_2 pellets. Animals in group II showed an increase in plasma lipids, and they demonstrated atheromatous lesions as well as expression of arginase I and II accompanied by a significant number of BrdU-positive cells in endothelial cells and intimal muscle cells, suggestive of an increase in cellular proliferation. There was significant expression of inducible NOS and increased staining of nitrotyrosine-positive areas. These were not observed in group I animals. In both groups, E_2 levels were low. In group III animals, E_2 supplementation led to a decrease in atheromatous lesions and BrdU-positive cells and reduced expression of both inducible NOS and arginase I and II accompanied by a decrease in nitrotyrosine staining. E_2 levels were increased. Our results suggest that E_2 was responsible for these effects, despite the animals being hyperlipidemic, similar to those in group II. Because arginase is responsible for cell proliferation by converting L-arginine to polyamines, our results indicate that expression of arginase may play an important role in cellular proliferation in atherosclerosis, and inhibition of arginase expression by E_2 may be another potential mechanism in attenuating atherogenesis.

arteriosclerosis | L-arginine | nitric oxide | endothelium | nitric-oxide synthase

Estrogens retard the development of atherosclerosis by attenuating the adhesion of circulating monocytes to endothelial cells and their subsequent migration to the subendothelial layer (1). Decreased expression of vascular cell adhesion molecule 1 (VCAM-1) and monocyte chemoattractant protein 1 (MCP-1) by estrogens may at least in part account for this effect (1–3). We (4, 5) and others (6) have demonstrated that estrogens increase nitric oxide (NO) production by endothelial cells, and it is now known that NO, either on its own (7) or produced after stimulation by estrogens (8), can attenuate the cytokine-induced expression of VCAM-1 (1) as well as MCP-1 (9, 10). Furthermore, the inhibition of NO production in animals administered an inhibitor of nitric-oxide synthase (NOS) results in potentiation of atherosclerotic lesions in high-cholesterol diet (HCD)-induced atherosclerosis (11) and also causes atherosclerotic coronary lesions, especially at microvascular levels in experimental animals (12). L-Arginine is a substrate for NOS, which catalyzes formation of N^{ω}-hydroxy-L-arginine as an intermediate that subsequently forms NO (13).

L-Arginine can be a substrate for NOS, which catalyzes its breakdown to release NO in endothelial cells. L-Arginine is also converted by arginase to ornithine, the only source of synthesis in mammalian cells of the polyamines putrescine, spermidine, and spermine, which are essential for cell proliferation and regulation of the cell cycle (14, 15) and, which are, therefore, proatherosclerotic. The exact mechanism by which polyamines increase cell proliferation is not known. In vertebrates there are two isoforms of arginase, both of which catalyze the conversion of arginine to ornithine and urea. They differ with regard to subcellular localization, tissue distribution, and certain enzymatic properties, reflecting the fact that different genes encode them (14, 15). Arginase I is expressed almost exclusively in the cytosol of liver cells, whereas arginase II is located within the mitochondrial matrix and is expressed at low levels in many tissues (15). Furthermore, citrulline, the end product of the NOS-mediated reaction, is converted to L-arginine by arginosuccinate synthetase and arginosuccinate lyase (16). The present work was, therefore, undertaken to assess whether arginase expression is increased in atherosclerotic lesions and to determine the modulating role of estrogen, if any, on this phenomenon.

Results

Blood Chemistry. All of the rabbits appeared to be healthy throughout the study. No significant differences in serum high-density lipoprotein (HDL)-cholesterol, total serum protein, or body weight existed among the three groups over the course of the study. In animals fed normal chow (group I), there was no difference in total cholesterol levels compared with basal values. The addition of 0.5% cholesterol to the diet (groups II and III) increased the total cholesterol levels significantly compared with the baseline value (Table 1).The treatment with E_2 did not significantly affect the plasma lipid levels in this study; however, it increased the plasma E_2 concentration up to physiological levels similar to that observed in ovary-intact rabbits (Table 1), as reported in ref. 2.

Histological Examination of Atherosclerosis. In animals fed normal chow (group I), no atheromatous lesions were observed. On the other hand, histological examination of the thoracic aortas of animals fed a HCD and who received a placebo pellet (group II) revealed more atheromatous lesions, as indicated by the mean percentage of luminal encroachment and the mean lesion area in the hypercholesterolemic than in the animals fed a HCD but who received E_2 pellets (group III). The area of atherosclerosis in the thoracic aorta was reduced by 70% in the E_2-treated group (group III) compared with the HCD group receiving placebo pellets (group II) (Fig. 1 *Left* and *Center*). The intima:media (I:M) ratios also decreased after E_2 treatment (group III vs. group II) (Fig. 1 *Right*).

Conflict of interest statement: No conflicts declared.

Abbreviations: E_2, 17β-estradiol; EDNO, endothelium-derived nitric oxide; eNOS, endothelial nitric-oxide synthase; HCD, high-cholesterol diet; I:M ratio, intima:media ratio; iNOS, inducible nitric-oxide synthase; MCP-1, monocyte chemoattractant protein 1; NOS, nitric-oxide synthase; NZW, New Zealand White; VCAM-1, vascular cell adhesion molecule 1.

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Table 1. Plasma lipid (total cholesterol, triglycerides, and HDL-cholesterol), total protein, and E_2 concentration in rabbits fed a standard diet (group I), HCD (group II), and HCD plus E_2 (group III)

Group	Total cholesterol, mg/dl	Triglycerides, mg/dl	HDL-cholesterol, mg/dl	Total protein, g/dl	E_2, pg/ml
I	80.2 ± 8.2	36.9 ± 5.1	30.4 ± 4.1	8.1 ± 1.0	15.8 ± 4.1
II	1,451.5 ± 120.5*	82.4 ± 15.6*	30.9 ± 5.2	7.8 ± 1.1	12.9 ± 2.5
III	1,298.8 ± 140.9*	74.5 ± 11.5*	35.2 ± 8.2	8.2 ± 1.1	40.0 ± 2†

Rabbits were treated with each condition for 15 weeks. Results are the mean ± SEM of 10 rabbits. *P* was measured by an unpaired Student *t* test. *, $P < 0.05$ versus group I (control); †, $P < 0.05$ versus group I (control) and group II (HCD).

BrdU-Positive Neointima Lesion. BrdU-positive cells, mainly composed of endothelial cells and intimal smooth muscle cells, were significantly decreased in group III compared with group II (Fig. 2).

Immunocytochemical Analysis. ***Smooth muscle cell α-actin, monocytes/macrophages, inducible NOS (iNOS), and nitrotyrosine (one of the reaction products of $ONOO^-$).*** The atheroma in the aorta was composed of many macrophages derived from foam cells and intimal smooth muscle cell proliferation (Fig. 3). A significant reduction in the atherosclerotic area, as well as a decrease in the relative number of macrophages, was observed in animals in the E_2-treated group in this study (Fig. 3 *Left*). The number of smooth muscle cell α-actin-positive cells was not changed between groups II and III (Fig. 3 *Right*). The iNOS and nitrotyrosine-positive areas were decreased in the E_2-treated group (group III) compared with the placebo group (group II) (Fig. 4). We observed iNOS expression in the T cells and macrophages in the advanced atherosclerotic plaque of the thoracic aortas of group II, consistent with previous data (17).

Arginase I, arginase II, and arginosuccinate synthetase. The atheroma in the aorta expressed a large amount of arginase I and II, as well as arginosuccinate synthase in animals in group II; however, the expression of arginase I and II, but not arginosuccinate synthase, decreased in the aortas from group III (Fig. 5).

Discussion

Endothelial dysfunction leading to expression of adhesion molecules plays an integral part in the initiation of atherosclerosis. Expression of adhesion molecules on the endothelial surface leads to adhesion of monocytes to endothelial cells, which is one of the early steps in the development of atherosclerosis (18). Endothelial dysfunction is also characterized by impaired endothelium-derived nitric oxide (EDNO) production and impaired endothelium-dependent vasodilation (19). Only low concentrations of NO are normally produced by the endothelial cells physiologically, which protect the endothelial cell and, therefore, play an important role in attenuating the onset of atherosclerosis (20, 21). On the other hand, once atherosclerosis develops, activated macrophages are present in the lesion area, and these macrophages express iNOS (17). This process leads to relatively high concentrations of NO as well as superoxide (O_2^-), leading to formation of peroxynitrite

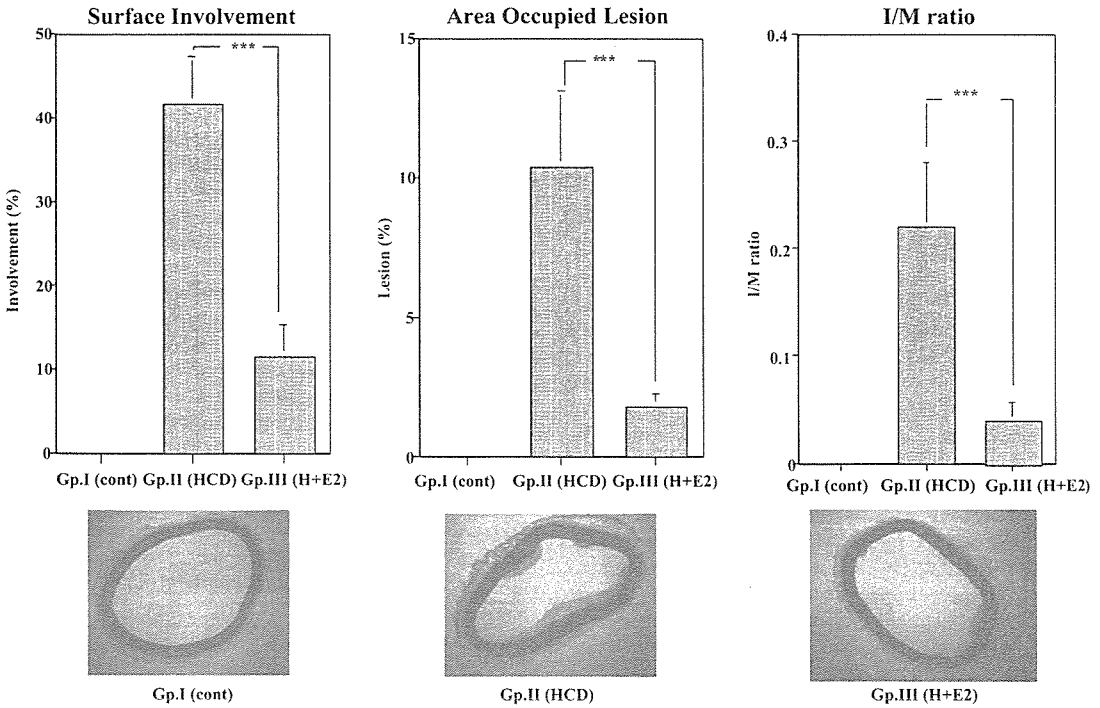

Fig. 1. Histological evaluation of the atherosclerotic area of thoracic aortas as indicated by the surface involvement, mean lesion area (percent of area occupied by lesion), and I:M ratio (*Upper*) and representative photographs (*Lower*). (*Upper Left*) Surface involvement of the atherosclerotic area of thoracic aortas from rabbits (group I, normal chow; group II, 0.5% HCD and placebo pellet; group III, 0.5% HCD and E_2 pellet). ***, $P < 0.001$. (*Upper Center*) Area occupied by the atherosclerotic area of thoracic aortas from the three groups of rabbits. ***, $P < 0.001$. (*Upper Right*) I:M ratio of thoracic aortas from three groups of rabbits. ***, $P < 0.001$. (*Lower*) Representative photographs of thoracic aortas from rabbits. (*Lower Left*) Group I. (*Lower Center*) Group II. (*Lower Right*) Group III. (Original magnification, ×40.)

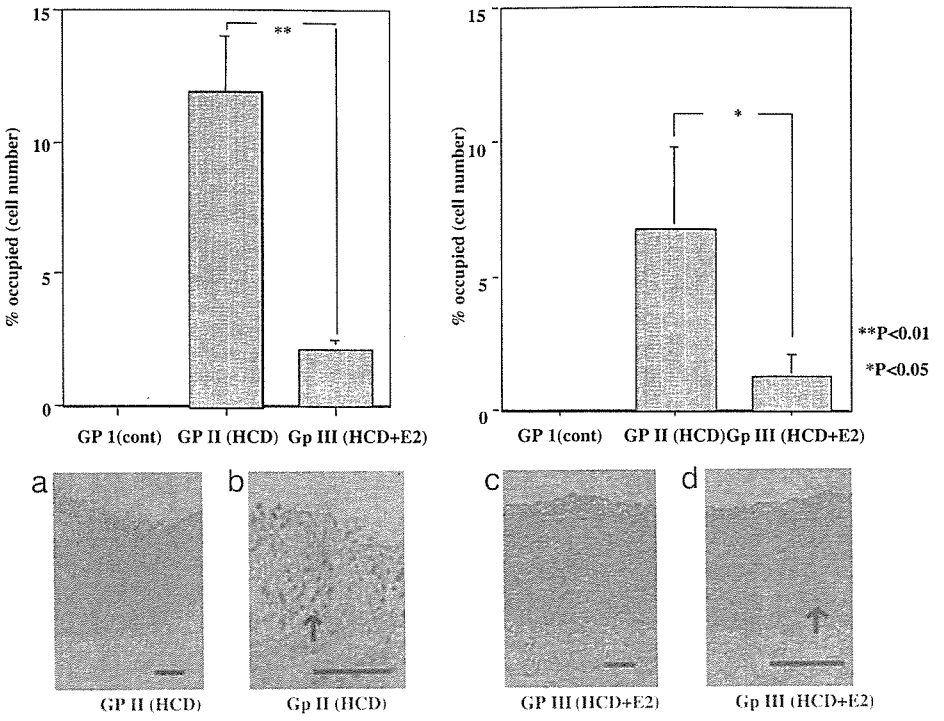

Fig. 2. Immunohistochemical analysis with the anti-BrdU-positive, neointimal area of the thoracic aortas of New Zealand White (NZW) rabbits from the atherosclerotic group. (*Upper Left*) Atherosclerotic area. (*Upper Right*) Nonatherosclerotic area. In group III, the number of positive cells was significantly decreased compared with group II. *, $P < 0.05$; **, $P < 0.01$. (*Lower*) Representative photographs of the thoracic aortas from rabbits. (*Lower Left a* and *b*) Group II. (*Lower Right c* and *d*) Group III. [Original magnification, ×100 (*a* and *c*); ×400 (*b* and *d*). Scale bars, 100 μm.] The arrows point to anti-BrdU-positive cells.

(ONOO⁻) in the lesion area, which is toxic to the endothelial cells (22, 23). L-Arginine is the precursor to NO (13, 22), and therefore, L-arginine should either have a protective or deleterious role with regard to atherosclerosis depending on whether endothelial NOS (eNOS) or iNOS is expressed.

Chronic administration of L-arginine to HCD rabbits enhanced the synthesis of EDNO and reduced or reversed the progression of intimal lesions (24, 25). In these studies, the animals initially received 0.5% a HCD for only 10 weeks. Subsequently, L-arginine or vehicle was administered for an additional 13 weeks while the HCD was continued. We demonstrated that administration of L-arginine led to regression of the preexisting intimal lesions. More recently, it has been demonstrated in humans that oral supplementation with large amounts (6–21 g/day) of L-arginine, the precursor to EDNO (26), as well as intake of a nutrient bar enriched with L-arginine (26), designed to enhance EDNO, improved flow-

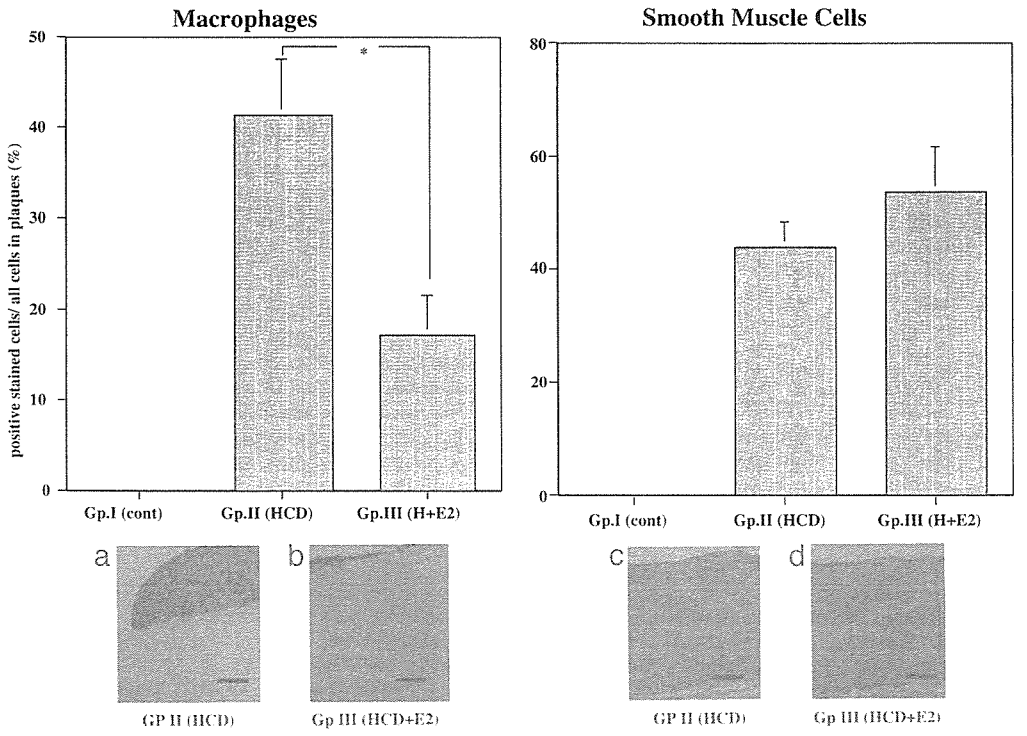

Fig. 3. Immunohistochemical analysis with anti-macrophage and anti-smooth muscle cell α-actin monoclonal antibody of thoracic aortas of NZW rabbits from the atherosclerotic group. (*Upper Left*) Area occupied by macrophages in subintimal atherosclerotic plaque of thoracic aortas of groups I, II, and III rabbits. *, $P < 0.05$. (*Upper Right*) Area occupied by smooth muscle cell α-actin in subintimal atherosclerotic plaque of thoracic aortas of groups I, II, and III rabbits. (*Lower*) Representative photographs of thoracic aortas from rabbits. (*Lower Left a*) Group II. (*Lower Left b*) Group III. Macrophages were detected in both the core and fibrous cap in a section stained with a monoclonal antibody against rabbit macrophages. (Original magnification, ×100.) (*Lower Right c*) Group II. (*Lower Right d*) Group III. Smooth muscle cell α-actins were detected in the media and subintimal atherosclerotic plaque area of thoracic aortas of groups II and III rabbits. No significant difference between groups II and III was observed. (Original magnification, ×100. Scale bars, 25 μm.)

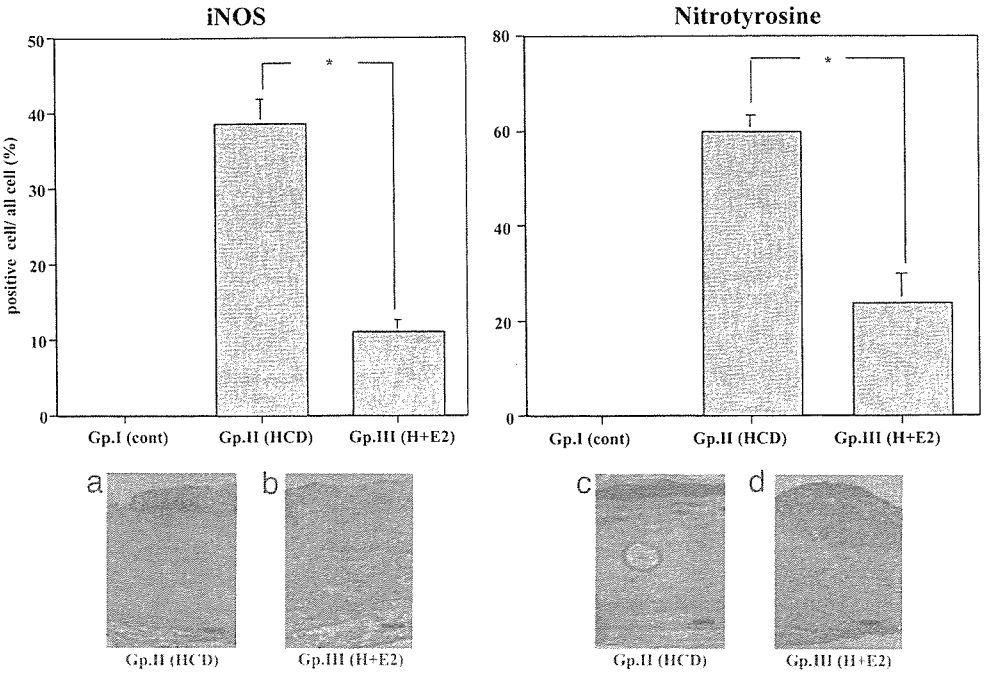

Fig. 4. Immunohistochemical analysis with anti-iNOS and anti-nitrotyrosine monoclonal antibody of thoracic aortas of NZW rabbits from atherosclerotic group. (*Upper*) Area occupied by iNOS-positive cells (*Left*) and nitrotyrosine-positive cells (*Right*) in subintimal atherosclerotic plaque of thoracic aortas of rabbits. *, $P < 0.05$. (*Lower*) Representative photographs of thoracic aortas from rabbits. (*Lower Left a*) Group II. (*Lower Left b*) Group III. iNOS was detected adjacent to the necrotic core. (Original magnification, ×100. Scale bars, 25 μm.) (*Lower Right c*) Group II. (*Lower Right d*) Group III. Nitrotyrosine was detected in the subintimal atherosclerotic plaque area of the thoracic aortas. (Original magnification, ×100. Scale bars, 25 μm.)

mediated endothelium-dependent vasodilation in hypercholesterolemic individuals. Although L-arginine is the precursor to EDNO, there are other actions of L-arginine independent of NO production that may also have been the cause of improved flow-mediated endothelium-dependent vasodilation in hypercholesterolemic individuals.

In our studies, when the animals were fed a HCD for a much longer period, i.e., 15 weeks, there was an 8-fold increase in arginase I and II expression along with the expression of iNOS compared with animals fed normal chow. Arginase II expression is also up-regulated in rheumatoid arthritis (27, 28) in a way similar to that seen in our studies on atherosclerosis. It has been suggested that in cells where both arginase II and iNOS activity occurs, there is a reciprocal regulation, suggesting that agents that induce arginase II could down-regulate the levels of NO and divert L-arginine metabolism toward cell proliferation and/or tissue regeneration (28). Similarly, the concomitant expression of iNOS as well as arginase can markedly reduce basal NO synthesis in endothelial cells because of a decrease in the intracellular arginine content in these cells (28). In our study, increased arginase expression most likely led to increased cellular proliferation and reduced endothelial cell NO formation, and thus, the protective effects that the endothelial-derived NO has on the atherosclerotic process were absent.

Similar to our results in rabbits, an increase in arginase II enzyme activity in apolipoprotein $E^{-/-}$ mice has been found by another group of investigators (29). Furthermore, in these mice, L-arginine induced vasoconstriction in segments of mouse aorta. The contraction induced by L-arginine was much more pronounced in atherosclerotic apolipoprotein $E^{-/-}$ mice compared with control animals. These contractions were converted to relaxations in the presence of an arginase inhibitor (29). On the basis of our results and those of others (29), it is possible to speculate that after expression of arginase in atherosclerotic lesions, L-arginine administration may have a deleterious effect on the atherosclerotic process rather than the beneficial effect obtained in the absence of arginase expression.

We (1) and others (30, 31) have previously demonstrated that estrogens may attenuate atherogenesis by increasing NO production by endothelial cells (4–6). It has also been shown that estrogens can decrease the expression of TNFα-stimulated VCAM-1 expression by a NO-mediated mechanism (8, 32). This action of estrogens in increasing eNOS would allow L-arginine to be directed to the L-arginine–NO pathway, leading to attenuation of initiation of atherosclerosis. Results from this study further indicate that estrogens attenuate the expression of arginase II, which would blunt the L-arginine–polyamine pathway and prevent cell proliferation. This action would then allow L-arginine to be directed to the L-arginine–NO pathway, thereby offering another potential mechanism by which estrogen attenuates atherogenesis. E_2 treatment did not affect the expression of arginosuccinate synthetase, indicating that estrogens do not affect the availability of L-arginine from citrulline.

The precise mechanism(s) by which E_2 attenuates the expression of arginase is not known, and it was not assessed in our study. E_2 can have varied effects on arginase activity. E_2 benzoate evoked a 3-fold elevation in the arginase activity of the dorsal prostate, in contrast to the decreased arginase activity in the ventral prostate after E_2 administration (33). However, the type of arginase was not indicated in the study. Further studies are needed to assess the mechanism(s) by which estrogen modulates arginase II activity.

In conclusion, the results from our studies indicate that after prolonged feeding of a HCD, arginase expression is increased in hyperlipidemic rabbits in the atherosclerotic lesion area, whereas the expression of arginase II is significantly reduced by simultaneous administration of E_2. The increase in arginase II activity may account for the associated cellular proliferation by diverting L-arginine to form polyamines, whereas E_2, by inhibiting arginase II expression, attenuates atherosclerosis by providing a substrate for eNOS to synthesize NO, which is atheroprotective. It is also possible that L-arginine may be beneficial in the early stages of atherosclerosis before the expression of arginase II, whereas it may have deleterious effects if administered later on when significant lesions have already developed, and arginase II, expressed as L-arginine is administered, would then lead to cell proliferation (34). Further studies are needed to assess whether the timing of L-arginine administration may be the key determining factor as to whether it would be atheroprotective or lead to deleterious effects.

Materials and Methods

Chemicals and Solutions. Monoclonal antibodies against smooth muscle cell α-actin (HHF35) and monocytes/macrophages

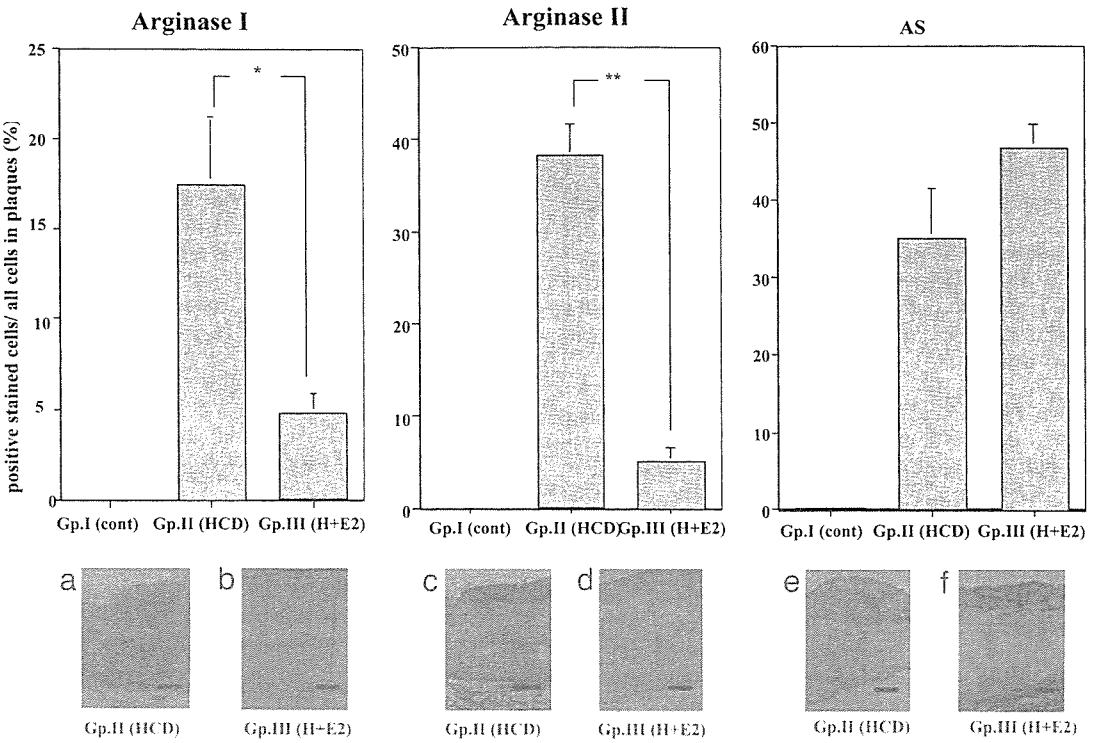

Fig. 5. Distribution of arginase I and II and arginosuccinate synthase in atherosclerotic aortas. (*Upper*) Immunohistochemical analysis with anti-arginase I and II and arginosuccinate synthetase (AS) antibody of thoracic aortas of NZW rabbits from the different groups. *, $P < 0.05$ and **, $P < 0.01$. (*Lower*) Immunohistochemical analysis with anti-arginase I [(*a*) group II and (*b*) group III)] and anti-arginase II [(*c*) group II and (*d*) group III] monoclonal antibodies and the anti-arginosuccinate synthetase monoclonal antibody [(e) group II and (*f*) group III] of the thoracic aortas of NZW rabbits from the atherosclerotic group. (Original magnification, ×100. Scale bars, 25 μm.)

(RAM11) were purchased from Enzo Diagnostics and DAKO, respectively. Antibodies against iNOS, nitrotyrosine, arginase I, and arginosuccinate synthase were all purchased from Transduction Laboratories (Lexington, KY). Antibodies against arginase II were gifts from M. Gotoh and M. Mori (Kumamoto University School of Medicine) (35). BrdU, a thymidine analog that labels newly synthesized DNA, was purchased from Sigma. Peroxidase-conjugated anti-BrdU was purchased from DAKO.

Animals. Female NZW rabbits weighing 3–3.5 kg were used. All animals were fed regular chow for 2 weeks. They were housed in individual cages and underwent oopherectomy with placement of either placebo or E_2 pellets (10 mg, 60-day release). Eight weeks after the placement of pellets, new E_2 or placebo pellets were placed, and the animals were killed at 15 weeks.

The method for oopherectomy was similar to that described in ref. 1. For diets, the animals were divided into three groups. Group I received normal chow. Group II received a 0.5% HCD for 15 weeks and a placebo pellet. Group III received a 0.5% HCD and an E_2 pellet. The pellets were replaced by new pellets at the end of 8 weeks to ensure that the animals had E_2 released from the pellets for the full duration of the study. At the end of the 15-week feeding period, animals were anesthetized. Anesthesia was initiated by with acepromazine (0.3 mg/kg i.v.) and ketamine (10 mg/kg i.v.) followed by isoflurane [2% (vol/vol) by inhalation]. For postoperative analgesia, the animals were also administered buprenorphine (0.03 mg/kg i.m.) twice each day for 2–3 days.

The animal protocol was formally approved by the Animal Research Committee of the University of California at Los Angeles and by the Nagoya University Graduate School of Medicine.

Determinations of Plasma Lipids and Estradiol Concentration. To assess lipid and E_2 levels, an aliquot of blood from each animal was collected into tubes containing EDTA. The total cholesterol and triglyceride levels were measured by enzymatic assays as described in refs. 36 and 37. The HDL-cholesterol was determined after precipitation with phosphotungstate/$MgCl_2$ (37). The plasma concentration of E_2 was examined as described in ref. 38.

Histological Evaluation of Aortic Atherosclerosis. After 15 weeks of feeding, the animals were euthanized with pentobarbital (100 mg/kg i.v.). The descending thoracic aorta was quickly dissected out, and adjoining segments were either snap frozen in liquid nitrogen or preserved in formaldehyde. Cross sections of the descending thoracic aorta were stained with hematoxylin/eosin (SRL, Tokyo) to examine the endothelial lining and with van Gieson's elastic stain (SRL) to determine the thickness of the intima. Morphometric analysis was performed as described by Weiner *et al.* (39). Briefly, the complete section of each block was projected onto a vertical surface with a projecting microscope. Six samples from each rabbit aorta were analyzed with the objective lens. The contours of the lumen and the internal elastic lamina were traced, and the tracings were digitized with a graphics tablet. The surface involvement by atherosclerotic lesion was calculated by dividing the lesion circumference by the circumference of the internal elastic lamina. The circumferences of the lesion area and normal area were defined as circumferences of each part of the internal elastic lamina. The area occupied by atherosclerotic lesions was defined as the percent area bounded by the lumen and the internal elastic lamina. The control luminal area was calculated from the perimeter of the internal elastic lamina as described in ref. 40. The I:M ratio was calculated (41). Data were transferred to a minicomputer (Macintosh iMac; Apple, San Jose, CA) for further analysis.

BrdU Incorporation and Immunohistochemistry. BrdU was administered at 18 h (100 mg/kg s.c. and 30 mg/kg i.v.) and 12 h (30 mg/kg

i.v.) before harvest. BrdU labeling was carried out on 5-μm frozen sections (42). Background staining was blocked by incubation with 5% normal goat serum for 30 min, and then the sections were incubated with a monoclonal antibody to BrdU (1:200; DAKO) at 4°C overnight followed by an alkaline phosphatase-conjugated goat anti-mouse IgG (1:200; Jackson ImmunoResearch) at room temperature for 1 h (42). The BrdU-labeled endothelial and smooth muscle cell nuclei, identified as elongated oval regions of immunoreactivity, were counted in five sequential sections from the thoracic artery of each rabbit. The percentage of BrdU-labeled endothelial cells was expressed as the ratio of vessels having BrdU-labeled endothelial and intimal smooth muscle cells to the total number of endothelial cells and intimal smooth muscle cell profiles per cross section (43).

Immunohistochemical Analysis. Cross sections of the descending thoracic aorta were deparaffinized with xylene and dehydrated with graded alcohol (17). The specimens were preincubated for 30 min with methanol containing 0.3% hydrogen peroxide and washed for 10 min with PBS. The specimens were permeabilized with 0.1% Triton X-100 in PBS for 20 min and washed with PBS. They were then blocked with normal horse serum for 1 h and incubated with primary monoclonal antibody (for smooth muscle cell α-actin, monocytes/macrophages, iNOS, nitrotyrosine, arginase I, arginase II, and arginosuccinate synthetase) diluted in PBS for 60 min, and washed again with PBS. Negative controls included substitution of irrelevant antibodies for the primary antiserum/antibody. A biotinylated rabbit anti-mouse IgG (1:500 dilution) was incubated for 30 min and washed with PBS followed by avidin–biotin peroxidase complex reagent (ABC kit; Vector Laboratories) incubation for 30 min. The result was a brown peroxidase reaction product of diaminobenzidine. The cell nuclei were counterstained with methyl green (17). In the negative controls, either PBS or irrelevant antibodies replaced the primary antiserum. Each field was scored for the number of positive stained cells against each antibody in plaques on slides, and all cells in the plaques were calculated and analyzed statistically as described in ref. 17. From each section, five digital images were obtained with a 3CCD color camera (JVC; Victor Company of Japan, Tokyo) and Leitz microscope. The intensity and distribution patterns of the staining reaction were evaluated by two blinded, independent observers (T.E. and T.M.) using a semiquantitative staining score (graded as 0 = none, 1 = weak, 2 = moderate, and 3 = strong staining). The cells whose mean scores were higher than 2 were recognized as positive staining cells.

Data Analysis. The results were expressed as the mean ± SEM. The SPSS/PC 6.01 software package (SPSS, Chicago) was used for collection, processing, and statistical analysis of all data. Statistical analysis was performed with the nonparametrical Wilcoxon signed-rank test for comparison of the means. The Spearman ρ coefficient was used to assess any significant correlations between the analyzed substances within the distinct groups. $P < 0.05$ was considered statistically significant.

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1. Nathan, L., Pervin, S., Singh, R., Rosenfeld, M. & Chaudhuri, G. (1999) *Circ. Res.* **85,** 377–385.
2. Pervin, S., Singh, R., Rosenfeld, M., Chaudhuri, G. & Nathan, L. (1998) *Arterioscler. Thromb. Vasc. Biol.* **18,** 1575–1582.
3. Simoncini, T., Maffei, S., Basta, G., Barsacchi, G., Genazzani, A. R., Liao, J. K. & De Caterina, R. (2000) *Circ. Res.* **87,** 19–25.
4. Hayashi, T., Fukuto, J. M., Ignarro, L. J. & Chaudhuri, G. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 11259–11263.
5. Hayashi, T., Yamada, K., Esaki, T., Ishikawa, T. & Iguchi, A. (1995) *Biochem. Biophys. Res. Commun.* **214,** 847–855.
6. Weiner, C. P., Lizasoain, I., Baylis, S. A., Knowles, R. G., Charles, I. G. & Moncada, S. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 5212–5216.
7. De Caterina, R., Libby, P., Peng, H. B., Thannickal, V. J., Rajavashisth, T. B., Gimbrone, M. A., Jr., Shin, W. S. & Liao, J. K. (1995) *J. Clin. Invest.* **96,** 60–68.
8. Mukherjee, T. K., Nathan, L., Dinh, H., Reddy, S. T. & Chaudhuri, G. (2003) *J. Biol. Chem.* **278,** 11746–11752.
9. Zeiher, A. M., Fisslthaler, B., Schray-Utz, B. & Busse, R. (1995) *Circ. Res.* **76,** 980–986.
10. Fleming, I. & Busse, R. (1995) *Adv. Pharmacol.* **34,** 187–206.
11. Naruse, K., Shimizu, K., Muramatsu, M., Toki, Y., Miyazaki, Y., Okumura, K., Hashimoto, H. & Ito, T. (1994) *Arterioscler. Thromb.* **14,** 746–752.
12. Quyyumi, A. A., Dakak, N., Andrews, N. P., Husain, S., Arora, S., Gilligan, D. M., Panza, J. A. & Cannon, R. O., III (1995) *J. Clin. Invest.* **95,** 1747–1755.
13. Fukuto, J. M. & Chaudhuri, G. (1995) *Annu. Rev. Pharmacol. Toxicol.* **35,** 165–194.
14. Jenkinson, C. P., Grody, W. W. & Cederbaum, S. D. (1996) *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **114,** 107–132.
15. Pegg, A. E. & McCann, P. P. (1982) *Am. J. Physiol.* **243,** C212–C221.
16. Hecker, M., Sessa, W. C., Harris, H. J., Anggard, E. E. & Vane, J. R. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 8612–8616.
17. Esaki, T., Hayashi, T., Yamada, K. & Iguchi, A. (1997) *Atherosclerosis (Shannon, Irel.)* **128,** 39–46.
18. Ross, R. (1999) *N. Engl. J. Med.* **340,** 115–126.
19. Chester, A. H., O'Neil, G. S., Moncada, S., Tadjkarimi, S. & Yacoub, M. H. (1990) *Lancet* **336,** 897–900.
20. Khan, B. V., Harrison, D. G., Olbrych, M. T., Alexander, R. W. & Medford, R. M. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 9114–9119.
21. Koh, K. K., Son, J. W., Ahn, J. Y., Lee, S. K., Hwang, H. Y., Kim, D. S., Jin, D. K., Ahn, T. H. & Shin, E. K. (2001) *Int. J. Cardiol.* **81,** 43–50.
22. Moncada, S. & Higgs, A. (1993) *N. Engl. J. Med.* **329,** 2002–2012.
23. Beckman, J. S. & Koppenol, W. H. (1996) *Am. J. Physiol.* **271,** C1424–C1437.
24. Cooke, J. P., Singer, A. H., Tsao, P., Zera, P., Rowan, R. A. & Billingham, M. E. (1992) *J. Clin. Invest.* **90,** 1168–1172.
25. Candipan, R. C., Wang, B. Y., Buitrago, R., Tsao, P. S. & Cooke, J. P. (1996) *Arterioscler. Thromb. Vasc. Biol.* **16,** 44–50.
26. Maxwell, A. J., Anderson, B., Zapien, M. P. & Cooke, J. P. (2000) *Cardiovasc. Drugs Ther.* **14,** 309–316.
27. Huang, L. W., Chang, K. L., Chen, C. J. & Liu, H. W. (2001) *Kaohsiung J. Med. Sci.* **17,** 358–363.
28. Corraliza, I. & Moncada, S. (2002) *J. Rheumatol.* **29,** 2261–2265.
29. Ming, X. F., Barandier, C., Viswambharan, H., Kwak, B. R., Mach, F., Mazzolai, L., Hayoz, D., Ruffieux, J., Rusconi, S., Montani, J. P. & Yang, Z. (2004) *Circulation* **110,** 3708–3714.
30. Clarkson, T. B., Hughes, C. L. & Klein, K. P. (1995) *Prog. Cardiovasc. Dis.* **38,** 189–198.
31. Alexandersen, P., Haarbo, J., Zandberg, P., Jespersen, J., Skouby, S. O. & Christiansen, C. (2003) *Hum. Reprod.* **18,** 1395–1403.
32. Hayashi, T., Esaki, T., Muto, E., Sumi, D., Thakur, N. K., Jayachandran, M. & Iguchi, A. (2000) *Arterioscler. Thromb. Vasc. Biol.* **20,** 1613–1621.
33. Yamanaka, H., Shimazaki, J., Imai, K., Sugiyama, Y. & Shida, K. (1975) *Endocrinol. Jpn.* **22,** 297–302.
34. Li, H., Meininger, C. J., Kelly, K. A., Hawker, J. R., Jr., Morris, S. M., Jr., & Wu, G. (2002) *Am. J. Physiol.* **282,** R64–R69.
35. Gotoh, M. & Mori, M. (1999) *J. Cell Biol.* **144,** 427–434.
36. Allain, C. C., Poon, L. S. & Chen, C. S. G. (1974) *Clin. Chem.* **20,** 470–473.
37. Lipid Research Clinics (1982) *Manual of Laboratory Operations* (Natl. Inst. Health, Bethesda), DHEW Publ. No. (NIH) 76-628, 2nd Ed., Vol. I.
38. Gaskell, S. J., Finlay, E. M. & Pike, A. W. (1980) *Biomed. Mass Spectrom.* **7,** 500–504.
39. Weiner, B. H., Ockene, I. S. & Hoogasian, J. J. (1986) *N. Engl. J. Med.* **315,** 841–845.
40. Hayashi, T., Fukuto, J. M., Ignarro, L. J. & Chaudhuri, G. (1995) *J. Cardiovasc. Pharmacol.* **26,** 792–802.
41. Kown, M. H., Yamaguchi, A., Jahncke, C. L., Miniati, D., Murata, S., Grunenfelder, J., Koransky, M. L., Rothbard, J. B. & Robbins, R. C. (2001) *J. Thorac. Cardiovasc. Surg.* **121,** 971–980.
42. Ehsan, A., Mann, M. J., Acqua, G. D., Tamura, K., Braun-Dullaeus, E. & Dzau, V. J. (2002) *Circulation* **105,** 1686–1692.
43. Virag, J. I. & Murry, C. E. (2003) *Am. J. Pathol.* **163,** 2433–2440.

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17β-Estradiol Antagonizes the Down-Regulation of Endothelial Nitric-Oxide Synthase and GTP Cyclohydrolase I by High Glucose: Relevance to Postmenopausal Diabetic Cardiovascular Disease

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ABSTRACT

In postmenopausal women, the risk of diabetic cardiovascular disease drastically increases compared with that of men or premenopausal women. However, the mechanism of this phenomenon has not yet been clarified. We hypothesized that the beneficial effects of estrogen on endothelial function may be relevant to protection against hyperglycemia-induced vascular derangement. Bovine aortic endothelial cells were incubated for 72 h in the presence and absence of the physiological concentration of 17β-estradiol (17β-E2) under normal and high-glucose conditions. The presence of 17β-E2 significantly counteracted the reduction in basal nitric oxide production under high-glucose conditions. This finding was associated with the recovery of endothelial nitric-oxide synthase (eNOS) protein expression, tetrahydrobiopterin (BH4) levels, and the activity and gene expression of GTP cyclohydrolase I (GTPCH-I), a rate-limiting enzyme for BH4 synthesis. Both the gene transfer of estrogen receptor α using adenovirus and treatment with the protein kinase C inhibitor bisindolylmaleimide I significantly enhanced the effects of 17β-E2 treatment under high-glucose conditions, whereas these effects were abolished by the estrogen receptor antagonist ICI 182,780 (faslodex). Transfection of small-interfering RNA targeting eNOS resulted in a marked reduction in GTPCH-I mRNA under both normal and high-glucose conditions, but this reduction was strongly reversed by 17β-E2. These results suggest that the activation of ERα with 17β-E2 can counteract high-glucose-induced down-regulation of eNOS and GTPCH-I in endothelial cells. Therefore, estrogen deficiency may result in an exaggeration of hyperglycemia-induced endothelial dysfunction, leading to the development of cardiovascular disease in postmenopausal diabetic women.

Impaired NO bioavailability results in endothelial dysfunction, which is thought to be a characteristic feature of vascular diseases such as diabetic macroangiopathy (Cai and Harrison, 2000; Calles-Escandon and Cipolla, 2001; Creager et al., 2003). NO plays a central role in maintaining vascular homeostasis through its effects on endothelial cells, smooth muscle cells, leukocytes, and platelets (Albrecht et al., 2003). eNOS is only fully functional in a dimeric form, and the functional activity of the eNOS dimer is dependent on the number of bound BH4 molecules. BH4 levels are principally regulated by de novo synthesis, in which GTPCH-I is a rate-limiting enzyme (Gesierich et al., 2003).

Several lines of evidence have suggested the link of excess vascular oxidative stress with impaired NO activity in patients with diabetes (Tesfamariam and Cohen, 1992; Hayashi et al., 2005). It has been reported that supplementation with BH4 improves endothelial-dependent vasodilation by increasing NO activity in patients with type II diabetes mellitus (Heitzer et al., 2000). Thus, hyperglycemia and insulin resistance may be pathogenic factors that lead to decreased vascular relaxation via an imbalance of NO/O_2^- due to a relative deficiency of BH4 in

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ABBREVIATIONS: NO, nitric oxide; BH4, tetrahydrobiopterin; eNOS, endothelial nitric-oxide synthase; GTPCH-I, GTP cyclohydrolase I; BAEC, bovine aortic endothelial cell; HRT, hormone replacement therapy; 17β-E2, 17β-estradiol; siRNA, small-interfering RNA; CS, calf serum; PCR, polymerase chain reaction; ER, estrogen receptor; NOS, nitric-oxide synthase; ANOVA, analysis of variance; ROS, reactive oxygen species; PKC, protein kinase C; ICI 182,780, 7α-[9-[(4,4,5,5,5-pentafluoropentylsulphinyl)-nonyl]-estra-1,3,5(10)-triene-3,17β-diol, faslodex; HG, high glucose; NG, normal glucose; Man, mannitol.

endothelial cells (Shinozaki et al., 1999). Previously, we reported that a high-glucose concentration enhanced oxidative stress by eNOS dysfunction and activation of NADPH oxidase in BAECs (Ding et al., 2004).

Diabetes and abnormal glucose tolerance are associated with increased risk of cardiovascular disease (Kannel and McGee, 1979). The effect of diabetes on cardiovascular disease seems to be worse in women than in men (Barrett-Connor et al., 1991). Because the risk of cardiovascular disease dramatically increases with age, especially after menopause (Gordon et al., 1978), the influence of postmenopausal hormone use on cardiovascular disease in postmenopausal women has been a subject of interest. Recent reports from Heart and Estrogen/Progestin Replacement Study (Kanaya et al., 2003) and Women's Health Initiative Hormone (Margolis et al., 2004) randomized trials indicate that postmenopausal hormone replacement therapy (HRT) reduces the risk of diabetes. Therefore, it remains important to gain a better understanding of the molecular mechanisms that confer cardiovascular-protective effects of hormone replacement therapy in postmenopausal women with diabetes.

Laboratory evidence suggests that the vascular endothelium is an important target of estrogen. It has been shown that estrogen activates eNOS via genomic and nongenomic mechanisms, leading to an increase in NO (Hayashi et al., 1995b; Simoncini et al., 2000). Thus, we hypothesized that the profound effect of estrogen on eNOS-associated endothelial function may be relevant for obtaining protection against hyperglycemia-induced vascular impairment. In the present study, we demonstrate that treatment with 17β-E2 at the physiological concentration can significantly counteract the down-regulation of eNOS and GTPCH-I in BAECs under high-glucose conditions. We found that 17β-E2 increased GTPCH-I expression independently of eNOS expression, despite the finding that GTPCH-I expression was strongly regulated by the presence of eNOS.

Materials and Methods

Materials. We used 17β-estradiol (Sigma-Aldrich, St. Louis, MO), D-glucose, D-mannitol (Wako Pure Chemicals, Osaka, Japan), bisindolylmaleimide I (Calbiochem, Darmstadt, Germany), Takara One Step RNA PCR kit (Takara, Kyoto, Japan), eNOS monoclonal antibody (BD Biosciences, San Jose, CA), estrogen receptor α antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), β-actin antibody (Abcam, Cambridge, UK), siGENOME set of four duplexes (siRNAs targeting eNOS) (GE Healthcare, Little Chalfont, Buckinghamshire, UK), and control (nonsil.) siRNA fluorescein (QIAGEN, Miami, FL). Lipofectamine 2000 and Opti-MEM were purchased from Invitrogen (Carlsbad, CA). ICI 182,780 was kindly provided by AstraZeneca Pharmaceuticals (Macclesfield, UK). GTPCH-I antibody was kindly provided from Prof. H. Ichinose (Tokyo Institute of Technology, Yokohama, Japan) who made it using recombinant full-length human GTPCH-I.

Cell Culture. BAECs were obtained from a fetal calf as described previously (Hayashi et al., 1995b) and cultured in Dulbecco's modified Eagle's medium (Invitrogen) with 10% (v/v) calf serum (CS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. Cells were allowed to reach 80% confluence, and they were then stimulated with different concentrations of D-glucose (5.5, 10.5, and 30.5 mM) as well as other reagents in phenol-red free Dulbecco's modified Eagle's medium with 2% (v/v) CS. We defined the control as the status under 5.5 mM glucose concentration, which is the same concentration as normal human plasma. Mannitol was used as a control to rule out the effect of osmotic pressure. Subconfluent cell monolayers were studied at six to eight passages.

Determination of Intracellular BH4 Levels and GTPCH-I Activity. BAECs were harvested with trypsin, pelleted by centrifugation, and frozen at −80°C. BH4 measurements were performed following the high-performance liquid chromatography procedure described by Fukushima and Nixon (1980). GTPCH-I activity was assayed as described by Sawada et al. (1986), based on the quantification of D-*erythro*-neopterin by high-performance liquid chromatography after the conversion of enzymaticaly formed D-*erythro*-7,8-dihydroneopterin triphosphate into D-*erythro*-neopterin by sequential iodine oxidation and dephosphorylation.

Reverse Transcription-Polymerase Chain Reaction Analysis of GTPCH-I mRNA and eNOS mRNA. Total RNA was isolated from BAECs with TRIzol reagent (Invitrogen) following the manufacturer's protocol. One microgram of total RNA was reverse-transcribed to cDNA using 200 U/μl Moloney murine leukemia virus reverse transcriptase, and the cDNA samples were analyzed for GTPCH-I and eNOS by PCR. PCR was performed in 25 μl of reaction volume containing 80.2 mM each of Ex-*Taq* polymerase buffer and deoxynucleotide phosphate as well as mixture, 81.5 mM $MgCl_2$, and 2.5 U of Ex-*Taq*DNA polymerase, oligonucleotide primers (0.5 μM each), and the cDNA. The programmed cycles were as follows: one cycle of 50°C × 30 min and 94°C × 2 min followed by 40 cycles of 94°C × 30 s, 60°C × 30 s, and 72°C × 1 min. The PCR-amplified product was analyzed by 1% agarose gel electrophoresis. The bovine GTPCH-I primer sequences were 5′-CCGCCTACTCGTCCATCCTGA-3′ (sense) and 3′-ACCTCGCATTACCATACACAT-5′ (antisense). The bovine eNOS primer sequences were 5′-CCGTGTCCAACATGCTGCT-3′ (sense) and 3′-ACCTCGCATTACCATACACAT-5′ (antisense). The bovine β-actin primer sequence were 5′-CGAGCATTCCCAAAGTTCTACAGTG-3′ (sense) and 3′-CTACATACTTCCGAAAACCAGGGG-5′ (antisense).

Western Blot Analysis of eNOS and Estrogen Receptor α Protein. Total protein was extracted by adding lysis buffer (10 mM Tris, pH 7.4, 1% SDS, and 1 mM sodium carbonate). Protein was quantified, and 10 μg of protein was loaded into each lane of 7.5% (12.5% for β-actin) polyacrylamide gel. The protein was electrophoresed and transferred to polyvinylidene difluoride membrane, blocked with 2% skimmed milk powder, and then incubated with primary antibody overnight at 4°C and horseradish peroxidase-conjugated anti-mouse IgG antibody for 1 h. The bands were developed in the dark on the film (Fuji Medical X-Ray film; Fuji Photo Film, Tokyo, Japan) and measured densitometrically by an NIH Image analyzer. Equal protein loading was confirmed by Coomassie Brilliant Blue and amido black staining of protein in each lane of the same blot.

Measurement of Nitrite. As described in our previous report (Ding et al., 2004), the concentration of nitrite:metabolites of NO in 10 μl of culture medium was determined with an automated NO detector high-performance liquid chromatography system (ENO10; EICOM, Kyoto, Japan), where nitrite was detected based on Griess reaction to form a purple azo dye, and then absorbance at 540 nm was detected. Values for nitrite level were normalized for cell protein. The incubated medium was not completely free from nitrite; therefore, an aliquot of medium underwent the same process as the medium obtained from the cultured cells. We usually used the nitrite value obtained with the medium alone as a blank, and it was subtracted from all the samples. Our preliminary study confirmed that coincubation with the NOS inhibitor N^G-nitro-L-arginine methyl ester at 1 mM decreased a nitrite level after subtraction to less than 5% of the previous subtracted value, indicating that nitrites in the medium where BAECs are present are mostly from NOS-mediated NO production.

Construction of an Adenovirus Vector Carrying ERα and Transfer into Cultured Endothelial Cells. Human ERα cDNA cloned into pBR322 was inserted into pAxCAwt. Recombinant adenoviruses were constructed by Takara (Kyoto, Japan). Adenoviruses carrying an *Escherichia coli lacZ* gene encoding a nucleus-

localized variant of β-galactosidase were also used. We grew 5×10^5 BAECs in a six-well plate for 24 h and then incubated cells with adenoviruses at a multiplicity of infection of 20 for 24 h.

Preparation of siRNAs and Transfection of siRNAs into Endothelial Cells. Four siRNAs targeting human eNOS with the following sense and antisense sequences were used: numbers 1, 5′-UGAAGCACCUGGAGAAUGAUU-3′ (sense) and 5′-PUCAUU-CUCCAGGUGCUUCAUU-3′ (antisense); 2, 5′-CGGAACAGCAC-AAGAGUUAUU-3′ (sense) and 5′-PUAACUCUUGUGCUGUUC-CGUU-3′ (antisense); 3, 5′-CGAGGAGACUUCCGAAUCUUU-3′ (sense) and 5′-PAGAUUCGGAAGUCUCCUCGUU-3′ (antisense); and 4, 5′-AGGAGAUGGUCAACUAUUUUU-3′ (sense) and 5′-PAAAUAGUUGACCAUCUCCUUU-3′ (antisense). Control (nonsil.) siRNA and fluorescein (20 μM) were used for negative control. The siRNAs were dissolved in siRNA buffer (20 mM KCl, 6.0 mM HEPES, pH 7.5, and 0.2 mM $MgCl_2$) to prepare a 20 μM stock solution. We grew 3×10^5 BAECs in a six-well plate for 24 h and then transfected with siRNAs in 1.5 ml of serum and antibiotics-free medium using Lipofectamine 2000. Five microliters of Lipofectamine 2000 in 245 μl of Opti-MEM was incubated at room temperature for 5 min and then added in 5 μl of siRNA stock solution in 245 μl of Opti-MEM. After 20-min incubation at room temperature, siRNAs-lipid complex solution was added to the cells in serum. After incubation for 6 h at 37°C, the medium was replaced with 2% CS and 1% penicillin (100 U/ml), streptomycin (100 μg/ml) contained medium and cultured for 48 h.

Statistical Analysis. Data were obtained from three or four different experiments. Results are expressed as the mean of independent experiments (mean ± S.D.). Overall differences between groups were analyzed using a two-way ANOVA followed by the Fisher's post hoc test for determining differences between means when more than two groups were compared. An independent t test was used when only two groups were compared. In all tests, a probability level of $P < 0.05$ was used as the decision rule for significance testing.

Results

Effects of a High-Glucose Levels and 17β-E2 on BH4 Levels and GTPCH-I Activity and Expression. Abnormally low levels of cofactor BH4 may be a factor involved in eNOS dysfunction; therefore, we measured BH4 levels in BAECs cultured for 72 h under high-glucose conditions. Under the two high-glucose conditions (10.5 and 30.5 mM), BH4 levels were significantly decreased by 20% compared with those under normal glucose conditions (5.5 mM) (Fig. 1A). In contrast, no change was observed in the number of cells with exposure to high glucose [95.3 ± 4.8 and 92.2 ± 6.1% of the control (5.5 mM glucose) with 10.5 and 30.5 mM glucose, respectively]. To evaluate whether 17β-E2 is effective at ameliorating eNOS dysfunction, BAECs were treated with 17β-E2. The addition of 17β-E2 (1 nM) significantly increased BH4 levels not only under normal but also under high-glucose conditions (Fig. 1A). Even under hyperglycemic conditions, almost the same amount of BH4 was obtained as under normal glucose conditions. To rule out an osmotic effect, we added 25 mM mannitol to 5.5 mM glucose and 20 mM mannitol to 10.5 mM glucose. It was confirmed that mannitol did not affect BH4 levels [102.3 ± 2.4 and 107.5 ± 6.2% of control (5.5 mM glucose) with and without 17β-E2, respectively] or GTPCH-I activity (98.3 ± 2.9 and 103.5 ± 6.1% of the control 5.5 mM glucose with and without 17β-E2, respectively).

Furthermore, we measured the activity and mRNA expression of GTPCH-I, a rate-limiting enzyme required for BH4 synthesis. GTPCH-I activity also decreased under high glucose conditions and was increased by 17β-E2 (Fig. 1B). The basal expression level of GTPCH-I mRNA under high-glucose conditions did not significantly differ from that under normal glucose conditions. Treatment with 17β-E2 resulted in a significant increase in GTPCH-I mRNA under both normal and high-glucose conditions (Fig. 1C). Interestingly, a more marked effect of 17β-E2 treatment on GTPCH-I mRNA was observed under high-glucose conditions.

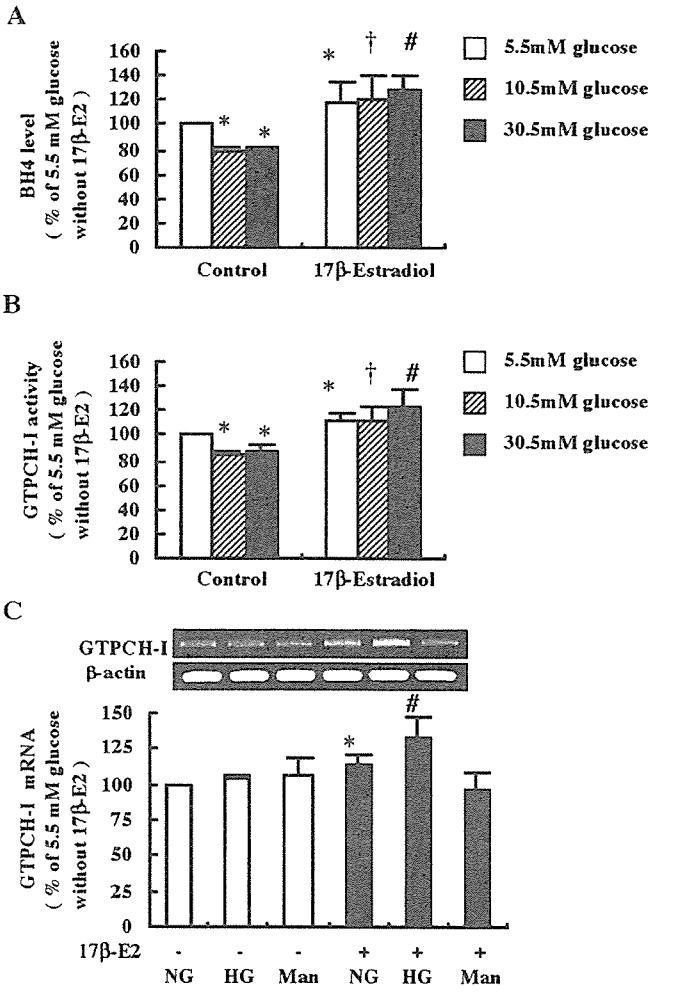

Fig. 1. Effect of 17β-E2 on BH4 levels (A), GTPCH-I activity (B), and GTPCH-I mRNA expression (C) in BAECs under different glucose levels. Cells were incubated for 72 h with different concentrations of D-glucose (5.5, 10.5, or 30.5 mM) in the absence or presence of 1 nM 17β-E2. Mannitol was added to rule out an osmotic pressure effect. It was confirmed that mannitol exerted no effect on either BH4 levels or GTPCH-I activity. In C, representative reverse transcription-PCR data are shown in the top image. Note that there is no apparent difference in β-actin mRNA, which was used as an internal control. Each result is expressed as the percentage of the value compared with that of the band obtained under normal (5.5 mM) glucose conditions without 17β-E2. *, $P < 0.05$ compared with normal (5.5 mM) glucose in the absence of 17β-E2. †, $P < 0.05$ compared with samples treated with 10.5 mM glucose in the absence of 17β-E2. #, $P < 0.05$ compared with samples treated with 30.5 mM glucose in the absence of 17β-E2 (A and B) or with those treated at a normal glucose concentration in the presence of 17β-E2 (C), as determined by ANOVA followed by Fisher's post hoc test. HG, high (30.5 mM) glucose; Man, mannitol (25 mM); NG, normal glucose.

Effects of High Glucose and 17β-E2 on eNOS Protein Expression. To determine whether a longer period of exposure to high glucose can affect the levels of expression of eNOS, we analyzed the expression levels of eNOS protein in

BAECs cultured for 72 h under high-glucose conditions. In this study, a high-glucose concentration was found to reduce the level of expression of eNOS protein, with a decrease of 19% at 10.5 mM and of 36% at 30.5 mM (Fig. 2A) after 72 h. We previously reported that 17β-E2 (10^{-10}~10^{-8} M) increases eNOS expression in BAECs (Hayashi et al., 1995b). In this study, 1 nM 17β-E2 caused 1.4- and 2.1-fold increases in eNOS protein expression under normal and high-glucose conditions, respectively (Fig. 2B). Interestingly, an even more marked effect of 17β-E2 treatment on eNOS protein expression was observed under high-glucose conditions; this effect was similar to that of 17β-E2 treatment on the expression of GTPCH-I, which is involved in the synthesis of cofactor BH4.

Effects of High-Glucose Levels and 17β-E2 on the Production of Nitrite. Given the finding that the expression levels of BH4 and eNOS protein were low in BAECs cultured for 72 h under high-glucose conditions and that normal levels were restored by treatment with 17β-E2, we measured the levels of stable NO metabolites, nitrite and nitrate, in the culture medium. Under high-glucose conditions, nitrite production was decreased by 15% compared with that under normal glucose conditions, whereas the addition of 1 nM 17β-E2 restored nitrite production to normal glucose levels (Fig. 3). The decrease in nitrite levels in a high glucose environment may be associated with an increase in the production of ROS.

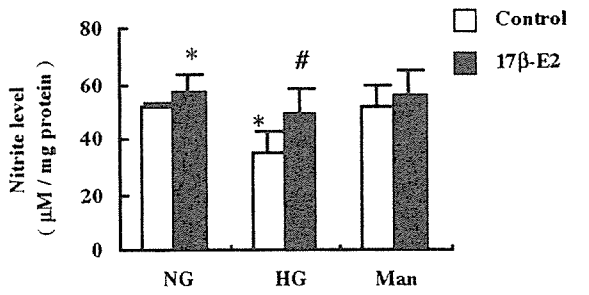

Fig. 3. Effect of 17β-E2 on the nitrite concentrations in the cultured medium of BAECs under normal and high-glucose conditions. Cells were incubated for 72 h with 5.5 or 30.5 mM glucose in the absence (open bar) or presence (solid bar) of 1 nM 17β-E2. The nitrite concentrations are expressed as percentage of the value obtained under normal glucose conditions in the absence of 17β-E2. *, $P < 0.05$ compared with normal glucose in the absence of 17β-E2. #, $P < 0.05$ versus high glucose in the absence of 17β-E2, by ANOVA followed by the Fisher's post hoc test. Each result is expressed as percentage of the value under normal (5.5 mM) glucose conditions. Man, 25 mM.

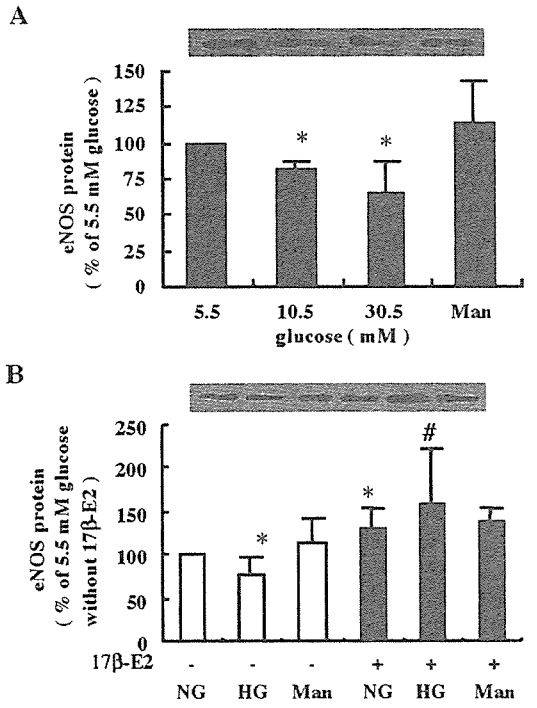

Fig. 2. A, changes in eNOS protein expression in BAECs under different glucose levels. B, effects of 17β-E2 on the high-glucose-induced decrease in eNOS protein expression in BAECs. Cells were incubated for 72 h with different concentrations of D-glucose (5.5, 10.5, or 30.5 mM) in the absence or presence of 1 nM 17β-E2. Mannitol (25 mM) was added to the samples to rule out an osmotic pressure effect. Each densitometric result is expressed as the relative percentage of the band obtained under abnormal glucose conditions compared with the band obtained under normal (5.5 mM) glucose conditions in the absence of 17β-E2 taken as 100%. Representative immunoblots are shown in the top trace of each panel. *, $P < 0.05$ compared with the sample exposed to a normal glucose concentration in the absence of 17β-E2. #, $P < 0.05$ compared with the sample exposed to a high (30.5 mM) glucose concentration and in the absence of 17β-E2, as determined by ANOVA, followed by Fisher's post hoc test.

Roles of ERα and Protein Kinase C in the Effects of 17β-E2 on eNOS under High-Glucose Conditions. To determine the mechanism underlying the effects of 17β-E2 under high-glucose conditions, we investigated the role of estrogen receptors and PKC. Two types of ER have been identified to date (ERα and ERβ) (Walter et al., 1985; Kuiper et al., 1996). ERα is known to have a protective effect on vascular tissues, and a deficiency in the expression of ERα in endothelial cells is known to accelerate atherosclerosis (Rubanyi et al., 1997; Sudhir et al., 1997). In contrast, ERβ is more widely present in tissues, and in females it is abundantly expressed in smooth muscle cells (Mendelsohn, 2002). In this study, we examined the role of ERα in eNOS expression in BAECs under high-glucose conditions. Although ERα protein levels tended to become lower under high-glucose conditions compared with those under normal glucose conditions, gene transfer of ERα resulted in a successful increase in ERα under both conditions (Fig. 4A). The reduction in eNOS protein expression by exposure to high levels of glucose was counteracted by gene transfer of ERα using adenovirus as well as by the administration of 17β-E2 (Fig. 4, A and B). The up-regulating effect of 17β-E2 was abolished by incubation of cells with ICI 182,780, a specific antagonist of ER, for 72 h (Fig. 4C). Furthermore, ICI 182,780 significantly decreased eNOS expression in the presence of 17β-E2 under normal glucose conditions. It is thought that PKC might be activated by hyperglycemia and thereby reduce eNOS expression (Srinivasan et al., 2004). The PKC inhibitor bisindolylmaleimide I significantly enhanced the protein expression of eNOS in the presence of 17β-E2 under high-glucose conditions without affecting that under normal glucose conditions (Fig. 4C).

Effect of siRNAs Targeting eNOS on GTPCH-I mRNA Levels under Normal and High-Glucose Conditions. To determine whether or not there is a correlation between the expression levels of eNOS and GTPCH-I, we used siRNA to specifically ablate eNOS mRNA in BAECs. Two of four siRNAs targeting eNOS (numbers 2 and 3) successfully silenced the expression of eNOS mRNA (Fig. 5A) and protein (Fig. 5C) compared with that of the negative control (nonsil.) siRNA 48 h after transfection. The expression levels of GT-

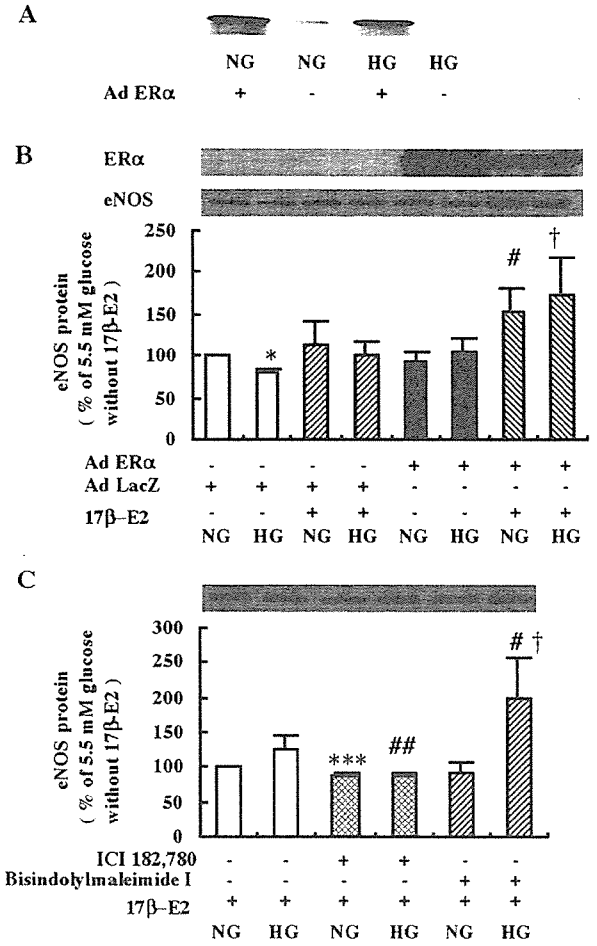

Fig. 4. A, effects of ERα overexpression by gene transfer of adenovirus vector (Ad ERα) on ERα protein expression in BAECs under normal and high-glucose conditions. Where indicated, cells were treated with 1 nM 17β-E2. B, effects of ERα overexpression by gene transfer of Ad ERα on eNOS protein expression in BAECs under normal and high-glucose conditions. The cells transfected with adenovirus carrying an *E. coli* lacZ gene (Ad LacZ) were served as control. Where indicated, cells were treated with 1 nM 17β-E2. *, $P < 0.05$ compared with normal glucose, Ad LacZ transfection. #, $P < 0.05$ compared with normal glucose, Ad LacZ transfection in the presence of 17β-E2. †, $P < 0.05$ compared with high-glucose, Ad LacZ transfection, by ANOVA followed by the Fisher's post hoc test. C, effects of ICI 182,780 and bisindolylmaleimide I on eNOS protein expression in BAECs treated with 17β-E2 under normal and high-glucose conditions. Cells were incubated for 72 h with 1 μM ICI 182,780 and 3 μM bisindolylmaleimide I. ***, $P < 0.001$ compared with normal glucose in the presence of 17β-E2. #, $P < 0.05$; ##, $P < 0.01$ compared with high glucose in the presence of 17β-E2. ‡, $P < 0.05$ compared with normal glucose in the presence of 17β-E2 and bisindolylmaleimide I, by ANOVA followed by the Fisher's post hoc test. In both A and B, each result is expressed as percentage of the control value under normal glucose conditions. Representative immunoblots are shown in the top trace of each panel. Note that ERα is usually detected as a very faint band. HG, 30.5 mM; NG, 5.5 mM.

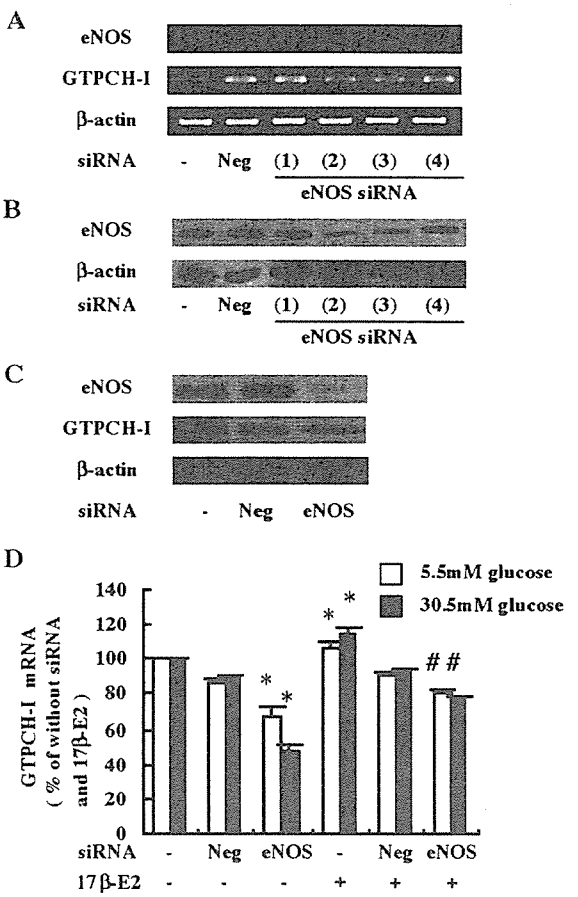

Fig. 5. Effect of siRNAs targeting eNOS on GTPCH-I mRNA expression in BAECs. A, representative reverse transcription-PCR data showing that two of four eNOS siRNAs (numbers 2 and 3) successfully eliminated eNOS mRNA 48 h after transfection and reduced GTPCH-I mRNA. Neg, negative control. B, representative immunoblot data showing no detection of eNOS protein 48 h after transfection of successful eNOS siRNA (numbers 2 and 3). C, representative immunoblot data showing no detection of eNOS protein and GTPCH-1 protein 48 h after transfection of successful eNOS siRNA. D, modulation by 1 nM 17β-E2 of the effect of successful eNOS siRNA (2) on the mRNA expression level of GTPCH-I in BAECs under normal (5.5 mM) and high (30.5 mM)-glucose conditions. *, $P < 0.05$ compared with control. #, $P < 0.05$ compared with transfection of eNOS siRNA in the absence of 17β-E2, by ANOVA followed by the Fisher's post hoc test.

PCH-I mRNA were significantly down-regulated by the elimination of eNOS mRNA (Fig. 5A). This finding suggests that the expression of GTPCH-I is greatly affected by the presence of eNOS. Under normal and high-glucose conditions, the transfection of eNOS siRNA (number 2) decreased GTPCH-I mRNA by 34 and 54%, respectively. The addition of 17β-E2 slightly but significantly increased GTPCH-I mRNA expression under normal and high-glucose conditions. Although the ablation of eNOS by siRNA markedly depressed GTPCH-I expression under both normal and high-glucose conditions, the presence of 17β-E2 strongly reversed this depression (Fig. 5B).

Discussion

In the present study, the impairment of endothelial function under hyperglycemic conditions was detected as a decrease in NO production resulting from the down-regulation of eNOS expression, BH4 levels, and the GTPCH-I expression and activity in BAECs. This decrease in NO production could lead to increased oxidative stress and impaired vascular dilation. Thus, it is most likely that an enhancement of BH4 synthesis and NO bioavailability may be important in preventing diabetic cardiovascular complications. Previously, we reported that under high-glucose conditions, the activation of NADPH oxidase and the uncoupling of eNOS due to a BH4 deficiency increased ROS (Ding et al., 2004).

We inferred that 17β-E2 may reverse hyperglycemia-in-

duced endothelial dysfunction, because premenopausal women have a lower incidence of cardiovascular disease than do postmenopausal women and men of a similar age (Gordon et al., 1978; Fujishima et al., 1996). Our present results showed that treatment with the physiological concentration of 17β-E2 (1 nM) in endothelial cells simultaneously exposed to high levels of glucose increased not only the eNOS expression level but also intracellular BH4 as well as GTPCH-I activity and gene expression levels. Interestingly, 17β-E2 was found to be more effective under high-glucose conditions. The characteristic effects of 17β-E2 on hyperglycemia have been noted in a previous clinical study (Colacurci et al., 1998), although the underlying mechanisms have not yet been investigated. It has been reported that 17β-E2 elevates mRNA levels of GTPCH-I and intracellular BH4 levels in microvascular endothelial cells in the brain (Serova et al., 2004). However, the overall pattern of effects of 17β-E2 on BH4 and GTPCH-I observed in that study seems to differ somewhat from the pattern observed in BAECs in the present study, based on differences between microvessels and muscular and elastic arteries. Statins (3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors) and insulin have also been shown to increase both BH4 levels and GTPCH-I activity in endothelial cells (Ishii et al., 2001; Hattori et al., 2003). However, to the best of our knowledge, the present study is the first to indicate that the beneficial effect of 17β-E2 on eNOS under high-glucose conditions may be associated with a recovery of GTPCH-I in endothelial cells.

There are several reports showing the profound effect of high levels of glucose on eNOS expression. Shrinivasan et al. (2004) have documented that, in human aortic endothelial cells, acute elevated glucose (4 h) led to the up-regulation of eNOS expression; however, chronic elevated glucose (1~7 days) was associated with decreases in eNOS expression and nitrite production as well as with increases in the mitochondrial production of ROS. Our previous study demonstrated that high-glucose exposure for 24 h significantly increased eNOS mRNA expression (Ding et al., 2004). Noyman et al. (2002) have found reduced levels of eNOS expression and activity with a decrease in insulin sensitivity in BAECs cultured under high-glucose conditions for 2 weeks. In contrast, one previous study revealed that eNOS expression and NO production are increased in human aortic endothelial cells cultured under high-glucose conditions for 2 weeks. Possible reasons for this apparent discrepancy may include the use of different types of endothelial cells and different culture conditions. In agreement with recent results (Noyman et al., 2002; Srinivasan et al., 2004), the present results revealed decreases in eNOS protein expression and nitrite production in BAECs cultured under high-glucose conditions for 1~3 days.

This study supports our previous reports showing a gender difference in NO production in the aortas of animals and humans (Hayashi et al., 1992, 1995a, 2002). We have reported that atherosclerosis developed more slowly in female rabbits than in male rabbits fed a high-cholesterol diet, which may have been caused by a difference in the basal release as well as the stimulated release of NO (Hayashi et al., 1995a,b, 2000, 2002). The results of the present study also suggest that estrogen contributes to the improvement of endothelial function and provides protection against the progression of atherosclerosis. We have also shown that basal eNOS can be activated by 17β-E2 at physiological concentrations (10^{-12}~10^{-8} M) in young cells (passaged fewer than six times), whereas ERα is decreased in old cells (passaged more than 12 times) (Hayashi et al., 1995b). We preliminarily observed that the expression of ERα at passages 6 to 8 tended to be lower under high-glucose than under normal glucose conditions (Fig. 4A). Accordingly, the gene transfer of ERα may be important for allowing estrogen to act effectively against the type of endothelial dysfunction caused by aging and hyperglycemia. Treatment with 17β-E2 following the gene transfer of ERα using adenovirus resulted in a large increase in eNOS expression in BAECs. This effect was abolished by the addition of ICI 182,780, an ER antagonist, which confirmed the involvement of genomic activity.

PKC may be yet another important factor in eNOS regulation in hyperglycemia. Ohara et al. (1995) have shown that PKC inhibits eNOS expression in BAECs. It has been suggested that the decrease in eNOS expression observed in hyperglycemia is mediated by PKC and mitochondrial ROS via the activation of oxidative stress transcription factor AP-1 (Srinivasan et al., 2004). eNOS activated by the phosphorylation of Ser1177 through the phosphatidylinositol 3-kinase/Akt pathway is inactivated by the phosphorylation of Thr495 and dephosphorylation of Ser1177 through PKC (Matsubara et al., 2003). Hyperglycemia seems to alter the signaling pathway activating eNOS via modification at the Akt site (Du et al., 2001) and the activation of PKC (Ishii et al., 2001; Naruse et al., 2006). In the present study, the PKC inhibitor bisindolylmaleimide I enhanced the effects of 17β-E2 on eNOS expression under high-glucose conditions, suggesting that the recovery effect of 17β-E2 on high-glucose-induced eNOS down-regulation is regulated by PKC.

Furthermore, this study is the first to demonstrate that eNOS expression levels affect GTPCH-I expression levels in endothelial cells. We thus found that transfection with siRNA-targeting eNOS reduced the expression of GTPCH-I mRNA. This suggests that the decrease in eNOS observed under pathological conditions such as inflammation, atherosclerosis, or diabetes accelerates endothelial dysfunction by decreasing levels of the cofactor BH4. However, even when eNOS siRNA was transfected, 17β-E2 was capable of increasing GTPCH-I expression levels. Thus, this effect of 17β-E2 was more clearly demonstrated in an experiment using eNOS siRNA, in which 17β-E2 enhanced the level of GTPCH-I mRNA expression, regardless of whether GTPCH-I was down-regulated by high-glucose conditions.

In diabetes, elevated blood glucose, altered insulin signaling, increased ROS, enhanced PKC, and inflammation can together or separately lead to a decrease in NO bioavailability (Endemann and Schiffrin, 2004). High-glucose levels reduced eNOS expression, GTPCH-I expression, and BH4 levels in BAECs. Finally, accelerated NO degradation by ROS might result in a decline in NO bioavailability, although we failed to confirm the possible involvement of oxidative stress in the high-glucose-induced decrease in eNOS expression in BAECs. Importantly, 17β-E2 treatment ameliorated these endothelial cell abnormalities under high-glucose conditions, which seemed to be related to the activation of ERα and masking by PKC.

The results of the present study may contribute to an understanding of the anti-atherogenic effects of estrogen in preventing endothelial dysfunction in diabetes mellitus (Fig.

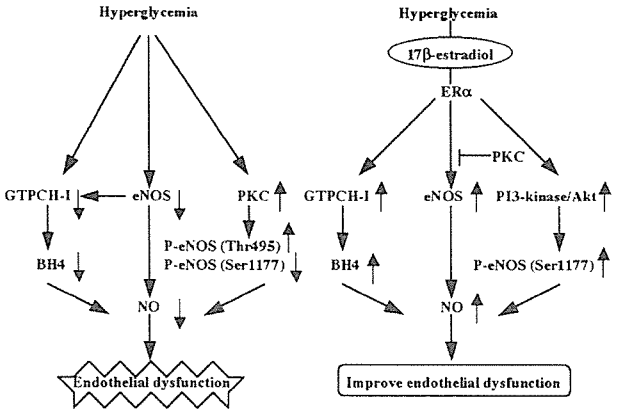

Fig. 6. Diagram of the possible mechanism underlying the effect of 17β-E2 on hyperglycemia-induced endothelial dysfunction. Left, hyperglycemia would cause endothelial dysfunction by decreasing eNOS expression and BH4 synthesis and by activating PKC. The decrease in eNOS expression could lead to a decrease in GTPCH-I expression. PKC may inactivate eNOS possibly due to phosphorylation of Thr495 and dephosphorylation of Ser1177. Right, 17β-E2 via activation of ERα could increase eNOS expression and BH4 synthesis even in hyperglycemia, and activate eNOS by phosphorylating Ser1177 through the phosphatidylinositol 3 (PI3)-kinase/Akt pathway. The increase in eNOS expression by 17β-E2 may be masked by PKC.

6). If estrogen also prevents endothelial NO dysfunction under high-glucose conditions in humans, premenopausal diabetic women may be protected against the progression of atherosclerosis. Moreover, the prevalence of coronary heart disease in premenopausal women is much lower than that in the same generation of males or that of postmenopausal women. HRT in postmenopausal diabetic women may provide the benefit of reduced risk of developing cardiovascular disease. However, it should be noted that WHI (Pradhan et al., 2002) and Women's Health Initiative Memory Study (Brinton, 2005) indicated the risk associated with HRT (0.625 mg of conjugated equine estrogen and 2.5 mg of medroxyprogesterone acetate per day) for stroke, coronary heart disease, and Alzheimer's disease, even in diabetic patients. However, most of the patients in the study had another coronary risk factor such as obesity (66%), smoking habit (50%), and hypertension (35%). Moreover, the data from the WHI from patients without a uterus who had been prescribed only conjugated equine estrogen showed no increase in coronary heart disease. It is generally thought that estrogen may be atheroprotective (Hayashi et al., 2002); however, in most clinical cases, concomitant administration of progesterone, which masks the beneficial effects of estrogen, is necessary to prevent uterine cancer. Therefore, we cannot conclude that estrogen has a negative effect, simply based on the results of the WHI study. The present study conclusively demonstrates that the activation of ERα with 17β-E2 can counteract the high-glucose-induced down-regulation of eNOS and GTPCH-I in endothelial cells. Therefore, estrogen may be of potential use in the reversal of hyperglycemia-induced endothelial dysfunction, in turn retarding the development of cardiovascular disease in postmenopausal diabetic women. The clinical implications of the present findings will need to be addressed in future studies.

References

Albrecht EW, Stegeman CA, Herringa P, Henning RH, and Goor HV (2003) Protective role of endothelial nitric oxide synthase. *J Pathol* **199:**8–17.

Brinton RD (2005) Investigative models for determining hormone therapy-induced outcomes in brain: evidence in support of a healthy cell bias of estrogen action. *Ann NY Acad Sci* **1052:**57–74.

Barrett-Connor EL, Cohn BA, Wingard DL, and Edelstein SL (1991) Why is diabetes mellitus a stronger risk factor for fatal ischemic heart disease in women than in men? The Rancho Bernardo Study. *J Am Med Assoc* **265:**627–631.

Cai H and Harrison DG (2000) Endothelial dysfunction in cardiovascular disease: the role of oxidant stress. *Circ Res* **87:**840–844.

Calles-Escandon J and Cipolla M (2001) Diabetes and endothelial dysfunction: a clinical perspective. *Endocr Rev* **22:**36–52.

Colacurci N, Zarcone R, Mollo A, Russo G, Passaro M, de Seta L, and de Franciscis P (1998) Effects of hormone replacement therapy on glucose metabolism. *Phanminerva Med* **40:**18–21.

Creager MA, Luscher TF, Beckman JA, and Cosentino F (2003) Diabetes and vascular disease: pathophysiology, clinical consequences, and medical therapy: part I. *Circulation* **108:**1527–1532.

Ding QF, Hayashi T, Packiasamy AR, Miyazaki A, Fukatsu A, Shiraishi H, Nomura T, and Iguchi A (2004) The effects of high glucose on NO and O_2^- through endothelial GTPCH1 and NADPH oxidase. *Life Sci* **75:**3185–3194.

Du XL, Edelstein D, Dimmeler S, Ju Q, Sui C, and Brownlee M (2001) Hyperglycemia inhibits endothelial nitric oxide synthase activity by posttranslational modification at the Akt site. *J Clin Investig* **108:**1341–1348.

Endemann DH and Schiffrin EL (2004) Nitric oxide, oxidative excess, and vascular complications of diabetes mellitus. *Curr Hypertens Rep* **6:**85–89.

Fujishima M, Kiyohara Y, Kato I, Ohmura T, Iwamoto H, Nakayama K, Ohmori S, and Yoshitake T (1996) Diabetes and cardiovascular disease in a prospective population survey in Japan. The Hisayama Study. *Diabetes* **45:**S14–S16.

Fukushima T and Nixon JC (1980) Analysis of reduced forms of biopterin in biological tissue and fluids. *Anal Biochem* **102:**176–188.

Gesierich A, Niroomand F, and Tiefenbacher CP (2003) Role of human GTP cyclohydrolase I and its regulatory protein in tetrahydrobiopterin metabolism. *Basic Res Cardiol* **98:**69–75.

Gordon T, Kannel WB, Hjortland MC, and McNamara PM (1978) Menopause and coronary heart disease. The Framingham Study. *Ann Intern Med* **89:**157–161.

Hattori Y, Nakanishi N, Akimoto K, Yoshida M, and Kasai K (2003) HMG-CoA reductase inhibitor increases GTP cyclohydrolase I mRNA and tetrahydrobiopterin in vascular endothelial cells. *Arterioscler Thromb Vasc Biol* **23:**176–182.

Hayashi T, Fukuto JM, Ignarro LJ, and Chaudhuri G (1992) Basal release of nitric oxide from aortic rings is greater in female rabbits than in male rabbits: implications for atherosclerosis. *Proc Natl Acad Sci USA* **89:**11259–11263.

Hayashi T, Fukuto JM, Ignarro LJ, and Chaudhuri G (1995a) Gender differences in atherosclerosis formation. Possible role of nitric oxide. *J Cardiovasc Pharmacol* **26:**792–802.

Hayashi T, Jayachandran M, Sumi D, Thakur NK, Esaki T, Muto E, Kano H, Asai Y, and Iguchi A (2000) Physiological concentration of 17beta-estradiol retards the progression of severe atherosclerosis induced by a high-cholesterol diet plus balloon catheter injury: role of NO. *Arterioscler Thromb Vasc Biol* **20:**1613–1621.

Hayashi T, Juliet PA, Kano-Hayashi H, Tsunekawa T, Dingqunfang D, Sumi D, Matsui-Hirai H, Fukatsu A, and Iguchi A (2005) NADPH oxidase inhibitor, apocynin, restores the impaired endothelial-dependent and -independent responses and scavenges superoxide anion in rats with type 2 diabetes complicated by NO dysfunction. *Diabetes Obes Metab* **7:**334–343.

Hayashi T, Kano H, Sumi D, Iguchi A, Ito I, and Endo H (2002) The long-term effect of estriol on endothelial function and bone mineral density in octogenarian women. *J Am Geriatr Soc* **50:**777–778.

Hayashi T, Yamada K, Esaki T, Kuzuya M, Satake S, Ishikawa T, Hidaka H, and Iguchi A (1995b) Estrogen increases endothelial nitric oxide by a receptor-mediated system. *Biochem Biophys Res Commun* **214:**847–855.

Heitzer T, Krohn K, Albers S, and Meinnertz T (2000) Tetrahydrobiopterin improves endothelin-dependent vasodilation by increasing nitric oxide activity in patients with type II diabetes mellitus. *Diabetologia* **43:**1435–1438.

Ishii M, Shimizu S, Nagai T, Shiota K, Kiuchi Y, and Yamamoto T (2001) Stimulation of tetrahydrobiopterin synthesis induced by insulin: possible involvement of phosphatidylinositol 3-kinase. *Int J Biochem Cell Biol* **33:**65–73.

Kanaya AM, Herrington D, Vittinghoff E, Lin F, Grady D, Bittner V, Cayley JA, and Barrett-Connor E (2003) Glycemic effects of postmenopausal hormone therapy: the Heart and Estrogen/Progestin Replacement Study. A randomized double-blind, placebo-controlled trial. *Ann Intern Med* **138:**1–9.

Kannel WB and McGee DL (1979) Diabetes and glucose tolerance as risk factors for cardiovascular disease: the Framingham study. *Diabetes Care* **2:**120–126.

Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S, and Gustafsson JA (1996) Cloning of a novel receptor expressed in rat prostate and ovary. *Proc Natl Acad Sci USA* **93:**5925–5930.

Margolis KL, Bonds DE, Rodabough RJ, Tinker L, Phillips LS, Allen C, Bassford T, Burke G, Torrens J, Howard BV, et al. (2004) Effect of oestrogen plus progestin on the incidence of diabetes in postmenopausal women: results from the Women's Health Initiative Hormone Trial. *Diabetologia* **47:**1175–1187.

Matsubara M, Hayashi N, Jing T, and Titani K (2003) Regulation of endothelial nitric oxide synthase by protein kinase C. *J Biochem* **133:**773–781.

Mendelsohn ME (2002) Genomic and nongenomic effects of estrogen in the vasculature. *Am J Cardiol* **90:**3F–6F.

Naruse K, Rask-Madesen C, Takahara N, Ha SW, Suzuma K, Way KJ, Jacobs JR, Clermont AC, Ueki K, Ohshiro Y, et al. (2006) Activation of vascular protein kinase C-β inhibits Akt-dependent endothelial nitric oxide synthase function in obesity-associated insulin resistance. *Diabetes* **55:**691–698.

Noyman I, Marikovsky M, Sasson S, Stark AH, Bernath K, Seger R, and Madar Z (2002) Hyperglycemia reduces nitric oxide synthase and glycogen synthase activity in endothelial cells. *Nitric Oxide* **7:**187–193.

Ohara Y, Sayegh HS, Yami JJ, and Harrison DG (1995) Regulation of endothelial constitutive nitric oxide synthase by protein kinase C. *Hypertension* **25:**415–420.

Pradhan AD, Manson JE, Rossouw JE, Siscovick DS, Mouton CP, Rifai N, Wallace

RB, Jackson RD, Pettinger MB, and Ridker PM (2002) Inflammatory biomarkers, hormone replacement therapy, and incident coronary heart disease: prospective analysis from the Women's Health Initiative observational study. *J Am Med Assoc.* **288:**980–987.

Rubanyi GM, Freay AD, Kauser K, Sukovich D, Burton G, Lubahn DB, Couse JF, Curtis SW, and Korach KS (1997) Vascular estrogen receptor and endothelium-derived nitric oxide production in mouse aorta. Gender difference and effect of estrogen receptor gene disruption. *J Clin Investig* **99:**2429–2437.

Sawada M, Horikoshi T, Masada M, Akino M, Sugimoto T, Matsuura S, and Nagatsu T (1986) A sensitive assay of GTP cyclohydrolase I activity in rat and human tissues using radioimmunoassay of neopterin. *Anal Biochem* **154:**361–366.

Serova LI, Maharjan S, Huang A, Sun D, Kaley G, and Sabban EL (2004) Response of tyrosine hydroxylase and GTP cyclohydrolase I gene expression to estrogen in brain catecholaminergic regions varies with mode of administration. *Brain Res* **1015:**1–8.

Shinozaki K, Kashiwagi A, Nishio Y, Okamura T, Yoshida Y, Masada M, Toda N, and Kikkawa R (1999) Abnormal biopterin metabolism is a major cause of impaired endothelial dependent relaxation through nitric oxide/O_2^- imbalance in insulin–resistant rat aorta. *Diabetes* **48:**2437–2445.

Simoncini T, Hafezu-Moghadam A, Brazil DP, Ley K, Chin WW, and Liao JW (2000) Interaction of oeestrogen receptor with the regulatory subunit of phosphatidylinositol-OH kinase. *Nature (Lond)* **47:**538–541.

Srinivasan S, Hatley ME, Bolick DT, Palmer LA, Edelstein D, Brownlee M, and Hedrick CC (2004) Hyperglycaemia-induced superoxide production decreases eNOS expression via AP-1 activation in aortic endothelial cells. *Diabetologia* **47:**1727–1734.

Sudhir K, Chou TM, Chatterjee K, Smith EP, Williams TC, Kane JP, Malloy MJ, Korach KS, and Rubanyi GM (1997) Premature coronary artery disease associated with a disruptive mutation in the estrogen receptor gene in a man. *Circulation* **96:**3774–3777.

Tesfamariam B and Cohen RA (1992) Free radicals mediate endothelial cell dysfunction caused by elevated glucose. *Am J Physiol* **263:**H321–H326.

Walter P, Green S, Greene G, Krust A, Bornert JM, Jeltsch JM, Staub A, Jensen E, Scrace G, Waterfield M, et al. (1985) Cloning of the human estrogen receptor cDNA. *Proc Natl Acad Sci USA* **82:**7889–7893.

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Nitric oxide (NO) is a new clinical biomarker of survival in the elderly patients and its efficacy might be nearly equal to albumin

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Abstract

Background: For elderly patients, the consideration of prognostic factors is very important, but there have been few reports about the potential use of vasoactive substances as prognostic markers in the elderly.

Objective: We assessed endocrinological substances, such as plasma NO_x (metabolites of NO), as the prognostic marker in elderly. We compared their efficacy with that of such well-known markers as albumin and pro-inflammatory cytokines such as IL-6.

Methods: The patients were recruited consequently from the clinics of Nagoya University Hospital or related home care services facilities. One hundred and twenty seven elderly aged 65 and older were registered. Biochemical analyses such as albumin, total cholesterol, BNP, and NO_x were measured upon enrollment. The main outcome was the survival rate.

Results: Forty-six patients died during the follow-up period. Mann–Whitney's *U*-test showed that the levels of age, hemoglobin, total protein, serum albumin, serum creatinine, total cholesterol, HDL-cholesterol, LDL-cholesterol, high sensitive CRP, NO_x, IL-6, and TNF-α were significantly different between the living and deceased subjects. Among the dependent variables in the logistic regression analyses, only albumin and NO_x were significantly different. In the Kaplan–Meier analyses of mortality, the prognosis of patients in 3rd and 4th quartile of NO_x was significantly worse than that in 1st or 2nd quartile.

Conclusion: NO_x has potential both as a vascular marker and as a marker for predicting survival in elderly. In the latter role, it may be as effective as albumin.

Keywords: Nitric oxide; cGMP; Albumin; Biomarker; Elderly; Prognostic marker; Vascular functional marker

Many nations, including Japan, are experiencing rapid growth in their elderly populations. The main causes of death in Japanese elderly are heart disease, cerebro-vascular disease, and cancer. Several biochemical markers, such as albumin and cholesterol, have been identified as having prognostic value for mortality and hospitalization [1–3]. Recent studies also have indicated the potential role of the immune system in the pathophysiology of congestive heart failure (CHF) and malignancy [4,5]. Plasma levels of interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) also have been reported to be significant prognostic predictors in patients with CHF or malignancy) [6–8]. TNF-α induces adhesion molecule expression such as ICAM-1 on endothelial cells, which promotes the progression of atherosclerosis [9]. In other words, in older populations, peripheral blood markers of nutrition or inflammation (albumin, cholesterol, IL-6, and TNF-α) have been individually shown to be increased risk for mortality [2,10,11].

In elderly people, the rate of CHF is important for predicting mortality and hospitalization rates. Brain natriuretic peptide (BNP) is a good marker of CHF, because the plasma BNP concentration is elevated according to the severity of CHF [12–15]. Binding of BNP to its receptors

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initiates natriuretic and vasorelaxant activities through an elevation in intracellular cyclic guanosine monophosphate (cGMP) [16,17]. Nitric oxide (NO) is also an important vasoactive substance, because it exerts anti-atherogenic effects by inhibiting the migration or proliferation of monocytes or smooth muscle cells and vasodilation mainly by cGMP dependent mechanism [18]. We reported that NO regulates cGMP in patients with renal insufficiency [19]. NO may be a useful prognostic marker for patients suffering from atherosclerotic diseases such as cerebral strokes or myocardial infarction, although as yet there have been no reports investigating the use of NO in this capacity. The source of NO is not only endothelial cells (endothelial NO synthase; eNOS) but also macrophages or T cells (inducible NO synthase; iNOS) and some neuronal cells (neuronal NO synthase; nNOS). The plasma level of NO_x (nitrite plus nitrate, metabolites of NO) may reflect the status of eNOS and, to some extent, the status of iNOS. Because iNOS is activated in patients with inflammations such as sepsis, advanced stages of malignancy, or progressed atherosclerotic lesions, the NO_x level may have potential as a marker of malignancy as well as atherosclerotic diseases [20,21].

For elderly patients, the consideration of prognostic factors is very important, but there have been few reports about the potential use of vasoactive substances. Therefore, in this study, we evaluated whether measurements of plasma levels of vasoactive factors such as NO_x, cytokines such as IL-6, and well-known markers such as albumin were useful as prognostic factors in the elderly.

Methods

Study sample

One hundred and twenty seven elderly subjects (48 males and 79 females; mean age, 81.3 ± 7.5 years; range, 65–101 years) were enrolled on August on 2002. The study was approved by the Ethics Committee of Nagoya University Graduate School of Medicine and written informed consent was obtained from all patients. Patients were selected consecutively among our geriatric clinics and related home care services. In brief, 91 participants were presented at Department of Geriatrics, Nagoya University Hospital and the related hospital as outpatients (31 from their homes, 31 from geriatric nursing care units, and 29 from other facilities such as private homes for the aged) and 36 were in home care services facility. At the baseline examination, participants underwent a review of their medical history, a physical examination, and assessment of cardiovascular disease risk factors. On registration, they were not suffering with acute or evident heart failure or acute inflammation whose serum CRP is larger than 2 mg/dl. They were also not suffering with acute myocardial infarction or cerebral infarction within 3 months. We followed patients up to 2.8 years. All participants had a clinical visit each year of the study period, and their laboratory data were determined at each of these visits. We had telephone contact with the patients who could not have clinical visit, or their physicians.

Measurement

We measured fasting serum or plasma levels of biochemical products including lipids and plasma levels of neurohumoral factors and cytokines. Levels of general biochemical products were measured at SRL Laboratories, Tokyo, on an automated sequential multiple analyzer. Samples for the assay of plasma norepinephrine (NE), angiotensin-II, BNP, NO_x, cGMP, IL-6, and TNF-α levels were transferred to chilled disposable tubes containing EDTA-2Na. The blood samples were immediately placed on ice and centrifuged at −4 °C, and aliquots of plasma were immediately stored at −80 °C until assay. BNP levels were measured with a specific radioimmunoassay. NE levels were measured by HPLC. NO_x levels were measured using an NO detector-HPLC system (ENO10; Eicom Co., Kyoto, Japan) [22]. cGMP concentration was determined using a specific radioimmunoassay method (RPN226; Amersham, Buckinghamshire, England) [23]. Angiotensin-II levels were measured by radioimmunoassay. Both IL-6 and TNF-α measurements were performed using a commercially available radioimmunoassay kit (Quantikine HS; R&D Systems, Minneapolis, MN). Hypertension was defined as systolic BP ⩾ 140 mmHg, or diastolic BP ⩾ 90 mmHG or antihypertensive drugs were prescribed. Hyperlipidemia was defined as follows. Total cholesterol ⩾ 220 mg/dl or LDL cholesterol (total cholesterol − HDL cholesterol − triglyceride/5) ⩾ 140 mg/dl) or anti-hyperlipidemic drugs were prescribed. Diabetes mellitus was defined as in American Diabetes Society Guidelines [24] (in brief, fasting blood glucose ⩾ 126 mg/dl or hemoglobin A1C ⩾ 6.5 g/dl). Previously diagnosed hypertension, hyperlipidemia or diabetes were also included.

Statistical analysis

The results are presented as means ± SD. Values of $P < .05$ were considered to indicate statistical significance in all analyses. All statistical analyses were performed using Stat View software (SAS Institute Inc., Cary, NC). Characteristics of the survivors and the deceased subjects were compared using Mann–Whitney's *U*-test. Characteristics that were significantly different between the survivors and deceased by Mann–Whitney's *U*-test were further subjected to inherent multiple logistic regression analysis. As a result, adjusted odds ratios were calculated. Survival curves were calculated by the Kaplan–Meier method.

Results

Clinical characteristics

Table 1 shows the baseline characteristics of patients. There were no significant differences in age or coronary risk factors among the situations where the patients were