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REVIEW ARTICLE

Possibility of the regression of atherosclerosis through the prevention of endothelial senescence by the regulation of nitric oxide and free radical scavengers

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In the elderly, atherosclerotic diseases such as stroke and myocardial infarction occupy a major part of their causes of death and care. The elderly always have atherosclerosis in their aorta and other arteries and are exposed to risk of attacks. It is the elderly who should receive its safe, harmless and advanced treatment. Advanced stage of atherosclerosis in the elderly is progressed by complicated risk factors such as dyslipidemia and diabetes mellitus and specific risk factors for the elderly, aging (and menopause). Treatment of atherosclerotic disease may need special ones targeted for the elderly. Recent studies reported that frequencies of dyslipidemia were not decreased in the older oldest. In the elderly, impaired glucose tolerance occurrs and it progresses atherosclerosis. Endothelial dysfunction like impairment of nitric oxide (NO) bioavailability also progresses atherosclerosis. Although we tried to regress the high cholesterol diet-induced atherosclerosis in rabbit aorta with a normal diet with or without statin, regression could not be achieved. NO targeting gene therapy (adenovirus endothelial nitric oxide synthase [eNOS] gene vector) regressed 20% of atherosclerotic lesions through reduction of lipid contents, however, a more integrated strategy is important for complete regression. We paid attention to NO bioavailability and developed two ways of increasing it in atherosclerosis: citrulline therapy and arginase II inhibition by estrogen. Further, we found a close relation between atherosclerosis and endothelial senescence and that NO can prevent it, especially in a diabetic model. Taken together, regression of atherosclerosis can be achieved by not only regulation of various risk factors but regulation of the cross-talk of NO and free radicals. **Geriatr Gerontol Int 2010; 10: 115–130.**

Keywords: cellular senescence, endothelial cells, endothelial nitric oxide synthase, hyperglycemia, nitric oxide.

Introduction

The elderly usually have initial stages of atherosclerosis in their aorta and other muscular arteries and are exposed to incident risk of onset of the severe diseases. Plasma low-density lipoprotein (LDL) cholesterol levels are usually four times higher than those of other mammals. Recent observed studies reported that the frequency of dyslipidemia was not decreased even in the

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older oldest when their activities of daily living (ADL) are maintained. Further, in the elderly, impaired glucose tolerance easily occurs and it is known to proceed the progression of atherosclerosis. The common injured or dysfunctional tissue by those risk factors is endothelium. Endothelial dysfunction such as impairment of nitric oxide (NO) bioavailability is also known to progress atherosclerosis. Oxidative stress is related to the various stages of cardiovascular diseases from the onset of underlying coronary risk factors to the final stage of acute coronary syndrome by thrombosis. It is important to understand the cross-talk and its failure between preventive factors represented by NO and pathological factors such as O_2^-. The result of the failure of cross-talk is a common denominator, but the mechanism by which failure occurs depends on the specific pathophysiological condition. Cellular senescence is characterized by permanent exit from the cell cycle. The occurrence of vascular endothelial cell senescence *in vivo*, and the senescent phenotype of endothelial cells can be transformed from anti-atherosclerotic to pro-atherosclerotic. In this review, we discuss the potential mechanisms underlying the ability of NO to prevent endothelial cell senescence and describe the possible changes in the NO-mediated anti-senescence effect under pathophysiological conditions, including oxidative stress and hyperglycemia. We provide insights into the potential of NO-based anti-senescence therapy for age-associated atherosclerosis.

Progression and retardation of atherosclerosis and NO

Response to injury hypothesis

The famous hypothesis by Russell Ross on atherosclerosis formation has been revised three times and established as a "response to injury hypothesis".[1-3] This theory asserts that reactive oxygen species (ROS) play an important role in the initiation and progression of atherosclerosis, especially through impairment of endothelium- and macrophage-derived foam-cell formation as an initial stage of atherosclerosis.[4] This process of atherosclerosis formation involves adhesion of blood monocytes to endothelial cells, chemotaxis of monocytes into the intra-endothelial space, and changes to macrophages and foam-cell formation by endocytosis of oxidized LDL. As a chemotactic response, smooth muscle cells migrate from the media into subendothelial spaces and proliferate. Several mechanisms participate in this.

Physiological and pathophysiological functions of NO

The vascular endothelium is the interface between the blood flow and the vascular wall. Endothelial cells regulate vascular tone and homeostasis by generating a number of autacoids. NO is a representative one regulating vascular tone, dilatation and homeostasis. Endothelial NO synthase (eNOS) expression is regulated by transcription (rate of eNOS gene transcription), stabilization (eNOS mRNA stability) and phosphorylation. As the eNOS promoter has multiple potential *cis*-regulatory DNA sequences, including SP1, AP1 and AP2, p53 binding regions and shear stress (or estrogen) response elements, hydrogen peroxide, protein kinase C (PKC) and estrogen can increase eNOS transcriptional regulation, and tumor necrosis factor (TNF)-α can decrease it.[5-7]

Endothelial NO synthase mRNA has a long half-life at baseline (10–35 h), and post-transcriptional regulation may be more important ([8] Searles CD Transcriptional and posttranscriptional regulation of endothelial nitric oxide synthase expression. Am J Physiol Cell Physiol. 2006;291:C803-16.). eNOS mRNA stability could be increased by statins or estrogen and decreased by Rho GTPase.[8,9]

Phosphorylation of eNOS at Ser-1177, Ser-635 and Ser-617 undergoes stimulatory regulation, while phosphorylation at Thr-495 and Ser-116 shows inhibitory regulation. Phosphorylation at Ser-1177 is catalyzed by different signaling pathways, including phosphatidylinositol 3-kinase (PI3-K)/Akt, adenosine monophosphate (AMP)-activated protein kinase (AMPK), and cyclic AMP-dependent protein kinase.[10] Shear stress, vascular endothelial growth factor, insulin and estrogen can regulate eNOS activity through the PI3K/Akt pathway. A recent report has indicated the presence of the AMPK/Akt/eNOS pathway in human umbilical venous endothelial cells (HUVEC).[11]

Shear stress caused by blood flow induces both NO production from endothelial cells (basal NO release)[12] and NO from activated platelets.[13] Under regular balanced conditions, NO prevents activated platelet adhesion to the endothelium and formation of thrombosis. NO prevents vascular smooth muscle cell proliferation[14] and monocyte chemotaxis.[15] These findings indicate that endothelium-derived NO maintains vascular homeostasis and retards the progression of atherosclerosis. Further, NO may potentially have an antioxidant effect.[16]

NO and atherosclerosis formation

As mentioned above, all processes of atherosclerosis formation have been shown to be inhibited by NO.[17] NO works to prevent the endothelial dysfunction. NO-dependent vascular endothelial relaxation responses become impaired in the early stages of atherosclerosis, causing hyperlipidemia and diabetes mellitus.[18] This impairment of NO-dependent relaxation is owing to the decreased release of NO from NO synthase

and the decrease in NO bioavailability caused by increased release of $O2^-$ from, for example, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, in endothelial cells, which inactivate NO. The basal NO release and half-life were investigated by pre- and co-treatment with an NO synthase inhibitor such as $N^{(G)}$-monomethyl L-arginine and superoxide dismutase (SOD).[19]

The NO-producing enzyme, eNOS, and its mRNA increase from stimulation such as by hyperlipemia. Under immunoelectronmicroscopy of atherosclerotic plaques, eNOS was found to be distributed in the cytoplasm not the cell membrane. In the endothelium of atherosclerotic vessels, the palmitoylation- and myristoylation-mediated movement of NO to the cell membrane to produce NO is affected, and uncoupling is thought to occur.[20] Uncoupling, by the depletion of coenzymes of NOS such as tetrahydrobiopterin (BH4) (diabetes) or substrates of NOS such as L-arginine (hyperlipemia), impairs the formation of the eNOS dimer, the active form of the enzyme. In addition, it is known that ROS production from enzymes such as NADPH oxidase is activated in arteriosclerosis.[21]

Low-density lipoprotein is oxidized and blood monocytes become macrophages having scavenger receptors, such as SR-A, LOX-1 and SR-PSOX, which catch oxidized LDL.[22] Subsequently, macrophages form foam cells. The lipid peroxide generation reaction is thought to be important, as well as the occurrence of chain reactions, as fatty acid chains are converted to various free radicals by the action of $O2^-$, and HO radicals, and these radicals continue lipid peroxidation on other fatty acid chains. Further, oxidized LDL induces chemoattractants such as vascular cell adhesion molecule-1 (VCAM-1), a vascular cell adhesion molecule, and it regulates the expression of matrix metalloproteases and their inhibitors, tissue inhibitors of metalloprotease and peroxisome proliferator-activated receptor-γ.[23] NO caused partial regression of atherosclerosis. Evidence has accumulated to identify the effect of NO on advanced atherosclerosis indirectly through diet or statin or directly through eNOS gene transfer (Fig. 1).[24] There is the possibility of NO causING not only retardation but also regression of atherosclerosis.

Progression of atherosclerosis by NO bioavailability

There are thought to be several ways in which free radicals pose atherosclerotic risks. First, there is the direct relationship between free radicals and atherosclerosis; second, is their relation to thrombosis formation; and third, the relation to other risk factors for cardiovascular diseases and atherosclerosis, specifically, diabetes mellitus.

Effect of ROS and NO on coronary risk factors: diabetes

As coronary risk factors, dyslipidemia, hypertension, diabetes, menopause and aging, among others, have been reported. Dyslipidemia has been mentioned above and this and the following sections deal with other risk factors, mainly diabetes, menopause and aging.

Chronic hyperglycemia activates the pathway of sorbitol, glucosamine and diacylglycerol-PKC, raises the nicotinamide adenine dinucleotide (NAD)/NAD (intracellular redox abnormality), and causes overproduction of advanced glycation end-product. These abnormalities are related to each other and cause functional or morphological abnormality of the blood vessels. Such vascular lesions are chronic and progressive, and are reversible in the early stage of atherosclerosis, but become irreversible after a certain point. As for the molecular mechanisms, increase in H_2O_2, O_2^-, and OH radicals occurs with modification of the protein and bridge formation by a non-enzymatic glycated reaction, and it is supposed that ROS including lipid radicals increase through a series of chain reactions. In addition, it has been reported that overproduction of ROS depends on the activation of ROS-producing enzymes such as NADPH oxidase and eNOS uncoupling by hyperglycemia.[25] Where there is insulin resistance as the pathophysiological base of diabetes mellitus, vascular lesions are formed by overproduction of ROS.[26] Therefore, vascular endothelial dysfunction represented by the decreased bioavailability of NO might relate to an increase in O_2^- (e.g. from NADPH oxidase) and uncoupling of eNOS.

Prevention and treatment of diabetic vasculopathy by targeting cancellation of ROS

The importance and efficacy of the strict control of risk factors other than plasma glucose levels, such as plasma lipid levels or blood pressure, has been suggested by large-scale clinical trials such as the UK Prospective Diabetes Study (UKPDS) and Scandinavian Simvastatin Survival Study (4S). It is assumed in the USA that the complication of diabetes carries a similar risk as a history of coronary heart disease for cardiovascular disease, and this means the strict control of all coronary risk factors.[27] We paid attention to the direct action of statins on the blood vessel wall. We administrated the usual dose of statin to elderly Japanese individuals with diabetes (>65 years old; mean 69.3 ± 3.4 years, serum total cholesterol levels 200–260 mg/dL), and with independent ADL, for 3 days.[28] We did not observe a significant change in plasma lipid levels with statin treatment; however, flow-mediated endothelial NO-dependent vascular dilatation in the forearm increased after statin treatment for

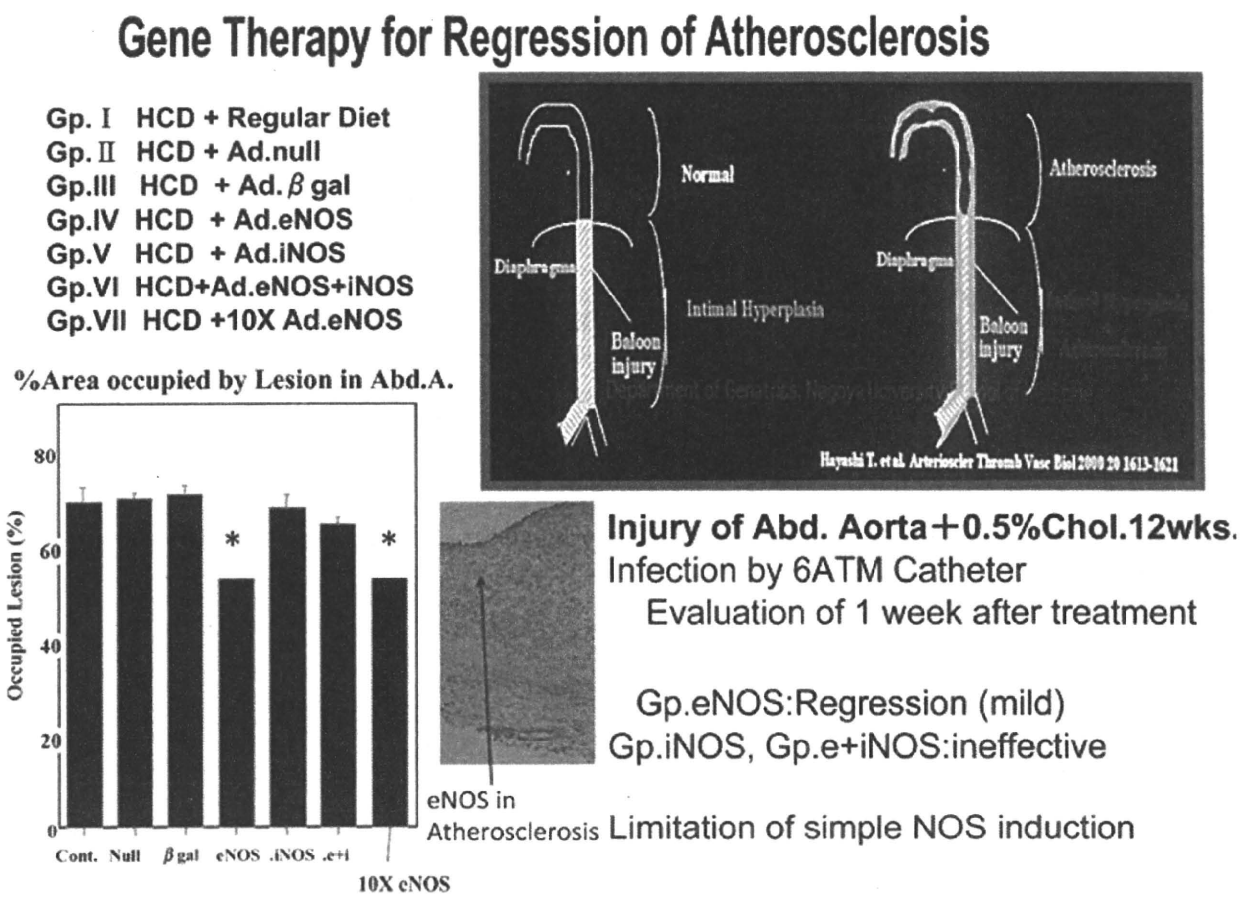

Figure 1 Gene transfer of endothelial nitric oxide synthase (eNOS) in an advanced lesion of atherosclerosis.[24] Histological evaluation of the atherosclerotic area of the aorta as indicated by the mean lesion area (% occupied lesion, left) and the intima/medial ratio (I/M ratio, right). Gp cont: no treatment; Gp null: Ad.null; Gp eNOS: Ad.eNOS; Gp iNOS: Ad.iNOS; Gp e+i: Ad.eNOS plus Ad.iNOS; Gp 10× eNOS: 10 times amount of Ad.eNOS. Ad., adeno-virus vector; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase. Lower: a section stained with hematoxylin–eosin from Gp cont and Gp eNOS. (Original magnification, ×40. Bar = 200 μm.)

3 days while it remained constant in control patients. Plasma levels of isoprostane, a marker of oxidative stress, also decreased with statin treatment. We suggest that these results indicate the pleiotropic effect without a change in plasma lipid levels to be one mechanism of the anti-atherosclerotic actions of statin through an increase in NO bioavailability. Patients with multiple coronary risk factors, including patients with metabolic syndrome, often show insulin resistance and very often suffer from hypertension and dyslipidemia (type IIb or IV). As diabetic coronary artery lesions were often observed in post-menopausal women as much as in men, and include multiple longer stenosis lesions, treatment is sometimes difficult. Information from these findings may help prevent the progression of diabetic coronary artery lesions.

Effect of ROS and NO on coronary risk factors: menopause and sex steroids

Although hormone replacement therapy (HRT) was reported to decreases coronary events in postmenopausal women in observational studies such as the Nurse Health Study, the Heart and Estrogen Replacement Study and Women's Health Initiative (WHI) showed that HRT carries a risk of thrombotic complications.[29–31] Interestingly, in the subsequent report of the WHI on patients after hysterectomy, estrogen showed anti-atherosclerotic effects, and HRT is still popular in Korea because the gene polymorphism relating to thrombosis, such as the Leiden mutation (coagulation factor V), is very rare in Asia races.[32,33] The other mechanism of the anti-atherosclerotic effect of estrogen, the direct action of estradiol on the vessel wall, has

basal NO production under high glucose. This finding was associated with the recovery of eNOS protein expression, BH4 levels, and the activity and gene expression of GTPCH-I. Both the gene transfer of estrogen receptor using adenovirus and treatment with the PKC inhibitor bisindolylmaleimide I significantly enhanced the effects of E2 treatment under high glucose, whereas these effects were abolished by the estrogen receptor antagonist ICI 182 780. Transfection of small interfering (si)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 E2. These results suggest that the activation of estrogen receptors (ER) by E2 can counteract high-glucose-induced downregulation 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.

Effect of estrogen on pathway of NO-modulating role in arginase II expression as an atheroprotective mechanism

Estrogens retard the development of atherosclerosis. We[40,43] have demonstrated that estrogens increase NO production by endothelial cells, and it is now known that NO can attenuate the cytokine-induced expression of VCAM-1 as well as monocyte chemotactic protein-1 (MCP-1).[44] L-Arginine is a substrate for NOS, which catalyzes formation of N ω-hydroxy-L-arginine as an intermediate that subsequently forms NO.[45]

L-Arginine is also converted by arginase to ornithine, the only source of synthesis in mammalian cells of the polyamines which are essential for cell proliferation,[46] and therefore, pro-atherosclerotic. 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 and tissue distribution.[47] Arginase I is expressed in the cytosol of liver cells, whereas arginase II is located within the mitochondrial matrix at low levels in many tissues.[48] Citrulline, the end-product of the NOS-mediated reaction, is converted to L-arginine by arginosuccinate synthetase and arginosuccinate lyase.[49,50] We evaluated the effects of a 0.5% cholesterol-enriched diet (HCD) on NOS and arginase expression, and the modulating role of E2 on this phenomenon.[51] Thirty oophorectomized rabbits were divided into three groups and treated for 15 weeks. Group I received normal chow; group II, HCD; and group III, HCD plus E2 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 5-bromodeoxyuridine (BrdU)-positive cells in endothelial cells and intimal muscle cells, suggestive of an increase in cellular proliferation. These were not observed in group I animals. In both groups, E2 levels were low. In group III animals, E2 supplementation led to a decrease in atheromatous lesions and BrdU-positive cells, and reduced expression of both inducible nitric oxide synthase (iNOS) and arginase I and II, accompanied by a decrease in nitrotyrosine staining. E2 levels were increased. Our results suggest that E2 was responsible for these effects, despite the animals being hyperlipidemic, similar to those in group II. Thus, expression of arginase may play an important role in cellular proliferation in atherosclerosis, and inhibition of arginase expression by E2 may be another potential mechanism in attenuating atherogenesis in addition to activation of the L-citrulline–L-arginine cycle (Fig. 3).[50]

Cross-talk of NO and free radical, especially NADPH oxidase-derived superoxide

Monocytes from patients with type 2 diabetes produce increased levels of superoxide anions compared to control subjects.[52] The NADPH oxidase pathway is the main source of O_2^- generation in monocytes/macrophages. Activation of the NADPH oxidase complex in the plasma membrane can lead to cell-mediated oxidation of LDL in macrophages.[53] Activation of the NADPH oxidase complex from a resting state to full superoxide-generating activity requires chemical modification and translocation of subunits, the polypeptides p47phox and p67phox.[54] The level of p47phox translocation to the membrane is increased with high glucose concentrations.[55] Caveolae are small, functionally important membrane invaginations found on the surface of many different cell types.[56] Caveolin is a cholesterol-binding protein and can form stable oligomers in the membrane. Caveolin-1 (Cav-1) has a critical role in the development of atherosclerosis.[57] Cav-1 is associated with cholesteryl ester uptake from LDL (but not oxidized LDL) and with cholesterol reverse transport by high-density lipoprotein in THP-1 macrophages. Insulin signaling for metabolic control depends on intact caveolae, and destruction of caveolae disrupts insulin-stimulated phosphorylation of insulin receptor substrate-1. We evaluated the relationship between the expression of Cav-1 and the number of caveolae in macrophages under conditions of high glucose concentration.[57] Increased superoxide production, induction of iNOS and decreased Cav-1 were observed in a concentration-dependent manner in THP-1-derived macrophages with high glucose. Co-localization of the NADPH oxidase component, p47phox, and caveolin was confirmed by confocal microscopy. An atomic force microscopy study showed

been studied vigorously. We demonstrated that estradiol retards the progression of severe atherosclerosis in rabbits. More than 50% of its anti-atherosclerotic effects might be due to this direct action.[34] Twenty-four octogenarian women (mean age 80.3 ± 3.5 years) were administered 1 g/day CaC12 with (HRT group; $n = 12$) or without (control group; $n = 12$) 2 mg/day E3 (Mochida Pharmaceutical, Tokyo, Japan) for 110 weeks.[35] They were active and had no history of ischemic cardiovascular disease. The changes in diameter of the right brachial artery were measured during reactive hyperemia as percentage flow-mediated dilatation (%FMD) and after sublingual nitroglycerin spray (300 μg), which causes endothelium-independent vasodilatation (percentage nitroglycerin-induced dilatation: %NTG-D). Serum concentrations of total cholesterol, triglyceride and apoproteins B100, C2 and E were unchanged in all patients. The %FMD in the HRT group increased during the study period. No difference in %NTG-D was demonstrated in the two groups. Plasma nitrite/nitrate ($NO2^-/NO3^-$) and cyclic guanosine monophosphate (cGMP) levels also were increased by HRT. An E3-induced improvement in endothelial function was shown in octogenarian women.

17β-Estradiol antagonizes the downregulation of eNOS and glutamyl transpeptidase (GTP) cyclohydrolase I by high glucose: relevance to postmenopausal diabetic cardiovascular diseases

Impaired NO bioavailability results in endothelial dysfunction, a characteristic feature of vascular diseases such as diabetic macroangiopathy.[21] 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 GTP cyclohydrolase I (GTPCH-I) is a rate-limiting enzyme.[36] Supplementation with BH4 improves endothelial-dependent vasodilation by increasing NO activity in patients with type II diabetes mellitus.[37] Previously, we reported that a high glucose concentration enhanced oxidative stress by eNOS dysfunction and activation of NADPH oxidase in bovine aortic endothelial cells (BAEC).[38]

The risk of cardiovascular disease dramatically increases with age, especially after menopause.[39] Estrogen activates eNOS through genomic and non-genomic mechanisms, leading to an increase in NO.[40,41]

We hypothesized that the beneficial effects of estrogen on endothelial function may be relevant to protection against hyperglycemia-induced vascular derangement (Fig. 2).[42] BAEC were incubated for 72 h in the presence and absence of the physiological concentration of E2 under normal and high-glucose conditions. E2 significantly counteracted the reduction in

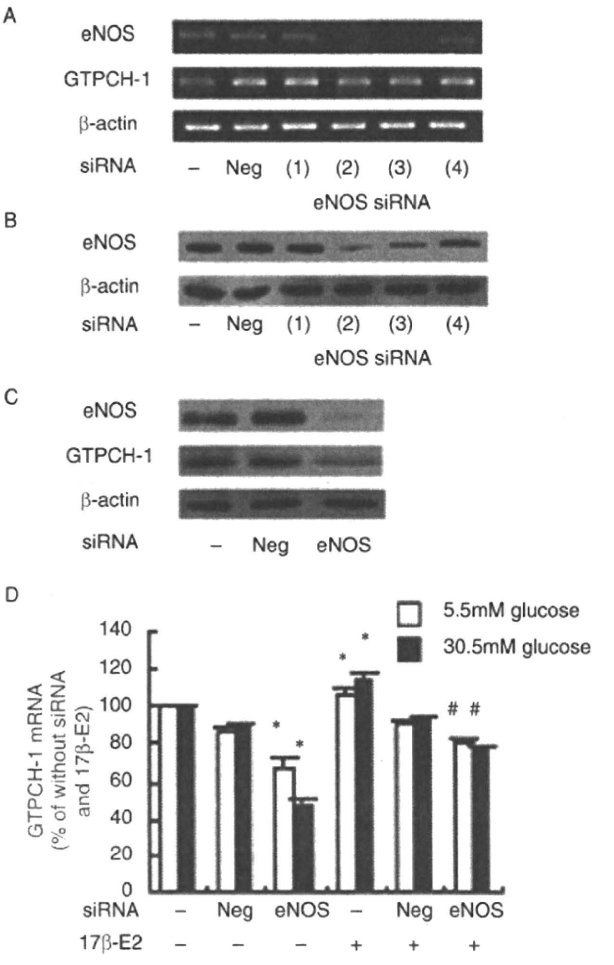

Figure 2 17β-Estradiol (E2) antagonizes the downregulation of endothelial nitric oxide synthase (eNOS) and glutamyl transpeptidase cyclohydrolase I (GTPCH-I) by high glucose: relevance to postmenopausal diabetic cardiovascular disease. Effect of small interfering RNA (siRNA) targeting eNOS on GTPCH-I mRNA expression in bovine aortic endothelial cells (BAEC). (a) Representative reverse transcription polymerase chain reaction data showing that two of four eNOS siRNA (nos 2 and 3) successfully eliminated eNOS mRNA 48 h after transfection and reduced GTPCH-I mRNA. The other two eNOS siRNA did not eliminate eNOS mRNA enough, and we abandoned use of them (nos 1 and 4). Neg, negative control. (b) Representative immunoblot data showing no detection of eNOS protein 48 h after transfection of successful eNOS siRNA (nos 2 and 3). Other candidates of eNOS siRNA (nos 1 and 4) did not eliminate eNOS protein expression. (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 1nM 17-E2 of the effect of successful eNOS siRNA (2) on the mRNA expression level of GTPCH-I in BAEC under normal (5.5 mmol/L) and high (30.5 mmol/L) glucose conditions. $*P < 0.05$ compared with control, $\#P < 0.05$ compared with transfection of eNOS siRNA in the absence of 17-E2, by ANOVA followed by Fisher's post-hoc test.

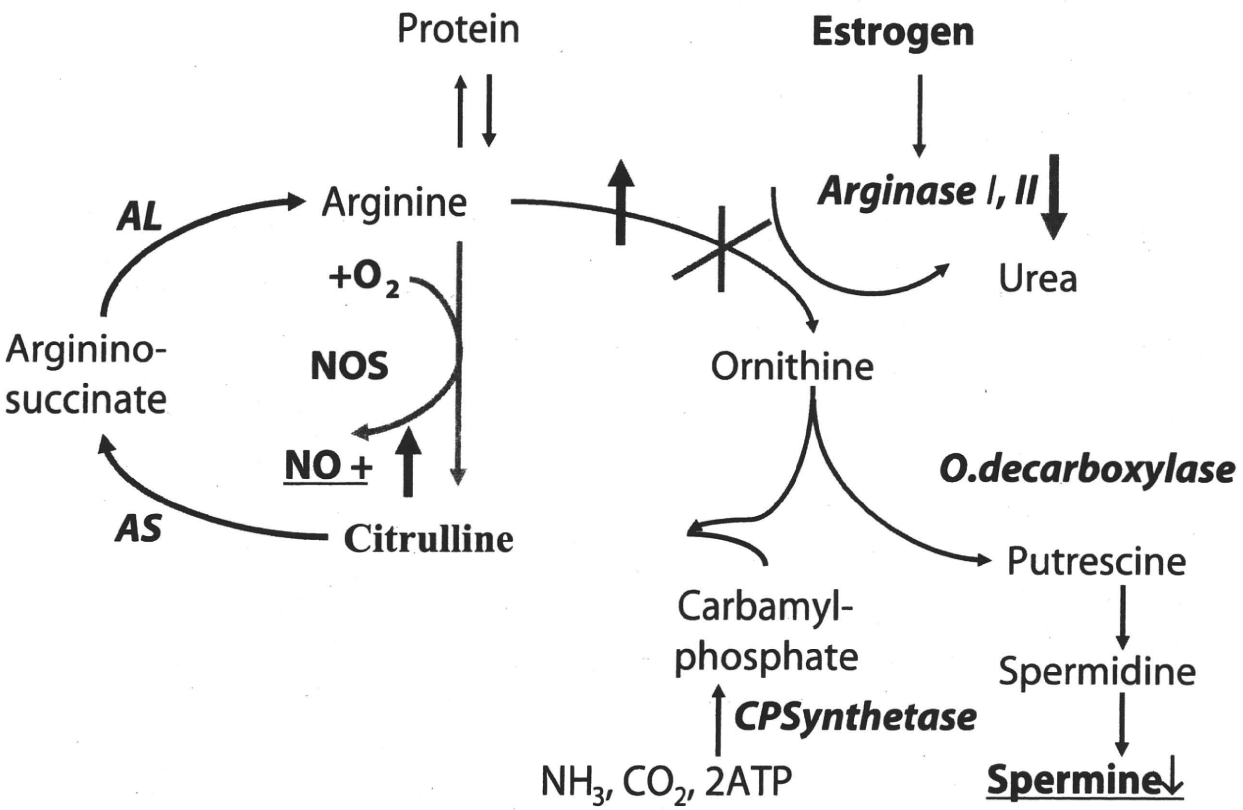

Figure 3 Modulating role of estradiol on arginase II expression in hyperlipidemic rabbits as an atheroprotective mechanism.[51] After prolonged feeding on a 0.5% cholesterol-enriched diet, arginase expression is increased in hyperlipidemic rabbits in the atherosclerotic lesion area, whereas the expression of arginase II is significantly reduced by simultaneous administration of E2. The increase in arginase II activity may account for the associated cellular proliferation by diverting L-arginine to form polyamines, whereas E2, by inhibiting arginase II expression, attenuates atherosclerosis by providing a substrate for endothelial nitric oxide synthase (eNOS) to synthesize nitric oxide (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 administrated later on when significant lesions have already developed, and arginase II, expressed as L-arginine is administrated, would then lead to cell proliferation. AL, argininosuccinate lyase; AS, argininosuccinate synthase; ATP, adenosine triphosphate.

that high glucose reduced the number and size of the caveolae. Taken together, high glucose environments suppress the levels of Cav-1 expression and reduce the number of caveolae.

NADPH oxidase inhibitor restores impaired endothelium-dependent and -independent responses, and scavenges superoxide anion in type 2 diabetes complicated by NO dysfunction

We investigated the effect of apocynin, an NADPH oxidase inhibitor, in the impairment of vascular responses in Otsuka Long-Evans Tokushima Fatty (OLETF) rats (type 2 diabetic rat model) with or without (w/wo) N(G)-nitro L-arginine methyl ester (L-NAME) treatment.[58] Male OLETF and littermate rats LETO were separated as follows: LETO w/wo apocynin (Gp C, Gp C-apo), OLETF w/wo apocynin (Gp DM, Gp DM-apo) and OLETF plus L-nitro arginine acetate ester w/wo apocynin (Gp DMLN, Gp DMLN-apo). Five days after, peritoneal macrophages were stimulated with thioglycolate. Two days after, they were evaluated. Plasma glucose and lipid levels remained unchanged. Acetylcholine-induced NO-dependent relaxation and nitroglycerin-induced NO-independent relaxation were improved in the Gp DMLN-apo, compared with the Gp DMLN. Tone-related basal NO release and plasma NO_2^- and NO_3^- tended to be lower in the Gp DM and Gp DMLN groups. It was restored by apocynin. This NADPH oxidase inhibitor restores the impairment of endothelial and non-endothelial function in diabetic angiopathy in OLETF without changing plasma glucose and lipid levels. NO and O_2^- may play a role in this process by decreasing TNF-α levels.

Vascular aging, NO and atherosclerosis

Effect of ROS and NO on coronary risk factors: aging

The free radical theory of aging attributes cellular pathology to the accumulation of ROS. The molecular interactions between ROS and reactive nitrogen species such as NO suggest that one effect of increased ROS is the disruption of protein S-nitrosylation, a ubiquitous form of post-translational signal transduction. ROS may not only damage cells but also disrupt widespread signaling pathways. We and many researchers have evaluated oxidative stress and aging.[59,60]

NO metabolites are associated with survival in older patients

The population of elderly persons and the proportion they make up of the whole population are increasing dramatically in many countries, such as Japan, the USA and China. Of the various causes of death in elderly patients, the ratios of heart disease and cerebrovascular disease increase with age. Several biochemical markers, such as albumin, cholesterol, interleukin-6 (IL-6), TNF-α and high-sensitivity C-reactive protein have been identified as having prognostic value for mortality and hospitalization.[61] A recent study based on functional assessment showed that impairment in ADL or cognition might affect lifespan. To assess the efficacy of various vascular endocrinological substances, such as plasma NO metabolites (NOx), as surrogate markers of survival in older patients (Fig. 4),[62] 150 patients aged 70 years and older were recruited consecutively from the outpatient clinics. Serum biochemical analyses, such as albumin and total cholesterol, and various prognostic markers, such as TNF-α, NOx, ADL and instrumental ADL, were evaluated on enrolment. The main outcome was survival rate over 2.75 years. Forty-nine patients died

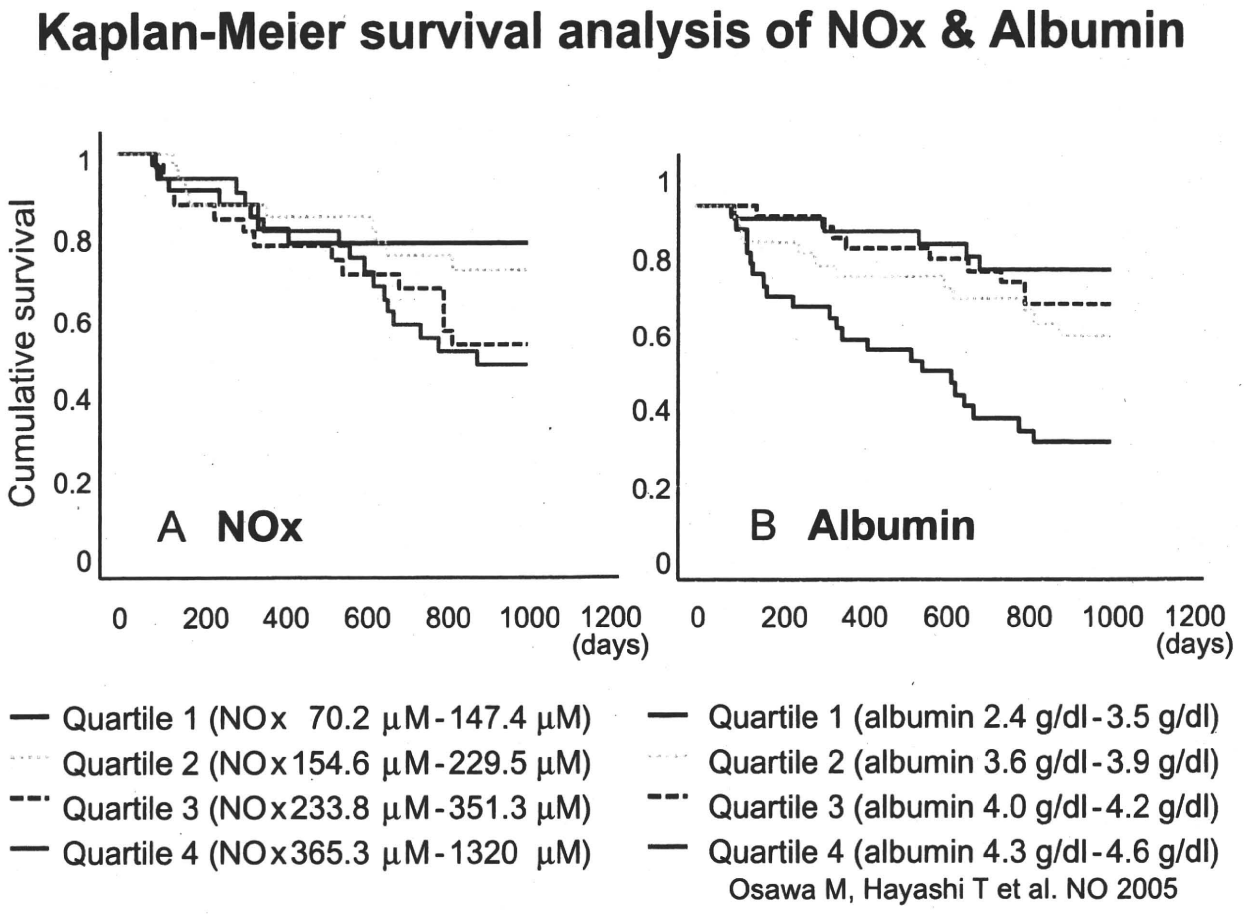

Figure 4 Nitric oxide (NO) metabolites are associated with survival in older patients.[62] Kaplan–Meier survival analysis. Circulating levels of NO metabolites (NOx) (a) and levels of albumin (b) were examined in relation to patient survival during follow up. For this analysis, circulating levels of NOx and those of albumin were arbitrarily divided into quartiles on registration.

during the follow-up period. Mann–Whitney *U*-test showed that hemoglobin, total protein, serum albumin, total cholesterol, high-density lipoprotein cholesterol, LDL cholesterol, high-sensitivity C-reactive protein, NOx, B-type natriuretic peptide, IL-6 and TNF-α levels; ADL; cognitive impairment; and depressive status were significantly different in subjects who survived compared to those who died. Of the dependent variables in the Cox proportional hazards regression analyses, only albumin, NOx, and ADL were significantly different. NO may be an effective marker; like albumin, which is a well-known marker.

Endothelial cellular senescence

Cellular senescence is generally defined as permanent exit from the cell cycle, which is accompanied by phenotypic changes in gene expression, morphology and function. Stopping of cell division in culture was first observed in normal human fibroblasts by Hayflick and Moorhead.[63] This type of senescence requires weeks or months of culture, has limited ability to divide when cultured *in vitro*, and is termed "replicative senescence". In the past decades, significant progress has been made in understanding the mechanisms underlying cellular senescence, and many findings linked senescence to the attrition of telomeres.[64] Senescence resulting from extended replication of cells is largely the consequence of the eventual dysfunction of telomeres, which leads to chromosomal instability. Telomere and telomerase regulate not only cellular lifespan but also organismal aging, and the senescence response can be derived from many stressful stimuli such as DNA damage,[65] oxidative stress[66] and oncogenic activation.[67] Human cells exposed to these sublethal stressors show a character of acute premature senescence within just a few days, which is usually connected to telomerase disorganization rather than telomere shortening per se. Such a limited proliferation of cells has been referred to as "stress-induced premature senescence" (SIPS).[68]

Many findings on cellular senescence are derived from studies of human fibroblasts and from some immortalized cell lines, and they may not be fully relevant to endothelial cells. Aging of healthy vascular endothelial cells leads to replicative senescence.[69] Further, endothelial cells may undergo SIPS under oxidative stress.[70] Senescent endothelial cells are characterized by decreased production of NO, changes in expression or phosphorylation of eNOS and increased expression of plasminogen activator inhibitor-1, and so forth.[71] Moreover, senescence-associated β-gal (SA-β-gal)-positive coronary endothelial cells predominantly localized on the luminal surface of atherosclerotic plaques have been shown to appear flattened and enlarged.[72,73] Endothelial senescence contributes to the pathogenesis of age-related vascular disorders.

Relationship between endothelial cell senescence and NO

The activity of eNOS and the production of NO are diminished in senescent human endothelial cells.[74] Vascular aging in rat aorta can be initiated by enhanced O_2^- production, followed by trapping NO and subsequent peroxynitrite formation.[75] Generation of nitrotyrosine (a marker of peroxynitrite and the reactive substances of NO and superoxide anion), expression of NADPH oxidase and activation of nuclear factor-κB were higher in endothelial cells of older than of younger people.[76] On the other hand, the plasma concentration of SOD decreases with age.[77] Atherogenic diseases such as hyperlipidemia and diabetes mellitus, characterized by increase in oxidative stress, show the decrease in bioavailability of NO and impairment of endothelial function. The increase in oxidative stress and impairment of NO bioavailability promote the progression of atherosclerosis synergistically. NADPH oxidases are a major source of O_2^- in both endothelial and smooth muscle cells of human vessels.[20] The expression of p22phox, an essential component of NADPH oxidase, in atherosclerosis was increased.[78] *In situ* ROS generation of directional coronary atherectomy specimens from patients with angina pectoris was closely correlated with the expression level of p22phox, indicating the functional importance of p22phox-based NADH/NADPH oxidase. Furthermore, the generation of ROS overlapped with the distribution of oxidized LDL and p22phox. The severity of atherosclerosis also correlates with NADPH oxidase subunit mRNA expression, providing a potential link between human atherosclerotic disease, oxidative stress and activity of NADPH oxidases.[79]

Nitric oxide acts in multiple ways to prevent the progression of atherosclerosis,[80] and in our previous studies gene transfer of eNOS or ingestion of certain NO-boosting substances, such as L-arginine and L-citrulline, displayed additive effects on the retardation of the progression of atherosclerosis and the partial regression of advanced atherosclerosis in rabbits.[24,37] Then, what is the effect of NO on cellular senescence? Little is known about the beneficial effects of NO on endothelial senescence. The first short report by Vasa *et al.*[81] showed the effect of NO donors on retardation of cellular senescence. Although this had a significant impact, the mechanism was not investigated fully. Furthermore, because of the development of tolerance to NO donors such as nitroglycerin, their long-term use would be difficult as we described elsewhere.[82] We investigated cellular senescence in human aorta obtained by autopsy and in

HUVEC.[72] We demonstrated cellular senescence with SA-β-gal-positive staining in atherosclerotic lesions of the luminal side of human thoracic aorta. No staining was detected in the non-atherosclerotic area and advanced atherosclerotic area, overlaying the necrotic core and ulcer-complicated lesion. In addition, when HUVEC were treated with the NO donor (Z)-1-(2-[2-aminoethyl]-N-[2-aminoethyl] amino) diazen-1-ium-1,2-diolate (DETA-NO), causing less cross-tolerance to nitroglycerin, SA-β-gal activity was reduced in a dose- and time-dependent manner (Fig. 5). Finally, we examined the effect of transfection with eNOS into HUVEC, which evidently decreased SA-β-gal activity.

NO delays telomere-dependent endothelial cell senescence

Telomeres are non-nucleosomal DNA–protein complexes at the ends of eukaryotic chromosomes that serve to protect chromosomes from fusion and degradation and to prevent initiation of the DNA damage response.[83] As a result of DNA replication, the termini of chromosomes are not duplicated completely, resulting in successive shortening of telomeres with each cell division.[64] Short telomeres may trigger the cellular senescence.[84] Telomeres are also involved in SIPS. This second pathway initiates not because of shortening, but

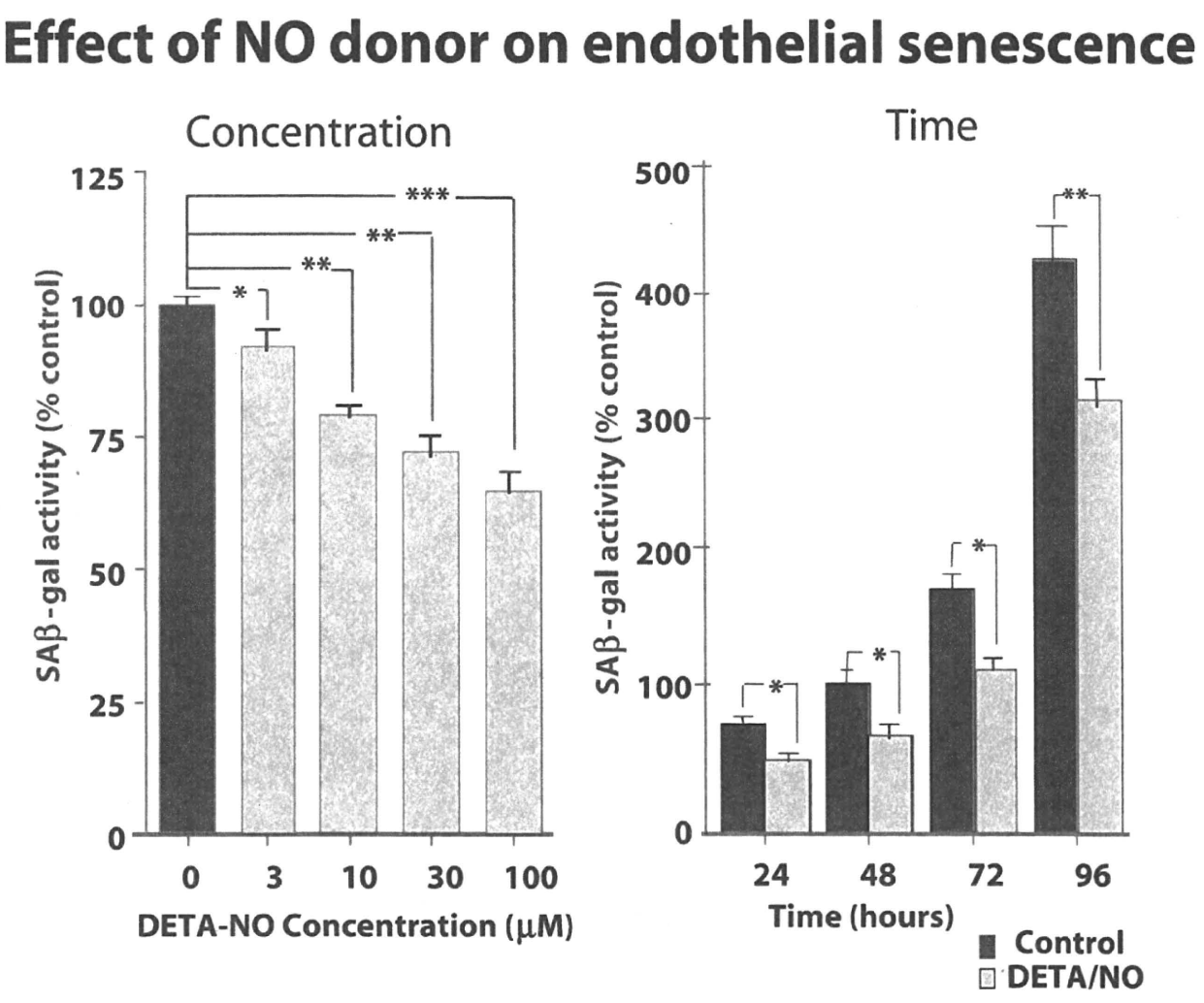

Figure 5 Endothelial cellular senescence is inhibited by nitric oxide (NO): implications in atherosclerosis. (Left) Concentration-dependent decrease in senescence-associated β-gal (SA-β-gal) activity in human umbilical venous endothelial cells (HUVEC) by NO donor (Z)-1-(2-[2-aminoethyl]-N-[2-aminoethyl] amino) diazen-1-ium-1,2-diolate (DETA-NO). HUVEC were treated with DETA-NO for 24 h. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.0001$ versus DETA-NO-untreated control. (Right) Time-dependent decrease in SA-β-gal activity in HUVEC by DETA-NO. HUVEC were treated with 10 μM DETA-NO for 24–96 h. $^{*}P < 0.05$, $^{**}P < 0.01$ versus the corresponding control. Control sample, treated for 48 h, is expressed as 100%.

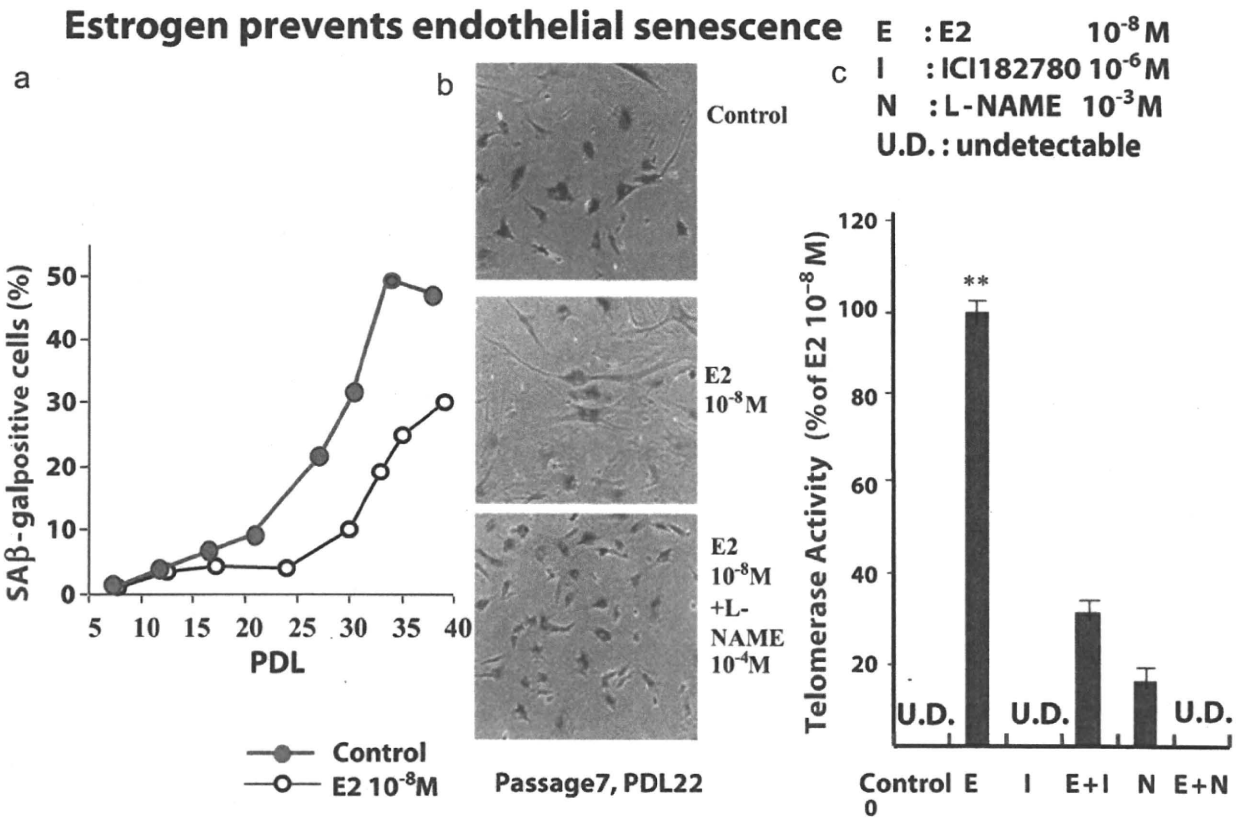

Figure 6 Endothelial cellular senescence is inhibited by nitric oxide (NO): implications in atherosclerosis associated with menopause. (a) The relative levels of senescence-associated β-gal (SA-β-gal)-positive staining cells in different population doubling length (PDL) when human umbilical venous endothelial cells (HUVEC) were untreated or treated with 10 h^{-8} M E2 for 24 h. Positive staining cells were evaluated by FACscan. (b) Representative photographs of SA-β-gal staining in control, 10^{-8} M E2-treated, and 10^{-8} M E2- and 10^{-4} M L-arginine methyl ester (L-NAME)-treated cells. Note that treatment with E2 decreased the number of SA-β-gal-positive cells, which was prevented by further treatment with L-NAME. Cells were used in PDL 22 at passage 7. (c) The effects of E2 (E, 10^{-8} M), ICI 182780 (I, 1 μM), and L-NAME (N, 1mM) on the telomere.

because of changes in telomere structure, including single-stranded overhang and function.[85] Telomerase-deficient mice have been developed and exhibit a reduced capacity for neovascularization.[86] We showed that transfection with eNOS into HEK293 cells or HUVEC for 48 h significantly increased telomerase activity.[72] Thus, increasing NO bioavailability or eNOS activity activates telomerase and delays endothelial cell senescence.

Estrogen, NO and endothelial senescence

The incidence of cardiovascular disease is lower in premenopausal women than in age-matched men and postmenopausal women.[87] The postmenopausal decline in estrogen causes a reduction in NO synthesis and an increase in ROS, scavenging NO from the blood flow, and accelerates the progression of atherosclerosis, eventually resulting in thrombosis formation. In HUVEC, we have demonstrated that E2 can reduce SA-β-gal activity, especially at the large population-doubling level (cellular senescent), and markedly activate telomerase (Fig. 6). As these effects could be inhibited by both the specific estrogen receptor antagonist ICI 182780 and L-NAME, the counteracting effect of E2 on endothelial cell senescence involves an eNOS/NO-dependent mechanism by means of activation of the ER.[51]

Moreover, plasma NO levels have been shown to be elevated by HRT.[88] Meanwhile, the plasma LDL-cholesterol level inversely relates to the plasma NO level with HRT,[89] and hyperlipidemia appears to be closely associated with a reduction in NO synthesis.[90] We propose that estrogen relates closely to prevention of endothelial cell senescence through NO, which may contribute to its anti-atherosclerotic effects.

High glucose, NO and endothelial cell senescence

We examined the effect of NO bioavailability on endothelial cell senescence under high glucose conditions.[73]

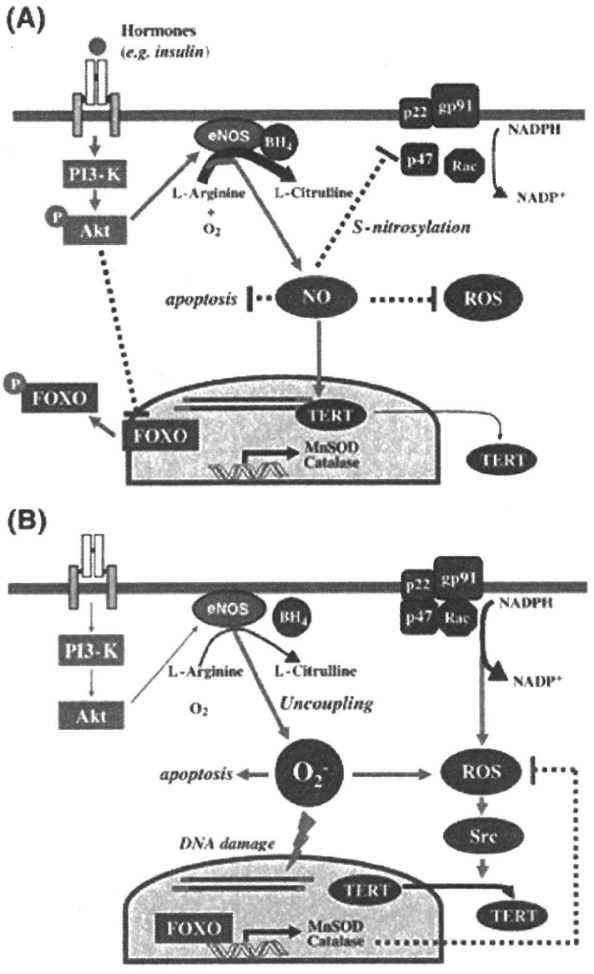

Figure 7 (a) Anti-senescence effect of nitric oxide (NO) in endothelial cells. Under normal homeostasis conditions, endothelial NO is synthesized from L-arginine and oxygen in a reaction catalyzed by endothelial NO synthase (eNOS). Insulin and estrogen activate eNOS expression through the phosphatidylinositol 3-kinase (PI3-K)/Akt pathway and/or direct effect through promoter or stabilization of eNOS mRNA. They may also inactivate nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. NO shows anti-apoptotic effects and suppresses reactive oxygen species (ROS) production by scavenging directly or preventing NADPH, which results in depletion of telomerase reverse transcriptase (TERT) export to the cytoplasm from the nucleus. Activation of the PI3-K/Akt pathway can also downregulate forkhead box O (FOXO), leading to decreased FOXO-dependent expression of anti-oxidant genes. Solid lines represent positive regulatory pathways. Dotted lines represent negative regulatory pathways. (b) Progress of endothelial senescence under pathological conditions. Both the decrease in eNOS expression by decline in the hormonal signal with age or insulin resistance and the increase in ROS production by endogenous or exogenous sources cause progression of endothelial senescence owing to an imbalance between NO and ROS. Additionally, reduction in BH4 under pathological conditions such as diabetes causes eNOS uncoupling. Then, stimulation of the PI3-K/Akt pathway causes not only downregulation of FOXO but also increased eNOS uncoupling, which may result in accelerating endothelial senescence. Solid lines represent positive regulatory pathways. Dotted lines represent negative regulatory pathways.

Exposure of HUVEC to high glucose for 24 h decreased the expression level of eNOS protein in a manner dependent on the concentration of glucose. In HUVEC cultured under high glucose for 3 days, nitrite or NOx (nitrite plus nitrate, marker of NO production) production was decreased and intracellular ROS generation was increased in a manner dependent on the concentration of glucose and cellular senescence was promoted. Treatment with L-arginine/L-citrulline and anti-oxidants (vitamin C and E), alone or in combination, led to a significant recovery of the decreased nitrite level and tended to prevent cellular senescence under high glucose conditions. eNOS transfection also delayed cellular senescence. Diabetic macroangiopathy can occur under almost the same conditions, with increased levels of ROS from NADPH oxidase and impaired NO production.[91] We speculate that not only BH4 but also L-arginine/L-citrulline, as well as anti-oxidants, are important in prevention of the endothelial senescence and development of diabetic macroangiopathy.

Mechanisms of the anti-senescent and anti-atherosclerotic effect of NO

The free radical theory of aging proposes that degenerative senescence may be largely attributable to the cumulative effect of ROS.[92] In endothelial cells, telomerase activity can be post-transcriptionally regulated by kinases such as PKC, ERK1/2 and Akt.[93] ROS formation leads to an increase in Src-family (a family of proto-oncogenic tyrosine kinases) kinase activation in aged endothelial cells, shortening telomeres and causing aging. The NO-mediated reducing effect on ROS may thus be owing to its direct ROS scavenging action and NADPH oxidase inhibitory action. NO can prevent endothelial cell apoptosis through the inhibition of caspase-3 by S-nitrosylation,[94] which may underlie the anti-senescent effect of NO in endothelial cells.

Our study has indicated that telomerase activity may be regulated by NO bioavailability.[73] Recent evidence indicates that the bulk of L-arginine may not be available for NO production, because free L-arginine for eNOS may be limited by uptake into plasmalemmal caveolae.[95] The pathway where L-citrulline is recycled to L-arginine is localized to the caveolae in mammalian cells including endothelial cells, and it may be the main source of available L-arginine.[50,51] This recycling pathway would play an important role in continuing to

produce NO in endothelial cells, especially in advanced atherosclerosis or diabetes mellitus.

The mechanisms underlying the ability of NO to prevent endothelial cell senescence and the possible changes in the NO-mediated anti-senescence effect under pathological conditions are schematically shown in Figure 7.

Forkhead box O (FOXO) transcription factors are involved in multiple signaling pathways and play critical roles in a number of physiological and pathological processes, including differentiation, proliferation and survival.[96] Constitutively active FOXO1 and FOXO3a repress eNOS protein expression and bind to the eNOS promoter. *In vivo*, FOXO3a deficiency increases eNOS expression and enhances postnatal vessel formation and maturation, suggesting an important role for FOXO transcription factors in the regulation of endothelial function in the adult.

Forkhead box O is a mammalian homolog of Daf-16, and activation of Akt leads to phosphorylation of FOXO, thereby inhibiting the transcription of anti-oxidant genes such as manganese SOD.

Conclusion

Endothelial dysfunction and cell loss underlie most of the major cardiovascular diseases. Experimental data point to an age-associated increase in senescent endothelial cells *in vivo*, and to a prevalence of this phenomenon in areas of the vasculature which are more susceptible to develop atherosclerosis. Thus, accumulating evidence implicates a critical role for endothelial cell senescence in the initiation and/or progression of atherosclerosis under coronary risk factors, such as diabetes mellitus, hypertension and dyslipidemia. As NO evidently delays cellular senescence, eNOS should play a pivotal role in the regulation of senescence of endothelial cells, and serve as a potential target of a novel prophylactic and/or therapeutic strategy of age-associated vascular disorders. Further understanding of the precise mechanisms underlying the NO-mediated delay in endothelial cell senescence will be required before eNOS-based anti-senescence therapy can be translated effectively into clinical practice.

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A hydroxymethylglutaryl coenzyme a reductase inhibitor improves endothelial function within 7 days in patients with chronic hemodialysis

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ABSTRACT

Background: Atherosclerosis-related diseases are leading causes of morbidity among patients undergoing hemodialysis. The effects of hydroxymethylglutaryl coenzyme A reductase inhibitors (statins) on the endothelial function of hemodialyzed patients are not known.

Methods and results: For 16 weeks, we prescribed simvastatin (low dose: 5 mg or moderate dose: 10 mg) to 28 patients (low dose: $n=14$, 61.2 ± 8.6 years, moderate dose: $n=14$, 60.8 ± 10.2 years) and chose 9 patients (61.5 ± 5.2 years) without prescriptions as controls. We compared the effects of statin on lipids, flow-mediated endothelium-dependent and nitroglycerin-induced endothelium-independent dilatation (%FMD, %NTD), and markers of oxidant stress and atherosclerosis. Serum HDL-cholesterol and triglycerides did not change significantly in any of the three groups; however, LDL-cholesterol was decreased at 16 weeks in both simvastatin groups. The %FMD and plasma NOx increased at 1 and 16 weeks in both statin groups, but not in the control group ($P<0.01$). The %NTD did not change. Oxidized LDL, VCAM-1, and 8-isoprostane decreased significantly after 16 weeks in both statin groups; however, TNF-α and interleukin 6 did not change. In the control group, no significant changes in these parameters were observed. Multiple regression analyses showed that the (short) period of hemodialysis and (young) age are significant factors associated with %FMD improvement.

Conclusions: A statin improved impaired endothelial function in the arteries of chronic dialysis patients, in part by enhancing NO bioavailability within one week. Improved endothelial function is in line with the anti-atherosclerotic effects observed in patients undergoing chronic hemodialysis.

1. Introduction

Atherosclerosis-related diseases such as myocardial infarction and ischemic heart disease-related heart failure are the leading causes of morbidity among patients undergoing hemodialysis in developed countries, such as the United States or Japan [1]. It is well known that lipid-lowering therapy, especially the use of hydroxymethylglutaryl coenzyme A reductase inhibitors (statins), decreases the risk of coronary events in both primary and secondary prevention [2,3]. The anti-atherosclerotic effects of statins are thought to be attributable to changes in plasma lipid levels (i.e., decreased LDL cholesterol and increased HDL cholesterol) [2,3]. We have reported that treatment of diabetic patients with a statin resulted in improved endothelial function before the appearance of its effects on lipids, in other words, in three days [4]. Statins are known to up-regulate endothelial nitric oxide synthase (eNOS) in cultured endothelium and in the endothelium of the aorta of rabbits fed a high-cholesterol diet [5,6]. The direct action of statins on the atherosclerotic arteries of rabbits, without lowering plasma lipids, has also been studied [6]. However, to our knowledge, there are no existing studies on this direct action in atherosclerotic arteries of patients undergoing hemodialysis. The present study focuses on the effect of statins on endothelial function, especially flow-mediated dilatation and nitric oxide (NO)-related endothelial function in hemodialytic atherosclerotic arteries of humans. We selected simvastatin, which is thought to have a long and strong tissue affinity [7]. Because we anticipated difficulty in improving endothelial function in patients undergoing hemodialysis, we examined two treatment groups, one of which received a low dose (5 mg/day, the usual dose in Japan) and the other a moderate dose of simvastatin (10 mg/day).

2. Materials and methods

2.1. Patients

Endothelial function was assessed in 37 hemodialysis patients (aged 60.6 ± 9.2 years, 17 males, and 20 females) with or without mild hyperlipidemia (LDL cholesterol, 95.9 ± 37.1 mg/dl, 72.9 to 172.8 mg/dl). The participants were ambulatory and were patients at our medical clinics (Souen Chuo Hospital, Sapporo, Japan; Nakashibetsu Public Hospital, Nakashibetsu, Japan; and Kyouritsu Hospital, Nagoya, Japan). They had not been prescribed

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Table 1
Biochemical profile, *$P<0.05$ between low and moderate dose of statin.

	Statin (low dose)	Statin (moderate dose)	Control
Male/female (number)	6/8	6/8	4/5
Age (y.o.)	61.2 (8.6)	60.8 (10.2)	61.5 (5.2)
BMI	24.9 (2.7)	24.7 (2.8)	25.0 (2.0)
Period of H.D. (months)	**32.4 (14.1)**	**64* (44.2)**	**51 (28.5)**
Origin of H.D. (%)			
Diabetes mellitus	57.1	43.0	55.5
Glomerulonephritis	21.5	21.5	22.2
Hypertension	**0**	**7.1***	**0**
Others	14.3	28.6	22.2
Complication (%)			
Ischemic heart disease	28.7	28.7	22.2
Hypertension	64.3	64.3	55.5
Diabetes mellitus	64.3	43.0	44.4
Smoking	21.5	14.3	22.2
Medication affecting endothelial functions (%)			
ACEis/ARBs	28.7	28.7	33.3
Other anti-hypertensive drugs	50.0	57.1	55.5
diuretics	21.5	21.5	22.2
nitrates	0	0	0
Anti-platelet/anti-coagulant	0	0	0
Plasma lipids			
Total chol. (mg/dl)	135.5 (36.0)	176.1 (46.1)	149.4 (44.4)
Triglyceride (mg/dl)	99.8 (57.4)	133.8 (62.0)	106.3 (40.1)
LDL chol. (mg/dl)	**83.2 (34.3)**	**118.0* (33.8)**	**91.1 (30.2)**
HDL chol. (mg/dl)	32.4 (11.7)	32.1 (6.2)	33.4 (7.8)
Cytokines and others			
sVCAM-1 (ng/ml mg prot.)	880.2 (168.2)	1000.4 (151.9)	919.2 (139.0)
TNFα (pg/ml)	**5.0 (1.9)**	**9.8* (5.4)**	**6.3 (3.2)**
IL-6 (pg/ml)	7.9 (3.3)	3.6 (1.4)	6.3 (2.6)
8epi ISP (ng mg protein/ml)	21.4 (6.4)	40.1(6.8)	29.1 (5.7)
Oxidized LDL (mg/ml)	24.3 (33.5)	29.4 (34.6)	27.0 (42.4)
Vascular and NO related profile			
Baseline diameter (mm)	3.01 (0.18)	2.94 (0.19)	2.95 (0.22)
Peak diameter (mm)	3.17 (0.25)	3.09 (0.25)	3.10 (0.24)
%FMD	5.6 (1.0)	4.8 (0.9)	5.2 (0.9)
GTN-induced peak diameter (mm)	3.31 (0.19)	3.24 (0.21)	3.26 (0.20)
%NTG-D	10.9 (1.1)	10.0 (0.9)	10.2 (0.9)
NOx (μM)	129.2 (16.8)	111.6 (18.1)	120.4 (15.1)

Bold emphasis and * show the significant differences between moderate dose of statin group and other two (low dose and control) groups.
The numbers are the mean ± SD, or the percent of each groups. *$P<0.05$ vs. data in low dose of statin treatment.
Abbreviations: Statin (low dose): simvastatin 5 mg/day group, statin (moderate dose): simvastatin 10 mg/day group. control: no prescription group.
BMI:Body Mass Index, H.D.:hemodialysis, ACE: angiotensin-converting enzyme, ARB: angiotensin receptor blocker, LDL: low-density lipoprotein, HDL: high-density lipoprotein, chol.:cholesterol, 8epi ISP: 8epiisoprostan, FMD: flow-mediated dilation, NTG-D: nitroglycerin-mediated dilation.

lipid-lowering drugs for at least 6 weeks prior to the study. None had suffered acute coronary events for at least three months prior to the study. Based on the plasma LDL levels of the patients, they were randomly assigned to treatment in the low-dose simvastatin group or the control group (baseline LDL< 100 mg/dl: 5 mg/d of simvastatin; $n = 14$, 6 men, LDL 82.3 ± 34.3 mg/dl and no prescription; $n = 9$, 4 men, LDL 91.1 ± 30.2 mg/dl) or to the moderate-dose simvastatin group (10 mg/day; $n = 14$, 6 men, baseline LDL< 100 mg/dl, LDL 118.0 ± 33.8 mg/dl). Prescription treatment lasted 16 weeks. All patients provided informed consent, agreed to the protocols, and were willing to participate in the study. Ineligible patients included those who had not taken any estrogen for >12 weeks. The study was approved by the ethics committee of Nagoya University Graduate School of Medicine. The participants had received hemodialysis for 4.1 ± 1.2 years, their average systolic and diastolic blood pressure was 127.4 ± 11.7/78.2 ± 9.8 mm Hg, and their complicated diseases included ischemic heart disease, hypertension, and diabetes mellitus (Table 1). Seven patients were smokers (Table 1). Their use of medications that can affect endothelial function, such as angiotensin converting enzyme inhibitor (ACEI), angiotensin receptor blockers (ARBs), other antihypertensive agents, and diuretics, is indicated in Table 1. Diabetic nephropathy was most frequent underlying renal disease. The patient profiles, including other backgrounds, are shown in Table 1. Patients in the moderate-dose group had received hemodialysis for a longer term, on average, and most of them suffered from hypertension. Between the low and moderate-dose groups and the controls, there were no significant differences among other parameters, including prescribed agents.

2.2. Vascular function

Flow-mediated dilatation (FMD) and dilatation by nitroglycerin were determined according to a method described previously [8]. Briefly, the diameter of the right brachial artery was measured by a high-resolution ultrasound cardiograph (SONOS 2000, Hewlett Packard). Blood pressure was monitored every 2 min. To produce reactive hyperemia, blood flow to the forearm was prevented by inflation of the cuff on the arm to 250 mm Hg for 5 min. The diameter was measured from the anterior to the posterior interface between the media and adventitia and was calculated from 3 cardiac cycles synchronized with the R-wave peaks on the ECG. The measurement obtained at 60 s after cuff release showed maximal dilatation. The diameter change was expressed as the percent change relative to the diameter during the initial resting scan (%FMD). Fifteen minutes later, a resting scan was recorded and a sublingual nitroglycerin spray (300 μg, Toa Eiyou Co.) was administered. Three minutes later, the last scan was performed. The diameter change was expressed as the percent dilatation by nitroglycerin (%NTD). In our study, the interobserver variability for repeated measurements of resting arterial diameter was 0.05 ± 0.02 mm. The intraobserver variability for repeated measurements of resting arterial diameter was 0.02 ± 0.02 mm. In other words, the reproducibility (<0.1% difference) of the %FMD was greater than 96.3%. Vascular function was studied before commencing treatment, and then after 1 week and 16 weeks of treatment; it was studied in the morning of the day of hemodialysis, and it was performed just before hemodialysis during overnight fast status.

2.3. Blood sampling

Blood sampling was performed on the morning of the ultrasound examination (day of hemodialysis) under overnight fast status. Serum total cholesterol, triglyceride, and HDL cholesterol concentrations were measured [9]. Plasma nitrite and nitrate levels ($NO2^-$ and $NO3^-$) were measured with an automated NO detector/high-performance liquid chromatography system (ENO10, Eicom Co., Kyoto, Japan), as previously reported [10]. In brief, nitrite and nitrate levels in the patient's plasma were separated by a reverse-phase separation column, and nitrate was reduced to nitrite in a reduction column. Nitrite was mixed with a Griess reagent, and the absorbance at 540 nm was measured by a flow-through spectrophotometer. The concentration of interleukin 6 (IL-6), tumor necrosis factor-α (TNF-α), soluble vascular cell adhesion molecule 1 (sVCAM-1), and 8-isoprostane (8-epi-prostaglandin F2) were assessed by ELISA kits (Cytoscreen Immunoassay Kit, Bioxytech 8-isoprostane assay kit, Oxis International, Inc). Plasma Ox-LDL was assayed using a Kyowa Medex MX kit (Kyowa Medex, Inc., Tokyo), which is a sandwich-type enzyme immunoassay using anti-oxidized phosphatidylcholine monoclonal antibody (DLH3) and anti-human apolipoprotein-B antibody [11,12].

2.4. Safety measures

All adverse events were recorded at each examination. Physical examinations, hematology, and serum chemistry assays (liver and renal function and creatine phosphokinase) were conducted throughout the study.

2.5. Statistical analyses

Data are presented as the mean ± SD of each group of patients. $P<0.05$ was considered to indicate statistical significance in all analyses. All statistical analyses were performed using JMP software (version 6, SAS Institute Inc., Cary, NC). Differences between categorical baseline characteristics were tested by the chi-square test. In addition, the parameters of interest were tested for statistical difference by ANOVA between the three different groups (low dose, moderate dose, and control groups).

Multivariate logistic regression analyses were done with adjustment for baseline characteristics such as sex.

3. Results

Table 1 shows the baseline profiles for each group. The period of hemodialysis, LDL cholesterol, and TNF-α were different between subjects receiving low (5 mg/day) doses of simvastatin or subjects in the control group and subjects receiving moderate (10 mg/day) doses of simvastatin (Table 1). No other differences were observed in the values for each condition shown in Table 1. Serum lipid concentrations (total cholesterol, triglycerides, and HDL cholesterol) remained unchanged in all patients in response to 1 week of treatment with simvastatin, and LDL cholesterol was decreased at 16 weeks in both statin groups (Table 2). The sVCAM-1 level decreased significantly at 1 week in both statin treatment groups (especially in the low-dose group), but not in the control group (Table 2). However, TNF-α and IL-6 did not change during the course of the experiment (Table 2). No abnormal data were noted in the other biochemical measures, including creatine phosphokinase levels, throughout the treatment term in either group (data not shown).

The endothelium-dependent flow-mediated dilatation (%FMD) in those receiving simvastatin significantly increased at 1 week in both statin groups (low dose: 5.7% to 8.7% in 1 week, $P<0.01$, and 10.1% in 16 weeks, $P<0.001$, moderate dose: 4.5% to 5.7% in 1 week, $P<0.01$, and 7.9% in 16 weeks, $P<0.001$ Fig. 1A). No difference in the response to nitroglycerin (%NTG) was demonstrated after 16 weeks of treatment (Fig. 1B). The %FMD

Table 2
Change of lipids and cyotokine concentrations by statin treatment.

Statin treatment	Low dose (5 mg/day)			Moderate dose (10 mg/day)			Control		
	Before	1 wk	16 wks	Before	1 wk	16 wks	Before	1 wk	16 wks
Total chol. (mg/dl)	135.5 ± 36.0	122.1 ± 46.3	120.3 ± 41.4	171.1 ± 46.1	151.0 ± 45.4	**128.2* ± 23.1**	149.4 ± 42.4	153.1 ± 49.1	152.5 ± 48.5
Triglycerides (mg/dl)	99.8 ± 57.4	95.2 ± 52.5	101.4 ± 48.5	133.8 ± 62.0	116.7 ± 55.7	103.0 ± 44.1	108.3 ± 40.6	113.2 ± 52.1	115.0 ± 54.3
HDL chol. (mg/dl)	32.4 ± 11.7	34.2 ± 12.8	35.3 ± 13.6	32.1 ± 6.2	31.8 ± 7.1	35.8 ± 9.4	33.1 ± 7.9	32.9 ± 7.2	33.6 ± 9.0
LDL chol. (mg/dl)	83.2 ± 34.3	71.1 ± 33.0	**64.7* ± 28.3**	109.6 ± 33.8	89.0 ± 33.7	**74.5* ± 30.6**	93.5 ± 31.2	94.1 ± 35.3	95.0 ± 32.8
sVCAM-1 (ng/ml mg protein)	880.2 ± 168.2	**686.2* ± 132.1**	**619.2* ± 210.4**	1027.4 ± 151.9	798.8 ± 115.4	**765.6* ± 115.1**	922.6 ± 137.2	938.2 ± 166.0	940.1 ± 169.1
TNFα (pg/ml)	5.0 ± 1.9	5.3 ± 2.3	4.5 ± 1.1	9.1 ± 5.4	9.0 ± 5.4	8.9 ± 3.1	6.6 ± 3.4	6.4 ± 3.7	6.4 ± 3.1
IL-6 (pg/ml)	7.9 ± 3.3	7.7 ± 2.5	7.9 ± 2.3	4.6 ± 1.4	4.9 ± 2.1	5.6 ± 2.0	6.5 ± 2.6	6.6 ± 2.5	6.4 ± 2.8

Bold emphasis and * show the significant differences vs. value in before treatment.
Low dose(5 mg/day): simvastatin 5 mg/day group, moderate dose(10 mg/day): simvastatin 10 mg/day group. Control: no prescription group. The numbers are the mean ± SD.
*$P<0.05$ vs. the value in before treatment. Abbreviations: low dose (5 mg/day): simvastatin 5 mg/day group, moderate dose/10 mg/day): simvastatin 10 mg/day group. Control: no prescription group.
LDL: low-density lipoprotein, HDL: high-density lipoprotein, chol.:cholesterol.
Before: before treatment, 1 wk; treatment with simvastatin for 1 week, 16 wks; treatment with simvastatin for 16 weeks.

after 16 weeks of treatment in both simvastatin groups tended to be higher than in patients in both groups at week 1 (Fig. 1A). In the control group, no changes were observed in %FMD or %NTG. There was no significant relationship between the degree of LDL lowering and improved endothelial function; this may suggest a direct effect of statin other than its lipid-lowering effect. However, the basal conditions, such as plasma LDL cholesterol and the period of hemodialysis, were different between the low and moderate statin groups (Table 1), and direct comparison between these two groups was difficult. The plasma nitrite/nitrate (NOx) levels also tended to become higher in patients receiving simvastatin (Fig. 2A), and low-dose simvastatin administration also caused an increase in NOx (mM) (120.4 ± 15.6 in 0 week, 77. 2 ± 5.2 in 1 week, 159.4 ± 7.61 in 16 weeks) ($P<0.05$). The 8-Epi-isoprostan (ng mg protein/ml)was decreased at 1 and 16 weeks in both statin treatment groups (low dose: 21.4 ± 6.4 in 0 week, 13. 2 ± 2.6 in 1 week, 10.1 ± 2.4 in 16 weeks), but it did not change in the control group (Fig. 2B). Oxidized LDL (mg/ml) was also decreased at 16 weeks in both statin groups (low dose: 245 ± 33.4 in 0 week, 220. 2 ± 19.2 in 1 week, 163.4 ± 17.6 in 16 weeks) ($P<0.05$ in data of 0 weeks or that of 8 weeks vs. that of 16 weeks) (Fig. 2C). However, oxidized LDL did not remain significantly lower after statin treatment when corrected for LDL cholesterol levels, and it is not evident whether a decrease in oxidized LDL or in LDL affects %FMD more. Considering dose differences with regard to the effect of statin treatment, no additional effect of a high dose of statin was observed in the levels of % FMD, NOx, sVCAM-1, 8-isoprostane, TNF-α, or IL-6. The effect of statin therapy on %FMD, NOx, and 8-epi-isoprostane tended to be greater in the low-dose group compared to the moderate-dose group without reaching the significance threshold. Since the effect of statin therapy on plasma cholesterol in this group was less pronounced, these data might favor a direct mechanism of statin therapy.

As the improvement of % FMD did not depend on (change of) LDL levels, we did multiple regression analyses inserting data in 0 weeks found to be significantly different between low dose and moderate dose groups. We found the (short) period of hemodialysis and (young) age are significant factors associated with %FMD improvement.

4. Discussion

The guidelines of the Japan Atherosclerosis Society (2007) and NCEP (2006) state that LDL should be below 120–130 mg/dl and HDL should be greater than 40 mg/dl [13,14] in individuals with end-stage renal disease. We tried to investigate the additional effect of statin on a further decrease in LDL and other effects in patients undergoing hemodialysis. Simvastatin improved the impaired endothelial function of dialysis patients by decreasing oxidized LDL, improving the lipid profile and, at least in part, enhancing NO bioavailability.

Various mechanisms other than lipid lowering have been proposed to account for the anti-atherosclerotic effects of statins, including antioxidant activity and enhanced NO activity, as direct effects of statins on cells comprising the vascular wall [4–6,10]. Statins increase eNOS activity both *in vitro* and *in vivo* [5,6,15]. Because NO has many

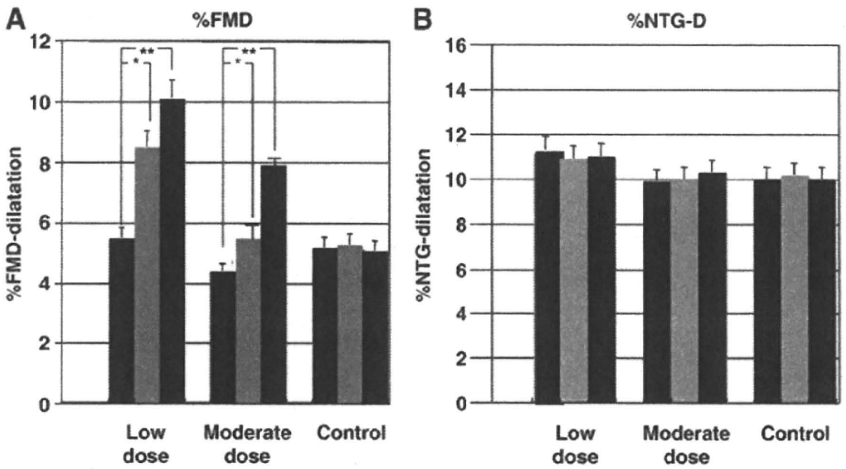

Fig. 1. A, Endothelial function assessed by measuring dilatation of the brachial artery using high-resolution vascular ultrasound in response to reactive hyperemia (FMD: endothelial-dependent flow mediated dilatation). The percent increase in vessel diameter induced by FMD (%FMD) is shown. *$P<0.05$. B, Endothelial-independent function assessed by measuring dilatation in response to sublingual nitroglycerin (NTG) infusion. The percent increase in diameter induced by nitroglycerin is shown (%NTG-D). No significant differences were observed following simvastatin treatment compared to before treatment. Data are expressed as the mean ± SD. Low dose: simvastatin 5 mg/day group, moderate dose: simvastatin 10 mg/day group. Control: no prescription group. The explanation of each bar graph in the group. Left; before treatment, middle; treatment with simvastatin for 1 week, and right; treatment with simvastatin for 16 weeks.

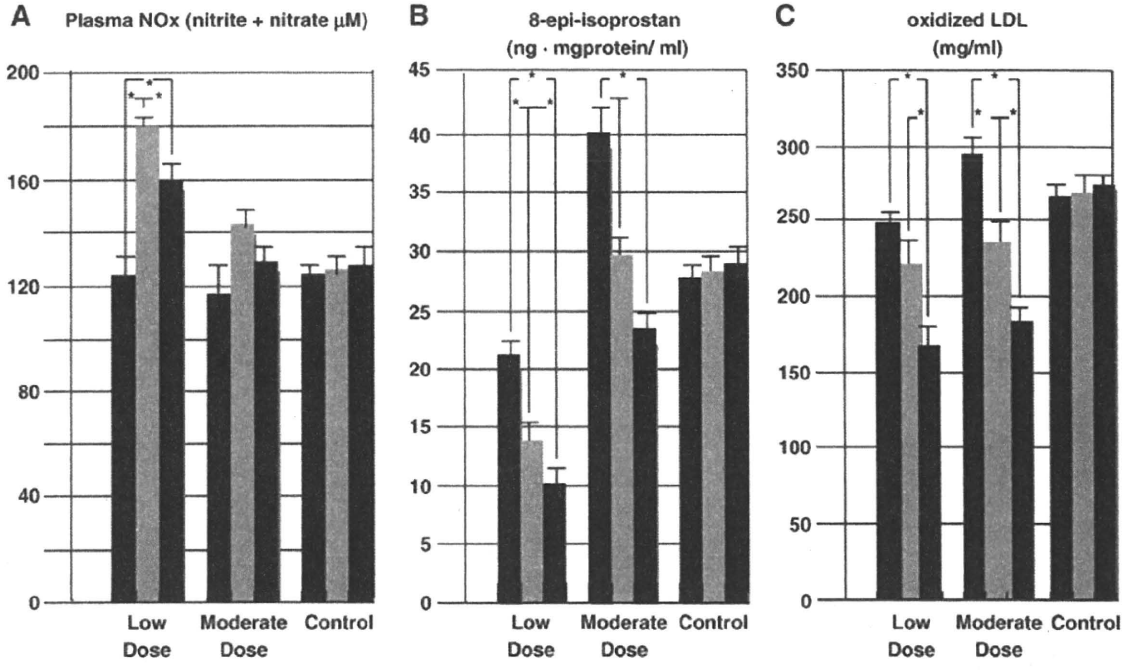

Fig. 2. Plasma concentration of nitrite/nitrate (NOx; A), isoprostane (B) or oxidized LDL (C) before and after simvastatin treatment or in the control group. Data are expressed as the mean ± SD. *$P<0.05$. Low dose: simvastatin 5 mg/day group, moderate dose: simvastatin 10 mg/day group. Control: no prescription group. The explanation of each bar graph in the group. Left; before treatment, middle; treatment with simvastatin for 1 week, and right; treatment with simvastatin for 16 weeks.

anti-atherosclerotic effects, such as inhibition of monocyte migration, the increased activity of eNOS in response to statins may partially explain its anti-atherosclerotic effects.

The %FMD has been studied extensively in recent years, and it is believed to reflect NO function in vessels [16]. Impairment of %FMD has been reported to precede coronary artery disease, and the %FMD is known to be low in atherosclerotic arteries [17]. Atherosclerosis is very severe in hemodialytic patients, regardless of their original diseases [18]. We noted that the %FMD in the patients evaluated in our study was low. The improvement in %FMD in hemodialysis patients through short-term statin treatment may be due to improved microvascular circumstances or blood fluidity rather than improvement in the atherosclerotic conduit vessel itself [19,20]. The fact that statistical analyses show that the period after introducing hemodialysis is a significant determinant may support this concept.

There are few reports on the effects of statin in patients undergoing hemodialysis [21–25]. Although the number of cardiac events may be reduced, total cardiovascular events, mortality, and total mortality were reported to be the same with or without statin [21–23]. In the present study, statin treatment does not seem to increase HDL cholesterol or reduce plasma triglycerides. Although this may be due to the specific composition of the study population under hemodialysis, it may remain a noteworthy observation and may be related to the effect of statin as mentioned above. Six weeks of atorvastatin treatment (40 mg/day) was reported to improve small artery compliance, but not FMD, in patients in stages 3–5 stages of CKD and hemodialysis [24], although the number of hemodialysis patients was small and a more detailed analysis may be necessary. Furthermore, five months of treatment with pravastatin in patients with chronic dialysis did not have a significant effect on surrogate markers of endothelial function, such as IL-6, sVCAM-1, sICAM-1, etc. Although the CKD and hemodialysis study [25] did not measure NO-related products and we cannot compare the data directly, the kind of statin and the condition of patients with dialysis, such as the period of dialysis, might account for the discrepancy. The difference in the present study's data with regard to the grade of FMD improvement between low and moderate-dose groups may support this explanation.

However, there is no information on the effect of the period of hemodialysis and endothelial function on the previous study's results [26]. Renal failure with or without hypercholesterolemia might further worsen endothelial function because of the presence of asymmetric dimethylarginine and/or uremic toxin [26]. Inhibition of arginine synthesis by urea is a mechanism of arginine deficiency in renal failure that leads to increased hydroxyl radical generation. However, the effect of uremic toxin should be decreased by hemodialysis. Endothelial function, as measured by %FMD, improved in the hemodialytic patients receiving simvastatin after only seven days, and the same trend was observed for plasma nitrite/nitrate, supporting the hypothesis that simvastatin improves endothelial NO function itself. This is the first report that an improvement in %FMD was observed in patients receiving hemodialysis.

In a thrombogenic event such as myocardial infarction, the thrombosis occurs due to impaired endothelial function and atherosclerosis caused by activation of cytokines or adhesion molecules such as VCAM-1 [26,27]. In the present study, VCAM-1, oxidized LDL, and isoprostane were decreased by statin. Improved endothelial function, such as increased %FMD, decreased adhesion molecules, and decreased free radicals may prevent vascular thrombogenic events [27–29]. When statins improve %FMD levels via a direct effect, an improvement in atherogenic molecules and free radicals as well as lipid lowering should result in further improvement of endothelial function. Although we reported improved %FMD in as short a time as three days [4], a short-term effect of statin independent of plasma lipid levels, the possibility of a pleiotropic effect without any relation to plasma lipid is interesting. Taken together, the detailed mechanism of the improvement in %FMD might be different between what has been previously reported for diabetics and what we determined in the present study for hemodialytic patients. In the present study, %FMD levels were greater after 16 weeks than after 7 days of treatment. The continuous improvement in %FMD levels after statin treatment for 16 weeks may mean that both mechanisms (direct and indirect effects) contribute to this action [5,6,30–32]. The data for 8-isoprostan, a marker of reactive oxygen species, support this hypothesis. Consequently, the bioavailability of