

Asymmetric Dimethylarginine Limits the Efficacy of Simvastatin Activating Endothelial Nitric Oxide Synthase

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Background—Asymmetric dimethylarginine (ADMA), an endogenous inhibitor of endothelial nitric oxide synthase (eNOS), is considered a risk factor for the pathogenesis of cardiovascular diseases. Simvastatin, a lipid-lowering drug with other pleiotropic effects, has been widely used for treatment of cardiovascular diseases. However, little is known about the effect and underlying molecular mechanisms of ADMA on the effectiveness of simvastatin in the vascular system.

Methods and Results—We conducted a prospective cohort study to enroll 648 consecutive patients with coronary artery disease for a follow-up period of 8 years. In patients with plasma ADMA level ≥ 0.49 $\mu\text{mol/L}$ (a cut-off value from receiver operating characteristic curve), statin treatment had no significant effect on cardiovascular events. We also conducted randomized, controlled studies using in vitro and in vivo models. In endothelial cells, treatment with ADMA (≥ 0.5 $\mu\text{mol/L}$) impaired simvastatin-induced nitric oxide (NO) production, endothelial NO synthase (eNOS) phosphorylation, and angiogenesis. In parallel, ADMA markedly increased the activity of NADPH oxidase (NOX) and production of reactive oxygen species (ROS). The detrimental effects of ADMA on simvastatin-induced NO production and angiogenesis were abolished by the antioxidant, *N*-acetylcysteine, NOX inhibitor, or apocynin or overexpression of dimethylarginine dimethylaminohydrolase 2 (DDAH-2). Moreover, in vivo, ADMA administration reduced Matrigel plug angiogenesis in wild-type mice and decreased simvastatin-induced eNOS phosphorylation in aortas of apolipoprotein E-deficient mice, but not endothelial DDAH-2-overexpressed aortas.

Conclusions—We conclude that ADMA may trigger NOX-ROS signaling, which leads to restricting the simvastatin-conferred protection of eNOS activation, NO production, and angiogenesis as well as the clinical outcome of cardiovascular events. (*J Am Heart Assoc.* 2016;5:e003327 doi: 10.1161/JAHA.116.003327)

Key Words: asymmetric dimethylarginine • endothelial nitric oxide synthase • NADPH oxidase • reactive oxygen species • simvastatin

Statins, the inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase, increase the hepatic expression of the low-density lipoprotein (LDL) receptor, which then decreases LDL levels by up to 50% in the circulation.^{1,2} In addition to the

cholesterol-lowering effect, statins also provide protection against cardiovascular diseases by other pleiotropic effects, such as antioxidation, anti-inflammation, and increased bioavailability of nitric oxide (NO).^{3–5} Treatment with statins promotes angiogenesis in ischemic limbs of normocholesterolemic animals.³ At cellular and molecular levels, simvastatin increases endothelial nitric oxide (eNOS) activity and leads to NO production in endothelial cells (ECs) by regulating a kinase-dependent pathway and protein-protein interaction.^{6,7} Despite many achievements of statins in treating cardiovascular complications,^{3,8–10} such treatment is not always effective in improving EC function and inflammation, with unclear mechanisms under some circumstances.^{11,12} Recently, asymmetric dimethylarginine (ADMA) has been implicated as a contributing factor to the lack of protective effect of statins.^{13,14}

ADMA, a circulating endogenous inhibitor of NOS by competing with L-arginine as the substrate, has been suggested as an important risk factor for cardiovascular diseases.^{13–15} Therefore, derangement of the L-arginine/NO pathway and increased oxidative stress by ADMA have been considered important contributing factors in the development

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of endothelial dysfunction.^{13–15} Epidemiological investigations have shown that the increased plasma level of ADMA may predict cardiovascular events in patients.^{16,17} Although the clinical efficacy of statin therapy in reducing cardiovascular mortality and morbidity has been well established by several clinical trials,^{1,2} whether ADMA impairs the statin-mediated benefit in EC dysfunction and cardiovascular diseases remains elusive.

Two isoforms of dimethylarginine dimethylaminohydrolase (DDAH) are responsible for metabolism of ADMA. DDAH-1 is predominately expressed in proximal tube kidney and in the liver, whereas DDAH-2 is the predominant isoform in vascular cells.^{18,19} Recent research found that overexpression of DDAH-1 reduced ADMA levels and angiotensin II–induced hypertension in mice.²⁰ Additionally, overexpression of DDAH-1 ameliorated atherosclerosis by lowering ADMA level in apolipoprotein E–deficient (apoE^{−/−}) mice.²¹ Moreover, overexpression of DDAH-2 improved endothelial dysfunction in normolipidemic, hyperlipidemic, and diabetic experimental animals.^{22,23} Therefore, homeostasis of the DDAH-ADMA system may play a crucial role in maintaining vascular function.

In the present study, we aimed to elucidate the role of ADMA in simvastatin-activated eNOS-NO signaling and the possible molecular mechanisms in human aortic ECs (HAECs) and mice. We first aimed to examine the relation between plasma ADMA level and the beneficial effect of statins in patients with coronary artery disease (CAD). Second, we explored the effects of ADMA on simvastatin-mediated eNOS activation, NO production, and angiogenesis in HAECs, then investigated the effects on NADPH oxidase (NOX) activity and reactive oxygen species (ROS) production. Finally, we explored whether overexpression of DDAH-2 in ECs could prevent the ADMA-induced detrimental effects in ECs and mice.

Methods

Clinical Outcomes Studies

From July 2006 to June 2009, we enrolled 648 consecutive patients with CAD. Exclusion criteria included patients with liver cirrhosis, end-stage renal disease, acute or chronic infectious/inflammatory disease, malignancy with expected life span <1 year, and unstable hemodynamic status. Statin treatment was defined as using statins before and during follow-up. All patients were prospectively followed by a monthly office visit or by telephone and chart review for occurrence of first-ever primary endpoints, which included all-cause mortality, cardiovascular death, and major adverse cardiovascular/cerebral events (MACCEs; defined as cardiovascular death, nonfatal myocardial infarction and stroke).

Cardiovascular death was diagnosed as any death with definite cardiovascular cause or any death that was not clearly attributed to a noncardiovascular cause. Myocardial infarction (MI) was defined as the presence of significant new Q waves in at least 2 electrocardiography leads or symptoms compatible with MI associated with increased creatine kinase-MB fraction ≥ 3 times the upper reference limit. Stroke with neurological deficit was diagnosed by a neurologist on the basis of imaging study. The study protocol was approved by the institutional review board at Taipei Veterans General Hospital (Taipei, Taiwan), and informed written consent was obtained from each participant in accord with the ethical guidelines of the Declaration of Helsinki.

Laboratory Measurements

All medications, cigarette smoking, and consumption of beverages containing alcohol or caffeine were withdrawn for at least 12 hours. Fasting blood samples were collected with EDTA used as an anticoagulant and centrifuged at 900 g for 10 minutes at 4°C immediately after collection. Plasma samples were frozen at −70°C until analysis. Plasma L-arginine, symmetric dimethylarginine (SDMA), and ADMA concentrations were determined by high-performance liquid chromatography using precolumn derivatization with o-phthalaldehyde as described.¹⁷ The recovery rate for ADMA was >90%, and the within-assay and between-assay variation coefficients were not more than 7% and 8%, respectively.

Reagents

Simvastatin, ADMA, SDMA, mouse antibody for α -tubulin, and Griess reagent were from Sigma-Aldrich (St Louis, MO). Rabbit antibody for phospho-eNOS Ser1179 was from Cell Signaling Technology (Beverly, MA). Rabbit antibodies for eNOS and DDAH-2 were from Santa Cruz Biotechnology (Santa Cruz, CA). The cGMP enzyme immunoassay kit was from R&D Systems (Minneapolis, MN). ECL Cell Attachment Matrix was from Millipore (Bedford, MA). The EnzyChrom NADP⁺/NAD(P)H assay kit was from BioAssay Systems (Hayward, CA). Hydroethidine (DHE) and 2',7'-dichlorofluorescein diacetate (DCFH-DA) were from Molecular Probes (Eugene, OR).

Cell Culture

HAECs from PromoCell (Heidelberg, Germany) were cultured in Endothelial Cell Growth Medium MV supplemented with 100 unit/mL of penicillin and 100 μ g/mL of streptomycin (HyClone, Logan, UT) in a humidified 95% air/5% CO₂ incubator at 37°C.

Animals

The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996), and all animal experiments were approved by the animal care and utilization committee of the National Yang-Ming University (Taipei, Taiwan). Eight-week-old male C57BL/6 wild-type (WT) and apoE^{-/-} mice were purchased from The Jackson Laboratory (Bar Harbor, ME). EC-specific transgenic (Tg) mouse lines were established by microinjecting Tie2-DDAH2 plasmid into fertilized C57BL/6 eggs. Positive Tg mice were identified by polymerase chain reaction (PCR). Tie2-DDAH-2 Tg plasmid was created by modification from Tie2-Nox4 plasmid, which was kindly provided by Dr Junichi Sadoshima (Department of Cell Biology and Molecular Medicine, Cardiovascular Research Institute, Rutgers New Jersey Medical School, Newark, NJ).²⁴ To generate apoE^{-/-}EC-DDAH-2 Tg mice, EC-DDAH-2 Tg mice were crossed with the apoE^{-/-} background, and PCR of genomic DNA was used to confirm apoE^{-/-} and EC-DDAH-2 Tg genotypes. Mice were housed in barrier facilities maintained on a 12-hour/12-hour dark cycle. Temperature (22°C) and humidity (40–60%) of the vivarium were tightly controlled. Mice were group housed 3 to 4 per cage and fed a regular chow diet, which contained 4.5% fat by weight (0.02% cholesterol; Newco Distributors, Redwood, CA). Male apoE^{-/-} and apoE^{-/-}EC-DDAH-2 Tg mice at 4 months old received daily treatment with simvastatin (10 mg/kg body weight) and DMSO (vehicle control; n=8 mice/group) by gastric gavage or ADMA (5 mg/kg body weight) by intraperitoneal injection for 4 weeks. At the end of the experiment, mice were euthanized with CO₂ and aortas were harvested and stored at -80°C. Isolated aortas were homogenized and lysates were subjected to western blot analysis.

Adenoviral Construction and Infection

To generate the adenoviruses (Ad) expressing the human DDAH-2 (Ad-DDAH-2), the cDNA fragment containing the human DDAH-2 was subcloned into the shuttle plasmid pTRE-shuttle2 and recombined into adenoviral DNA according to the protocol provided (Clontech, Palo Alto, CA). For Ad-mediated gene transfer, HAECs were infected with Ad at a multiplicity of infection (MOI) of 12.5 to 50 for 24 hours before experiments.

Determination of NO Production and Intracellular cGMP

Accumulated nitrite (NO₂⁻), the stable breakdown product of NO, in culture media was measured by mixing an equal volume of Griess reagent, then incubating at room

temperature for 15 minutes. Azo dye production was analyzed by use of a SP-8001 UV/VIS spectrophotometer (Metertech, Taipei, Taiwan) with absorbance at 540 nm. Sodium nitrite was used as a standard. Intracellular levels of cGMP in ECs were assessed by use of an enzyme immunoassay kit and normalized to protein content as determined by the Bradford assay.

Protein Extraction and Western Blot Analysis

HAECs were lysed by SDS lysis buffer containing 1% Triton, 0.1% SDS, 0.2% sodium azide, 0.5% sodium deoxycholate, and proteinase inhibitors (1 mmol/L of PMSF, 10 µg/mL of aprotinin, and 1 µg/mL of leupeptin). Lysates were centrifuged at 13 800 g for 5 minutes, and the supernatant was collected. Extracted protein was quantified by protein assay. Aliquots (50 µg) of cellular lysates were separated by 8% SDS-PAGE and transferred to BioTrace PVDF membrane (Pall Corporation, Westborough, MA). After blocking with 5% skim milk, blots were incubated with primary antibodies, then corresponding secondary antibodies. Protein bands were detected by use of an enhanced chemiluminescence kit and quantified by use of ImageQuant 5.2 (Healthcare Bio-Sciences, Philadelphia, PA).

In Vitro Angiogenesis (Tube Formation) Assay

ECL Cell Attachment Matrix (Millipore) was added to 24-well plates and polymerized overnight at 37°C. Cells were seeded onto the layer of matrix gel and incubated with the indicated treatments for 4 hours. Tube formation was assessed by counting the number of branch points.

In Vivo Matrigel Plug Angiogenesis Assay

To induce formation of new blood vessels in vivo, Matrigel (8 mg/mL) was mixed with heparin (50 U/mL), simvastatin (10 µmol/L) with or without ADMA (0.5 µmol/L), then subcutaneously injected into mice. At day 7 postinjection, Matrigel plugs were removed and photographed. The hemoglobin assay was performed after Matrigel plugs were homogenized and incubated with Drabkin's reagent for 30 minutes at room temperature. The hemoglobin concentration was calculated at 540 nm.

Measurement of Intracellular ROS Levels

The membrane-permeable probe, DHE, and DCFH-DA (Molecular Probes, Eugene OR) were used to assess intracellular ROS levels. Oxidation of DHE by ROS, preferentially O₂^{·-}, forms red fluorescent ethidium, whereas oxidation of DCFH-DA by ROS, particularly H₂O₂, yields fluorescent 2',7'-dichlorofluorescein

(DCF). Briefly, HAECs were washed with PBS and incubated in cell medium containing 10 $\mu\text{mol/L}$ of Hektoen enteric agar or 20 $\mu\text{mol/L}$ of DCFH-DA at 37°C for 45 minutes. Subsequently, the cell medium containing DHE or DCFH-DA was removed and replaced with fresh medium. Cells were then incubated with simvastatin (10 $\mu\text{mol/L}$) with or without ADMA (0.5 $\mu\text{mol/L}$) for 10 minutes. Cells were washed twice with PBS and detached with trypsin/EDTA, and fluorescence intensity was analyzed by FACScan flow cytometry (Becton Dickinson, San Jose, CA) at 530-nm excitation and 620-nm emission for ethidium and 488-nm excitation and 530-nm emission for DCF.

Determination of NOX Activity

HAECs were incubated with simvastatin (10 $\mu\text{mol/L}$) with or without ADMA (0.5 $\mu\text{mol/L}$) for 10 minutes. Activity of NOX

was analyzed by use of the EnzyChrom NADP⁺/NADPH assay kit.

Statistical Analysis

Results are presented as the median and interquartile range (IQR) or 95% CI. The Mann–Whitney *U* test was used to compare 2 independent groups. Kruskal–Wallis analysis followed by Bonferroni post-hoc correction was used to account for multiple testing. Categorical data were compared by chi-square or Fisher’s exact test. Analysis of receiver operating characteristic (ROC) curve was performed to obtain the optimal cut-off value of ADMA, which was employed to divide ADMA levels into 2 tertiles. Actuarial event-free survival curves were estimated by the Kaplan–Meier method and compared by log-rank test. A forward step-wise Cox

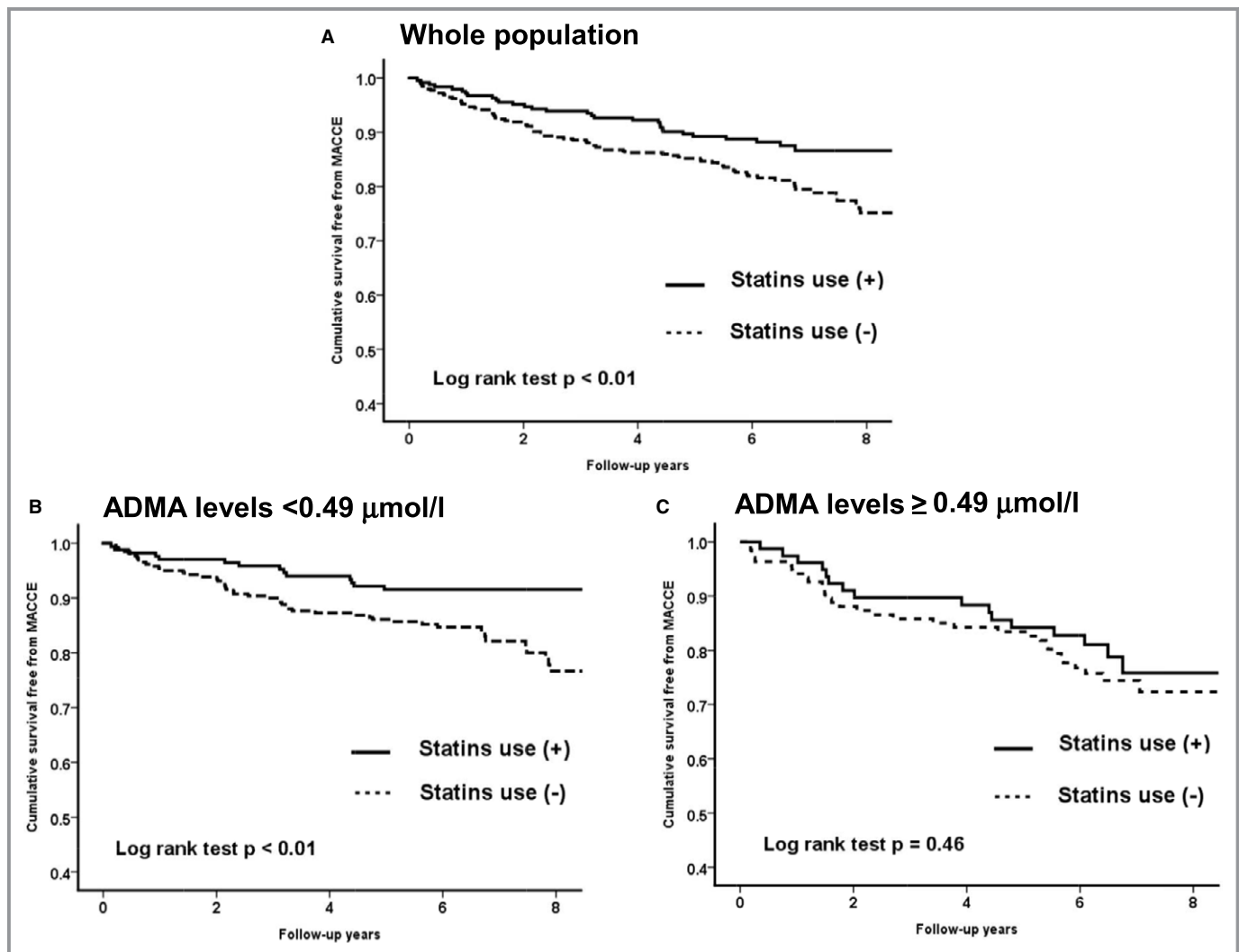


Figure 1. Kaplan–Meier survival analyses of major adverse cardiovascular/cerebrovascular events during follow-up by use or not of statins. A, In the whole population, (B) in patients with plasma ADMA levels $< 0.49 \mu\text{mol/L}$, and (C) in patients with plasma ADMA levels $\geq 0.49 \mu\text{mol/L}$. *P* values by log-rank test are shown. ADMA indicates asymmetric dimethylarginine; MACCE, major adverse cardiovascular/cerebral event.

Table 1. Baseline Characteristics

	Statins User (n=248)	Statins Nonuser (n=400)	P Value
Age, y	66±13	70±12	<0.01
Sex, male (%)	212 (86)	364 (91)	0.04
BMI, kg/m ²	26.8±3.9	25.4±3.5	<0.001
Hypertension (%)	194 (78)	310 (78)	0.85
Diabetes (%)	98 (40)	174 (44)	0.33
Smoking (%)	69 (28)	24 (16)	0.92
LVEF	51.5±11.4	50.8±12.0	0.45
Creatinine, mg/dL	1.3±0.8	1.3±0.7	0.64
Cholesterol, mg/dL			
Total	181.2±41.7	159.2±27.8	<0.01
LDL	114.8±38.8	97.3±23.8	<0.01
HDL	42.8±11.7	41.1±10.5	0.08
Triglyceride, mg/dL	158.7±90.8	139.5±90.6	0.01
eGFR, mL/min per 1.73 m ²	72.1±25.2	70.5±27.2	0.46
L-arginine, μmol/L	95.4±32.2	91.4±30.5	0.11
ADMA, μmol/L	0.47±0.11	0.47±0.10	0.63
SDMA, μmol/L	0.48±0.30	0.47±0.24	0.69

ADMA indicates asymmetric dimethylarginine; BMI, body mass index; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; LDL, low-density lipoprotein; LVEF, left ventricular ejection fraction; SDMA, symmetric dimethylarginine.

regression analysis was performed to identify independent predictors of long-term MACCE. Variables with significant differences ($P<0.05$) on univariate analysis were included in the multivariate Cox regression model to identify predictors that remained significant after adjustment for cofactors. $P<0.05$ was considered statistically significant. SPSS software (version 20.0; SPSS, Inc., Chicago, IL) was used for statistical analysis.

Results

Statin Treatment and Long-Term Clinical Outcomes

We enrolled 648 consecutive patients with CAD (576 males [88.9%]; mean age, 68.6±12.1 years) from July 2006 to June 2009. All patients were followed up for a median of 6.3 years (IQR, 5.5–7.9) without any lost to follow-up. During follow-up, there were 110 MACCEs (17.0%), including 70 cardiovascular deaths (11.1%); 248 patients (38.3%) had hypercholesterolemia and used statins, and although LDL cholesterol (LDL-C) levels were significantly reduced for patients not using statins (statins vs no statins: 114.8±38.7 vs 97.3±23.8 mg/dL; $P<0.01$), use of statins remained

significantly associated with a reduction in MACCEs by Kaplan–Meier analysis ($P<0.01$; Figure 1). Baseline characteristics of patients grouping according to the use of statins or not are provided in Table 1.

Further analysis by ROC curve revealed that the optimal cut-off value for ADMA was 0.49 μmol/L (sensitivity=0.418; specificity=0.716). We then subgrouped the whole population into 2 tertiles by plasma ADMA levels <0.49 and ≥0.49 μmol/L, and found that in patients with ADMA <0.49 μmol/L, use of statins remained associated with better clinical outcomes (log-rank test; $P<0.01$; Figure 1). In contrast, with plasma ADMA level ≥0.49 μmol/L, long-term MACCE did not differ in patients with and without statins (log-rank test; $P=0.46$), although the sample sizes of these 2 tertiles are different. On multivariate Cox regression analysis, statin treatment was associated with a reduction in MACCEs only in patients with lower ADMA tertiles (hazard ratio [HR], 0.72; 95% CI, 0.53–0.98, $P=0.035$), but not in those with the higher tertile. Statistical results from the univariate and multivariate Cox regression for the whole population as well as these 2 tertiles are presented in Table 2.

ADMA Impairs Simvastatin-Increased NO Bioavailability, eNOS Phosphorylation, and Tube Formation of ECs by Activating the ROS-NOX-Signaling Pathway

We then validated the effect of ADMA on simvastatin-increased NO bioavailability, eNOS phosphorylation, and angiogenesis in HAECs. HAECs were treated with concentrations of SDMA (0, 0.125, 0.25, 0.5, 1.0, and 2.0 μmol/L) or ADMA (0, 0.125, 0.25, 0.5, 1.0, and 2.0 μmol/L) in the presence of simvastatin. SDMA did not affect simvastatin-mediated production of NO and cGMP (Figure 2A). However, treatment with 0.5 to 2.0 μmol/L of ADMA inhibited simvastatin-mediated production of NO and cGMP, phosphorylated eNOS (Ser1179), and tube formation (Figure 2B through 2D), which suggests that ADMA had an inhibitory effect on the vascular benefits of statins in ECs. We then evaluated the molecular mechanism by which ADMA interferes with the beneficial effects of simvastatin in ECs. ROS production was increased with 0.5 μmol/L of ADMA as early as 5 minutes, with peak level at 15 minutes (Figure 3A and 3B). In addition, 0.5 μmol/L of ADMA increased NOX activity as early as 5 minutes, with peak level at 10 minutes (Figure 3C). Pretreatment with the antioxidant N-acetylcysteine (NAC) or NOX inhibitor apocynin (APO) totally abrogated ADMA-increased ROS production (Figure 3D). To provide further evidence that the ROS-NOX-signaling pathway is crucial in ADMA-impeded NO production, eNOS activation, and tube formation induced by simvastatin, we depleted NOX activity or ROS production by treatment with APO or NAC, respectively. The inhibitory effects of ADMA on

Table 2. Univariate and Multivariate Cox Regression Analyses of Risk Factors for Major Adverse Cardiovascular/Cerebrovascular Events

	Univariate Analysis		Multivariate Analysis	
	HR (95% CI)	P Value	HR (95% CI)	P Value
Total population, n=648				
Age	1.05 (1.03–1.07)	<0.001	1.04 (1.02–1.0)	<0.001
Sex	1.35 (0.65–2.78)	0.42	—	—
Hypertension	1.56 (0.93–2.62)	0.09	—	—
Diabetes	1.59 (1.09–2.32)	0.016	1.55 (1.05–2.28)	0.027
eGFR	0.98 (0.98–0.99)	<0.001	0.99 (0.98–1.00)	0.032
Use of statins	0.78 (0.63–0.96)	0.02	—	—
ADMA <0.49 μmol/L, n=432				
Age	1.04 (1.02–1.07)	0.001	1.03 (1.00–1.06)	0.053
Sex	1.89 (0.68–5.18)	0.22	—	—
Hypertension	1.69 (0.86–3.33)	0.13	—	—
Diabetes	1.55 (0.94–2.56)	0.09	—	—
eGFR	0.98 (0.97–0.99)	0.001	0.99 (0.98–1.00)	0.043
Use of statins	0.66 (0.49–0.89)	0.007	0.72 (0.53–0.98)	0.035
ADMA ≥0.49 μmol/L, n=216				
Age	1.08 (1.04–1.21)	<0.001	1.09 (1.05–1.13)	<0.001
Sex	1.43 (0.51–3.97)	0.50	—	—
Hypertension	1.13 (0.51–2.52)	0.76	—	—
Diabetes	1.69 (0.96–2.97)	0.07	2.01 (1.14–3.55)	0.016
eGFR	0.98 (0.97–1.00)	0.006	—	—
Use of statins	0.90 (0.66–1.21)	0.47	—	—

ADMA indicates asymmetric dimethylarginine; eGFR, estimated glomerular filtration rate; HR, hazard ratio.

simvastatin-increased production of NO and cGMP and tube formation were blunted with NAC or APO (Figure 4A through 4C). Thus, the ROS-NOX-signaling pathway may play an important role in regulating ADMA-mediated restriction of the beneficial effects of simvastatin in ECs.

Overexpression of DDAH-2 Abolishes ADMA-Induced Impairment of Simvastatin-Mediated NO Production and Tube Formation in ECs

DDAH-2 plays a crucial role in regulating ADMA metabolism of ECs.^{24,25} We analyzed whether overexpression of DDAH-2 protects against ADMA-induced impairment of simvastatin-mediated NO production and tube formation. Infection with Ad expressing the human DDAH-2 (Ad-DDAH-2) increased the intracellular protein level of DDAH-2 (Figure 5A). In addition, overexpression of DDAH-2 diminished the ADMA-impaired level of nitrite and tube formation induced by simvastatin (Figure 5B and 5C).

Overexpression of DDAH-2 in ECs Alleviates ADMA-Impaired Angiogenesis by Simvastatin In Vivo

To confirm the in vitro findings, we used Matrigel plug assay to assess the protective effect of DDAH-2 on angiogenesis in vivo by use of WT and EC-DDAH-2 Tg mice. Treatment with simvastatin stimulated vascularization, as revealed by the hemoglobin content of Matrigel plugs in WT mice (Figure 6). ADMA in Matrigel plugs could significantly decrease simvastatin-induced hemoglobin content as compared with simvastatin alone. Moreover, hemoglobin content was further increased in Matrigel plugs in EC-DDAH-2 Tg mice as compared to WT mice with simvastatin treatment. The harmful effect of ADMA on simvastatin-induced angiogenesis was ablated in EC-DDAH-2 Tg mice as compared to WT mice. Thus, DDAH-2 may play a vital role in preventing the unfavorable effects of ADMA on statin-conferred benefits in the physiological function of ECs in vivo.

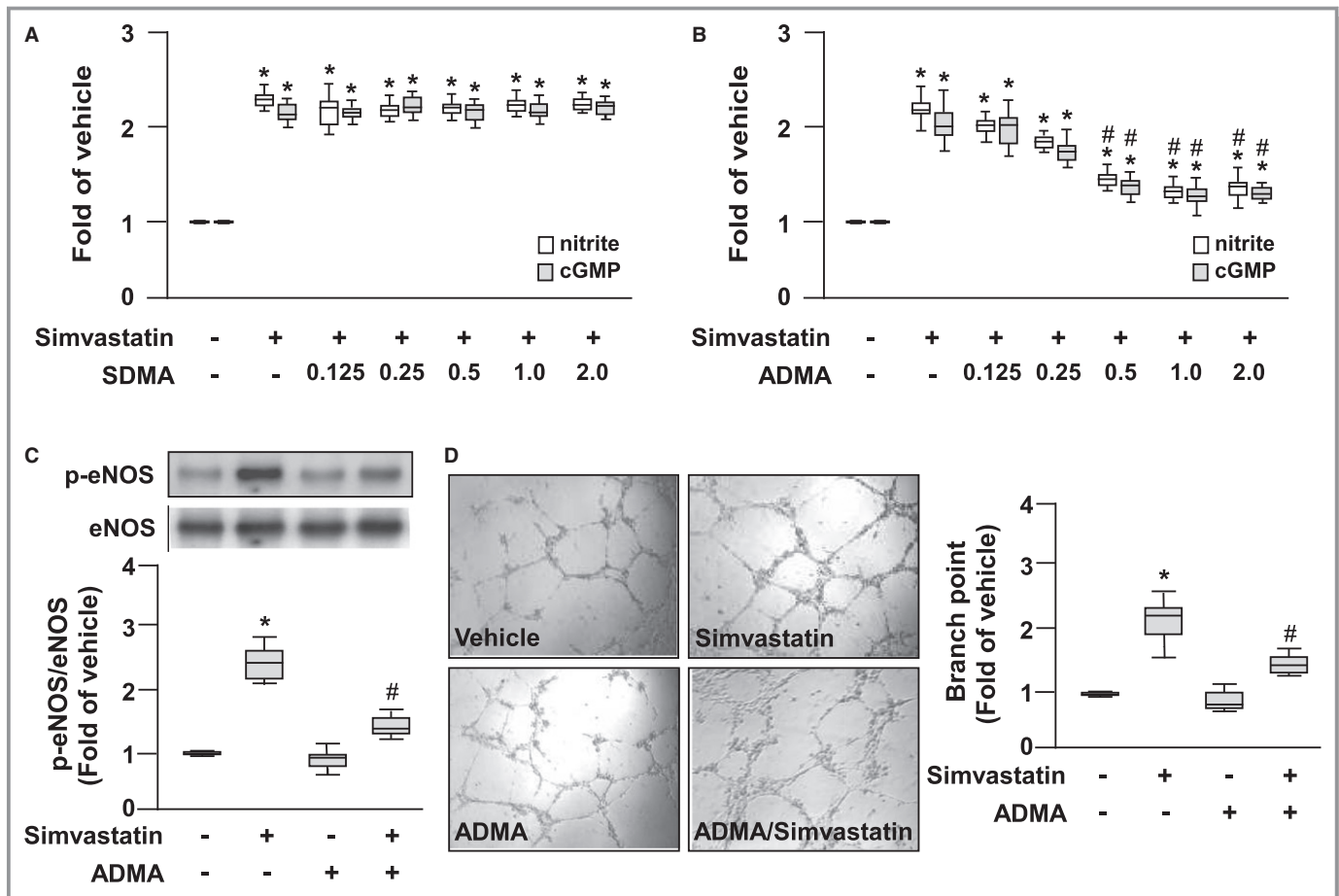


Figure 2. Effect of ADMA on simvastatin-mediated promotion of NO production, eNOS phosphorylation, and angiogenesis. A and B, Human aortic endothelial cells (HAECs) were pretreated with SDMA (0.125, 0.25, 0.5, 1.0, and 2.0 $\mu\text{mol/L}$) or ADMA (0.125, 0.25, 0.5, 1.0, and 2.0 $\mu\text{mol/L}$) for 30 minutes, then simvastatin (10 $\mu\text{mol/L}$) for 24 hours. Levels of nitrite in culture media and intracellular cGMP were measured by Griess assay and ELISA kits. C, After preincubation with ADMA, HAECs were treated with or without simvastatin (10 $\mu\text{mol/L}$) for 10 minutes. Western blot analysis of phosphorylated eNOS (p-eNOS) at Ser1179 and total eNOS level. D, Tube formation by HAECs; bar graphs indicate the fold increase in number of branch points in 5 randomly selected microscopy views. * $P < 0.05$ vs vehicle; # $P < 0.05$ vs simvastatin alone. ADMA indicates asymmetric dimethylarginine; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; SDMA, symmetric dimethylarginine.

Overexpression of DDAH-2 in ECs Prevents ADMA-Mediated Decrease in Simvastatin-Induced eNOS Phosphorylation in Aortas of apoE-Deficient Mice

To explore whether overexpression of DDAH-2 prevents ADMA-induced impairment of simvastatin-activated eNOS in aortas under hyperlipidemia, we determined the phosphorylated level of eNOS (Ser1179) in aortas of apoE^{-/-} mice and apoE^{-/-} EC-DDAH-2 Tg mice. Phosphorylated eNOS (Ser1179) level was significantly higher in simvastatin-treated than vehicle-treated apoE^{-/-} mice and apoE^{-/-} EC-DDAH-2 Tg mice (Figure 7). Additionally, treatment with ADMA abolished simvastatin-induced eNOS phosphorylation in aortas of apoE^{-/-} mice, but not apoE^{-/-} EC-DDAH-2 Tg mice (Figure 7).

Discussion

In this study, we found that patients with CAD show a reduction in long-term cardiovascular adverse events with statin treatment, as compared with no treatment, but these beneficial effects disappeared in those with the tertile of plasma ADMA $>0.49 \mu\text{mol/L}$. In addition, treatment with ADMA ($\geq 0.5 \mu\text{mol/L}$) impaired the simvastatin-increased NO production and eNOS phosphorylation as well as angiogenesis in ECs. Furthermore, ADMA markedly increased NOX activity and ROS production. The detrimental effects of ADMA on simvastatin-induced NO production and angiogenesis were abolished by treatment with the ROS scavenger NAC or NOX inhibitor apocynin or overexpression of DDAH-2. Finally, in vivo, administration of ADMA reduced Matrigel plug angiogenesis in WT mice and decreased simvastatin-

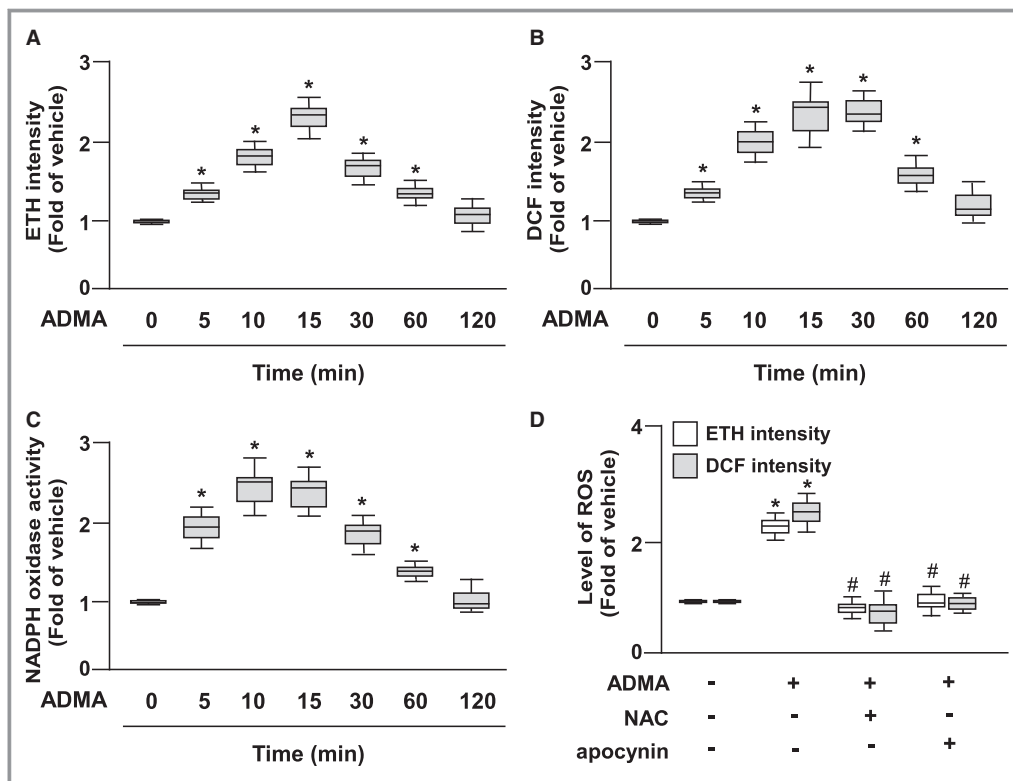


Figure 3. ADMA increases ROS production by the NADPH oxidase pathway. A through C, HAECs were treated with ADMA (0.5 $\mu\text{mol/L}$) for the indicated times. D, HAECs were pretreated with or without an ROS scavenger (NAC, 10 mmol/L) or an inhibitor of NAD(P)H oxidase (APO, 50 $\mu\text{mol/L}$) for 2 hours, then ADMA (0.5 $\mu\text{mol/L}$) for 15 minutes. Intracellular ROS levels and NADPH oxidase activities were analyzed by intensity of (A) red fluorescent DHE and (B) green fluorescent DCF and (C) $\text{NADP}^+/\text{NADPH}$ assay, respectively. * $P < 0.05$ vs vehicle; # $P < 0.05$ vs ADMA alone. ADMA indicates asymmetric dimethylarginine; DCF, dichlorofluorescein; ETH, ethidium homodimer; HAECs, human aortic endothelial cells; NAC, N-acetylcysteine; ROS, reactive oxygen species.

increased eNOS phosphorylation in aortas of apoE^{-/-} mice, but not EC-DDAH2-overexpressed aortas. ADMA may abolish simvastatin-elicited promotion of eNOS phosphorylation, NO production, angiogenesis, and probably the clinical benefits of statins, possibly by triggering NOX-ROS signaling.

Activation of eNOS-NO signaling is suggested to be a major mechanism of clinical therapeutic drugs in treating cardiovascular diseases.^{26–28} eNOS can be activated by physiological and metabolic stimuli, such as shear stress and clinical therapeutic drugs, and result in NO production. The protective effects of NO on the cardiovascular system are well documented^{29–31}; NO functionally inhibits platelet aggregation, leukocyte adhesion, and smooth muscle cell proliferation and maintains vascular tone, thereby maintaining cardiovascular homeostasis and preventing cardiovascular diseases.^{32–34} However, at the initial stage of several cardiovascular diseases, oxidative stress uncouples eNOS-derived NO, thereby increasing ONOO⁻ production, which deregulates eNOS activity and leads to disease progression.^{35–38} Statins can effectively lower cholesterol levels and reduce

cardiovascular events in patients with various risk profiles.^{1–5} They are reported to have antioxidant effects in vascular cells and platelets by inhibiting NOX2.^{39,40} Nevertheless, two thirds of statin-treated patients still experience adverse coronary events that have unclear mechanisms.^{41,42}

Notably, our clinical outcomes study showed that statins failed to protect against cardiovascular events in CAD patients with high plasma ADMA level (>0.49 $\mu\text{mol/L}$). Our in vitro studies further supported this notion because treatment with ADMA (≥ 0.5 $\mu\text{mol/L}$) markedly inhibited the beneficial effect of simvastatin on eNOS activation and NO production by activating NOX-ROS signaling in ECs, whereas lower concentrations of ADMA (<0.5 $\mu\text{mol/L}$) failed to do so. These findings agree with Janatuinen et al. and Böger et al., who found that plasma ADMA level could modify the statin-improved, endothelium-dependent vasodilation in humans.^{43,44} Janatuinen et al. reported that pravastatin improved adenosine-induced myocardial flow in young hypercholesterolemic subjects with low plasma ADMA level, but did not increase adenosine-induced myocardial flow in subjects with high ADMA level. Similarly,

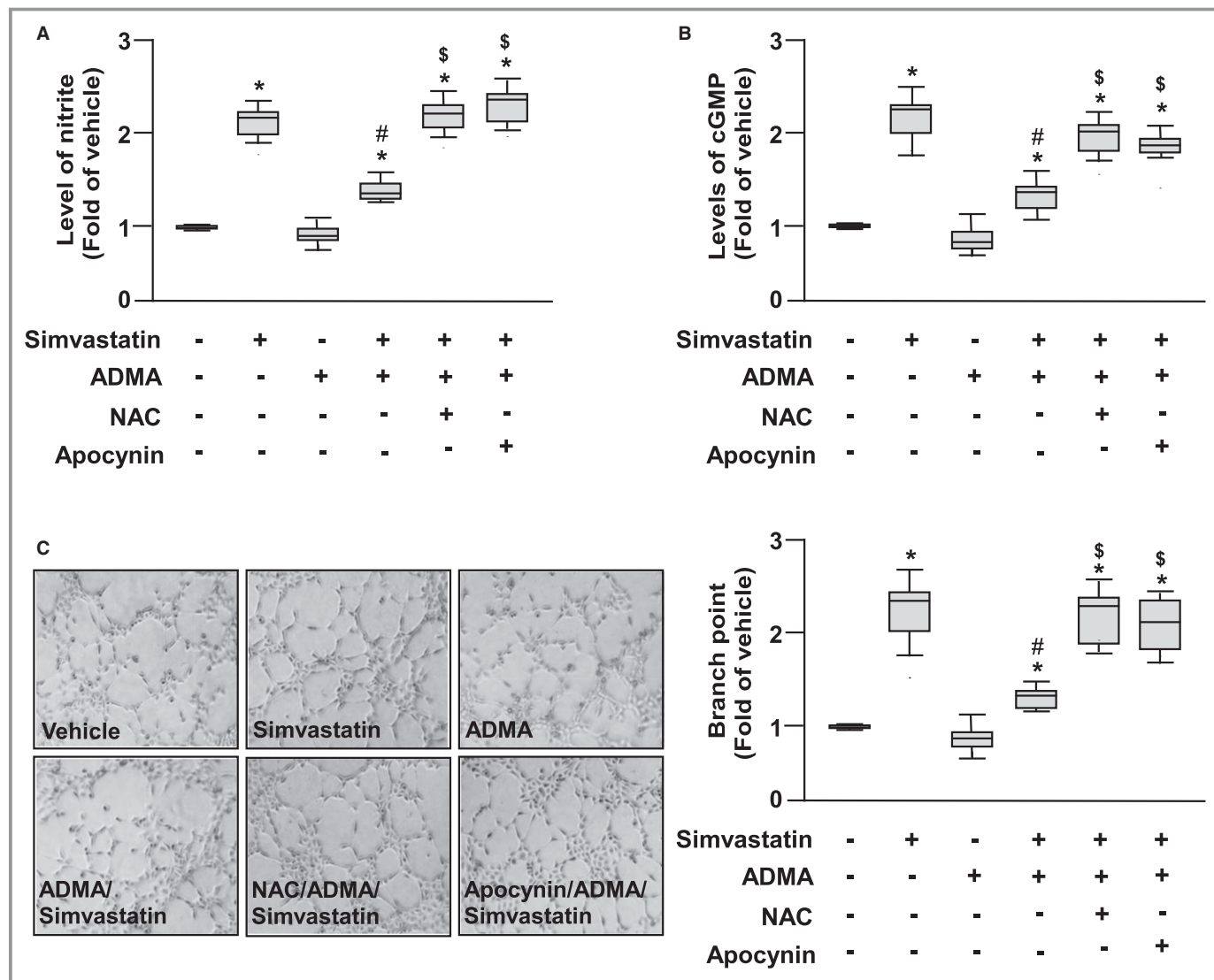


Figure 4. Inhibition of NOX-ROS signaling abrogates ADMA-impaired NO production and angiogenesis induced by simvastatin. HAECs were pretreated with or without NAC (10 mmol/L) or APO (50 μ mol/L) for 2 hours, then ADMA (0.5 μ mol/L) for 24 hours. A, Levels of nitrite in culture media and (B) intracellular cGMP measured by Griess assay or ELISA. C, HAECs were cultured in precoated ECL Cell Attachment Matrix (Millipore Bedford, MA) in the indicated treatments. Tube formation was visualized; bar graphs indicate fold increase in number of branch points in 5 randomly selected microscopy views. * P <0.05 vs vehicle; # P <0.05 vs simvastatin alone; \$ P <0.05 vs ADMA+simvastatin. ADMA indicates asymmetric dimethylarginine; APO, apocynin; HAECs, human aortic endothelial cells; NAC, N-acetylcysteine; NOX, NADPH oxidase; ROS, reactive oxygen species.

Böger et al. demonstrated that simvastatin did not enhance endothelial function in subjects with elevated ADMA. Furthermore, simvastatin and oral L-arginine combined improved endothelial function in these subjects. Interestingly, Speer et al. reported that the level of SDMA, the structural isomer of ADMA, is elevated in high-density lipoprotein (HDL) fraction of plasma from patients with chronic kidney dysfunction (CKD).⁴⁵ Notably, they also found that HDL from CKD patients or healthy HDL supplemented with SDMA not only loses the vasoprotective properties, but also changes toward a harmful lipoprotein that induces EC dysfunction, inflammation, and hypertension,

suggesting that SDMA may also play an important role in pathogenesis of cardiovascular diseases under CKD condition.⁴⁵ However, our results showed that there was no significant difference in plasma SDMA between statins users and statins nonusers in 3 groups (whole population group: 0.48 ± 0.30 vs 0.47 ± 0.24 ; $P=0.69$; plasma ADMA level ≥ 0.49 μ mol/L group: 0.48 ± 0.24 vs 0.49 ± 0.38 ; $P=0.79$; ADMA level <0.49 μ mol/L group: 0.47 ± 0.24 vs 0.47 ± 0.21 ; $P=0.91$). Cotreatment with SDMA did not affect simvastatin-induced NO production in ECs. The possible explanation for the discrepancy between our results and those of Speer et al. may

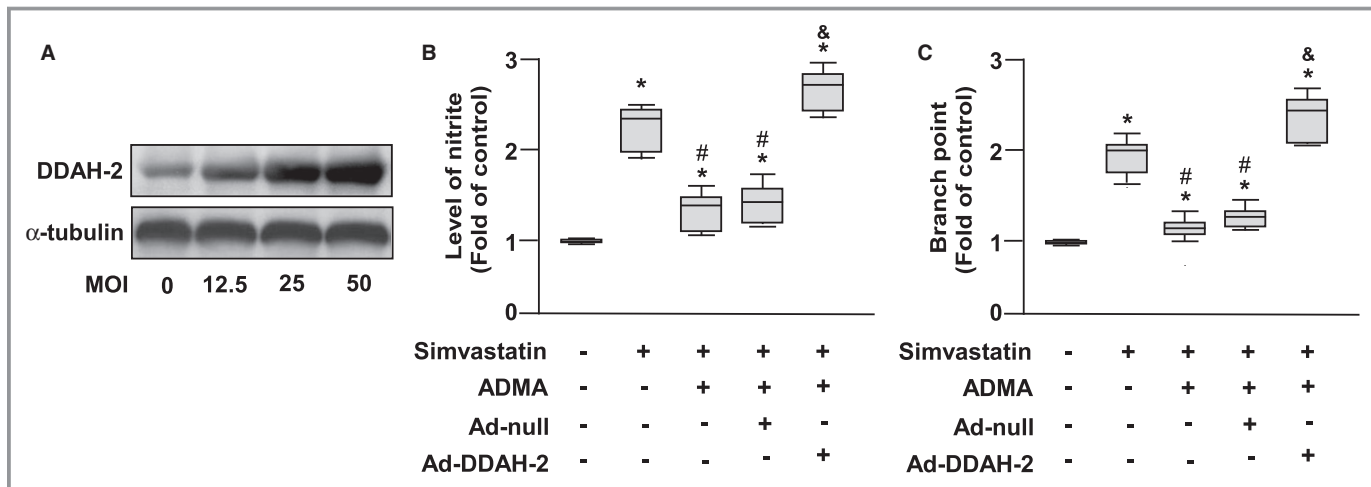


Figure 5. Overexpression of DDAH-2 abolishes ADMA-impaired NO production and angiogenesis induced by simvastatin. A, HAECs were infected with adenovirus (Ad) Ad-DDAH-2 (12.5–50 MOI) for 24 hours. Western blot analysis of the protein levels of DDAH-2 and α -tubulin. B, HAECs were infected with Ad-vector (Ad-null) or Ad-DDAH-2 (50 MOI) for 24 hours, then treated with ADMA (0.5 μ mol/L) for 30 minutes. Cells were then treated with simvastatin (10 μ mol/L) for 24 hours. Level of nitrite in culture media was measured by Griess assay. C, Treated HAECs were seeded in precoated ECL Cell Attachment Matrix (Millipore Bedford, MA). Tube formation was assessed; bar graphs indicate fold increase in number of branch points in 5 randomly selected microscopy views. * P <0.05 vs control group; # P <0.05 vs simvastatin alone; & P <0.05 vs ADMA+simvastatin. ADMA indicates asymmetric dimethylarginine; DDAH-2, dimethylarginine dimethylaminohydrolase 2; HEACs, human aortic endothelial cells; MOI, multiplicity of infection; NO, nitric oxide.

be attributed to the difference in pathological conditions or experimental protocols. Collectively, plasma ADMA level might have an impact on the beneficial effect of statins on long-term cardiovascular events. Monitoring plasma ADMA level might have clinical implications before initiation of statin treatment, and the clinical effects of statins for patients with increased ADMA level need to be further addressed.

ADMA is currently attracting considerable attention for its inhibition of eNOS activity and is considered a biomarker in

several cardiovascular and metabolic diseases, including hyperlipidemia, hypertension, and type 2 diabetes.^{13–17,46,47} Additionally, ADMA increases oxidative stress by uncoupling electron transport between NO synthase and L-arginine, which can lead to decreased production and availability of endothelium-derived NO.^{13–15} Furthermore, we have growing evidence for a contribution of ROS to ADMA-mediated pathological effects, so activation of NOX signaling might be required for ADMA involved in the pathogenesis of cardiovascular and

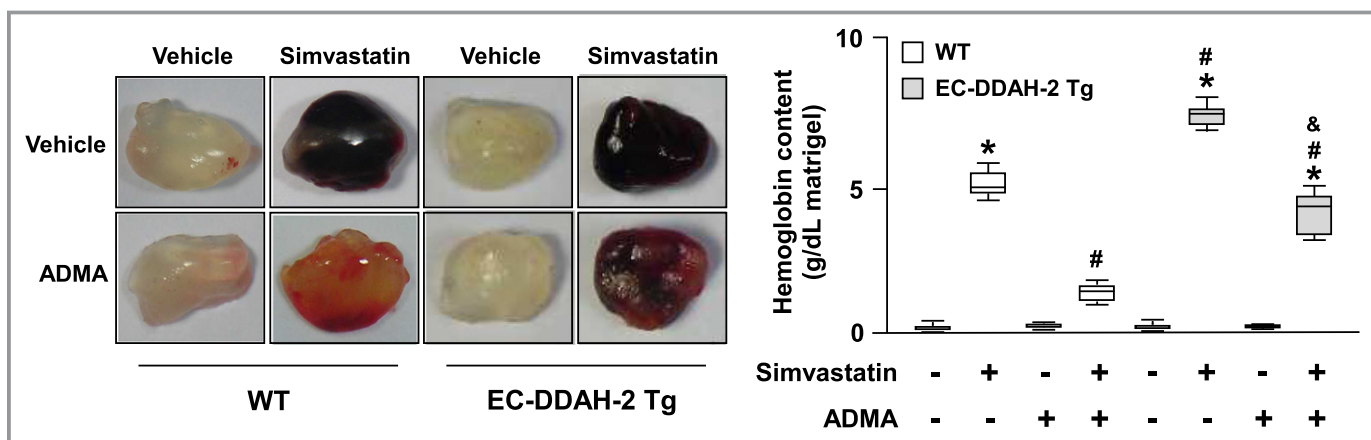


Figure 6. Overexpression of DDAH-2 in ECs reverses ADMA-impaired angiogenesis induced by simvastatin in vivo. Eight-week-old male wild-type (WT) or EC-DDAH-2 Tg mice were subcutaneously injected with Matrigel plugs with vehicle, simvastatin (10 μ mol/L) alone, or simvastatin (10 μ mol/L)+ADMA (0.5 μ mol/L). At 7 days postadministration, plugs were removed and photographed and hemoglobin content was analyzed. Data are mean \pm SD from 8 mice. * P <0.05 vs vehicle; # P <0.05 vs simvastatin; & P <0.05 vs WT with ADMA+simvastatin. ADMA indicates asymmetric dimethylarginine; DDAH-2, dimethylarginine dimethylaminohydrolase 2; ECs, endothelial cells; Tg, transgenic.

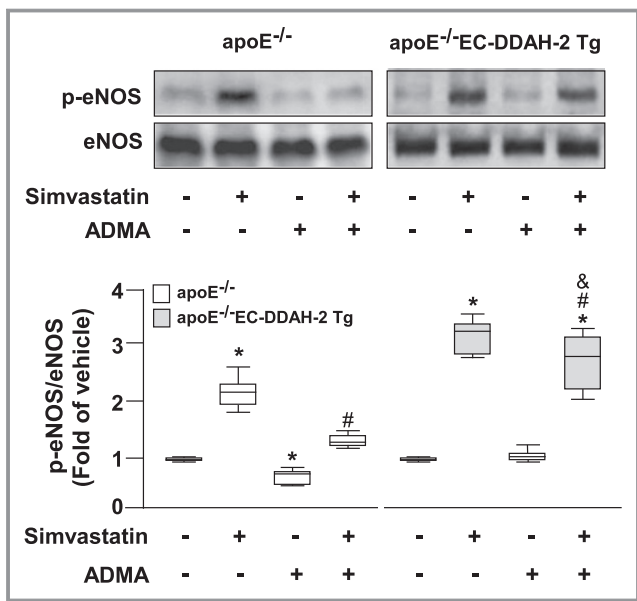


Figure 7. Overexpression of DDAH-2 in ECs prevents ADMA-mediated decrease in simvastatin-induced eNOS phosphorylation in aortas of apoE-deficient mice. Four-month-old male apoE^{-/-} or apoE^{-/-}EC-DDAH-2 Tg mice were treated daily with vehicle (DMSO), simvastatin (10 mg/kg) alone, ADMA (5 mg/kg), or ADMA+simvastatin. After 4 weeks, mice were killed and aortas were collected. Aortic lysates underwent western blot analysis to examine levels of phosphorylated eNOS (p-eNOS) (Ser1179) and total eNOS. Data are mean±SD from 8 mice. **P*<0.05 vs vehicle; #*P*<0.05 vs simvastatin; &*P*<0.05 vs WT with ADMA+simvastatin. ADMA indicates asymmetric dimethylarginine; DDAH-2, dimethylarginine dimethylaminohydrolase 2; ECs, endothelial cells; eNOS, endothelial nitric oxide synthase; Tg, transgenic.

metabolic disease.^{48–50} Recently, targeting vascular NOX-ROS signaling is considered a novel antioxidant strategy for inhibiting oxidative stress-induced pathological events.^{49,50} Statins are known to have an inhibitory effect on upstream signaling of NOX activation, which contributes to the clinical benefits in CAD patients.^{3,4,8} However, our results provide new evidence for the detrimental effect of ADMA on simvastatin-mediated NO production and tube formation, which was abolished by NOX inhibitor APO. These findings suggest that targeting NOX activation may be an applicable strategy in preventing the ADMA-mediated reduction in statin effects. However, we did not define the detailed molecular mechanism underlying the prevention by an antioxidant or NOX inhibitor of this detrimental effect. Nevertheless, whether antioxidation therapy can prevent the ADMA-mediated reduction in statin effects in clinical trials remains for further investigations. Given the negative impact of ADMA on development of many cardiometabolic diseases, whether ADMA also impairs the therapeutic efficacy of other eNOS-activated clinical drugs would be of interest.

As such, antioxidant therapy has been successful for the above cardiometabolic disorders by clinical trials or basical

science studies.^{51–55} Additionally, Keyamura et al. and Lee et al. reported that antioxidant coenzyme Q10 or probucol has an add-on effect on statin-conferred atheroprotection in CAD patients or cholesterol-fed experimental animals.^{56,57} Conversely, several large-scale, double, placebo-controlled trials have shown that antioxidative vitamin administration does not necessarily reduce CAD risk in patients even with coadministration with simvastatin.^{58–60} Much of this controversy probably results from shortages in experimental design of these studies. To appropriately evaluate the protective effects of antioxidation agents, including vitamins in CAD patients, the carefully designed studies regarding the intervention and dosage of antioxidant and applicable time interval in an appropriate selected patient population are warranted.

In addition to inhibiting ADMA-activated downstream NOX-ROS signaling, increasing the metabolic rate of ADMA may be a potent therapeutic strategy for preventing cardiovascular events.^{61,62} ADMA can be metabolized by DDAH-1 and DDAH-2, thereby increasing eNOS-derived NO bioavailability.^{19,20} Thus, DDAH deregulation, which causes ADMA accumulation, is also a risk factor for cardiovascular diseases.^{19,20} Hyperlipidemia deregulates DDAH activity, thereby leading to increased ADMA level, decreased NO production, and impaired EC function.⁶³ Overexpression of DDAH-1 can reduce ADMA levels and slow progression of atherosclerosis and hypertension.²¹ Because the DDAH-2 activity plays a predominant role in EC function,^{19,20} we used EC-specific overexpression of DDAH-2 to study the role of DDAH-2 in angiogenesis and aortic eNOS and found that adenovirus DDAH-2 overexpression in ECs blunted ADMA-mediated inhibition of simvastatin-elicited NO production and tube formation. Under ADMA challenge, EC-DDAH-2 Tg mice showed greater angiogenesis and aortic eNOS phosphorylation than did WT mice. These findings were consistent with previous reports that overexpression of DDAH-2 reverses endothelial dysfunction in ECs and experimental rodents.^{22–24} With relevance to vascular biology, therapeutic manipulation of DDAH-2 activity or expression may be a promising strategy for preventing EC dysfunction and eNOS-related cardiovascular diseases.

Our study contains several limitations. In our cohort study, the statins were used for clinical indications, namely, increased plasma LDL-C level. Because baseline LDL-C level remained significantly higher with statins, the beneficial effects of statins attributed to the LDL-C-lowering effect and their pleiotropic effects are difficult to conclude. A randomized, control trial might be warranted to determine the effect of statin use on plasma ADMA level. As well, we used only a gain-of-function strategy to investigate the protective role of DDAH-2 in ADMA-mediated unfavorable effects. A specific activator or inhibitor of DDAH-2 is not commercially available, so we could not study the significance of DDAH-2 in

translational or clinical medicine. However, our findings suggest that high plasma ADMA level may affect the effectiveness of statins for EC function and cardiovascular events.

In conclusion, our study provides new evidence that ADMA may be an independent risk factor of cardiometabolic diseases, and high plasma ADMA level may limit therapeutic efficacy of statins in patients with cardiovascular diseases. The molecular mechanism we reveal may provide advanced information for better understanding the pharmacological mechanism targeting the NOX-ROS-signaling pathway or DDAH-2 in ECs for treating ADMA-related cardiovascular or metabolic diseases.

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Disclosures

None.

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