

Nitric oxide (NO) derived from endothelial NO synthase (eNOS) is a critical signaling molecule in the vasculature, and has a range of anti-atherogenic effects¹. In eNOS/apolipoprotein E (ApoE) double-knockout mice, atherosclerosis is increased, suggesting a protective effect of eNOS-derived NO^{2,3}. However, certain vascular diseased states are associated with an increase rather than a decrease in the expression of eNOS^{4,5}. We have shown that endothelium-targeted over-expression of eNOS in ApoE-KO (ApoE-KO/eNOS-Tg) mice surprisingly resulted in decreased endothelial NO availability, increased vascular superoxide production, and accelerated atherosclerosis⁶.

These observations could be explained by a relative deficiency of the co-factor tetrahydrobiopterin (BH4) causing eNOS 'uncoupling'⁷, where the enzymatic reduction of molecular oxygen by eNOS is no longer coupled to L-arginine oxidation, resulting in production of superoxide rather than NO. In ApoE-KO/eNOS-Tg mice, dietary BH4 supplementation reduced superoxide production and increased NO availability, although it was unclear whether this was a general anti-oxidant effect of BH4 or a specific effect on eNOS coupling⁶. Indeed, dietary supplementation with the anti-oxidant vitamin C can also reduce vascular oxidative stress, increase BH4 levels, and was sufficient to improve the depressed endothelium-dependent relaxation in ApoE-KO mice fed a high fat diet⁸, although a specific effect on eNOS coupling was not investigated. These pre-clinical data suggest a potential role for vitamin C therapy in vascular disease, yet large scale clinical trials have failed to demonstrate an effect on major clinical endpoints in human atherosclerosis^{9,10}. It is possible that interventions targeted at specific redox mechanisms may be more effective than non-specific anti-oxidant strategies for treatment of vascular disease states.

Substantial evidence points to an important role for BH4 in regulating eNOS coupling in atherosclerosis. Indeed, even in the absence of vascular disease, eNOS over-expression in the endothelium without a concomitant increase in BH4 levels can result in eNOS uncoupling, but augmenting endothelial BH4 levels, by over-expression of the rate-limiting enzyme in BH4 biosynthesis, GTP-cyclohydrolase I (GCH), is able to restore eNOS/BH4 stoichiometry and eNOS coupling¹¹.

In this study, we sought to address the specific importance of uncoupled eNOS in atherosclerosis, and the potential mechanistic role for specific versus non-specific anti-oxidant strategies in restoring eNOS coupling. We used the ApoE-KO/eNOS-Tg mouse as a model of

eNOS uncoupling in atherosclerosis, and investigated the relationships between eNOS protein, BH4 availability and atherosclerotic plaque progression. Furthermore, we compared the strategy of directly augmenting endothelial BH4 levels by transgenic GCH over-expression, versus vitamin C supplementation.

Methods

Experimental design

The animals used in these experiments were offspring from breeding between ApoE-KO/eNOS-Tg mice (ApoE-KO mice over-expressing a bovine *eNOS* transgene in the endothelium) and ApoE-KO/GCH-Tg mice (ApoE-KO mice over-expressing a human *GCH* transgene in the endothelium), as previously reported in detail^{6,12}. The background strain was C57BL/6 mouse. ApoE-KO, ApoE-KO/eNOS-Tg and ApoE-KO/eNOS-Tg/GCH-Tg mice were generated and fed standard mouse chow from weaning. Experimental mice were euthanized at 16 weeks of age and organs used for histological and biochemical analysis. In parallel experiments, another group of ApoE-KO/eNOS-Tg mice were treated with 500 mg/kg body weight/day vitamin C dissolved in drinking water starting from 4 weeks of age until 16 weeks of age.

All animal experiments were conducted according to the Guidelines for Animal Experiments at Kobe University Graduate School of Medicine. All commercial drugs besides those mentioned specifically were purchased from Sigma Chemical Co. (MO).

Plasma lipid analyses, vitamin C levels and hemodynamic analyses

After overnight fasting, blood was collected by the cardiac puncture under anesthetic using pentobarbital sodium (80 mg/kg intraperitoneal injection). Plasma was obtained through centrifugation of the blood for 10 minutes at 5500 g at 4 °C and stored at -80 °C. Concentrations of plasma total cholesterol and triglyceride were determined by use of an automated chemistry analyzer. High density lipoprotein cholesterol levels were quantified by enzymatic reaction using a commercially-available kit (Wako, Osaka, Japan). Plasma concentrations of vitamin C were measured with reverse-phase high-performance liquid chromatography (HPLC).

Heart rate and systolic blood pressure were measured at 16 weeks of age using a tail cuff photoelectric device. All measurements were repeated six times for each mouse.

Measurement of vascular biopterin concentrations

Mice were anesthetized and the aorta sample was dissected from the ascending aorta to the iliac bifurcation. The dissected aorta was placed in a mixture of 0.5 M perchloric acid

containing 0.1 mM disodium EDTA, 0.1 mM Na₂S₂O₃ for protein separation and 0.1 mM ascorbic acid to prevent oxidation. Then the aorta was homogenized on ice in 200 µL homogenate buffer. After centrifugation (15000 g for 10 min) and filtration (0.45 µm pore size; Millex-HV Filter Unit, Millipore, Billerica, MA), we measured vascular BH₄ and dihydrobiopterin (BH₂) concentrations by HPLC developed by Tani et al¹³. Briefly, by post-column NaNO₂ oxidation with a reversed-phase ion-pair LC system, BH₄ and BH₂ were detected fluorometrically at wave lengths of 350 nm for excitation and 440 nm for emission (LC-10 series, Shimadzu, Kyoto, Japan). Protein concentrations of aortic homogenates were measured by the Bradford method and BH₄ or BH₂ concentration was corrected for protein concentration.

Analysis of eNOS protein

The levels of total eNOS protein in aorta homogenates were analyzed by western blotting, and in addition, to investigate the ratio of eNOS dimer to monomer, western blotting was performed by use of non-boiled lung homogenates and low-temperature SDS-page as previously described^{14, 15}.

Superoxide production in the aortic endothelium

First, we measured overall superoxide production from aortas. The aorta was cut into four pieces (5 mm length per each piece), which were incubated with the Cu-Zn superoxide dismutase inhibitor for 30 min at 37 °C. Then vascular superoxide production levels were measured by chemiluminescence with 10 µM lucigenin (bis-N-methylacridinium nitrate).

By this method, however, we cannot differentiate endothelium-derived superoxide from superoxide produced in other vascular cell components. We, therefore, measured *in situ* superoxide production from vessel wall by use of dihydroethidium (DHE; Molecular Probe, OR)¹⁶. Briefly, the unfixed tissues embedded in OCT were cut into 10 µm thick sections, and incubated with or without 5 mM N ω -nitro-L-arginine methyl ester (L-NAME) for 5 minutes at 37 °C, and then incubated with 2 µM DHE at 37°C for 10 minutes in a light-protected humidified chamber. The images were obtained with a laser scanning confocal microscope (Carl ZEISS, Germany). DHE fluorescence from high-power (x 200) images was quantified by automated image analysis using the Image J software (National Institutes of Health, MD). For quantification of endothelial cell ethidium fluorescence, fluorescence (intensity x area) was measured only on the luminal side of

the internal elastic lamina. For each vessel, mean fluorescence was calculated from 3 separate high-power fields taken in each sections of the vessel to produce n=1.

Atherosclerotic lesion assessment at the aortic sinus

At 16 weeks of age, mice were anesthetized and the aorta was perfused as above. The aorta was dissected from the middle of the left ventricle to the aortic arch, and fixed with 4% paraformaldehyde for 16 hours. The samples were cut in the ascending aorta, and the proximal samples containing the aortic sinus were embedded in OCT compounds (Tissue-Tek, CA). Five consecutive sections (10 μ m thickness), spanning 550 μ m of the aortic sinus, were collected from each mouse and stained with Sudan III. For quantitative analysis of atherosclerosis, the total lesion area of five separate sections from each mouse was obtained with the use of the Image J⁶.

Statistical Analysis

Data were expressed as means \pm SEM. One-way ANOVA or two-way ANOVA were used to compare the differences among the four experimental groups and then appropriate post hoc analysis with Bonferroni correction for each result was performed. Values of $p < 0.05$ were considered statistically significant.

Results

Plasma analyses and hemodynamics

Plasma total cholesterol, triglyceride and high density lipoprotein cholesterol levels were not different among the 4 groups (**Table 1**). Body weight was similar among the 4 groups in both gender mice. As previously reported ¹⁴, eNOS over-expression in the endothelium significantly decreased systolic blood pressure, but neither concomitant GCH over-expression nor vitamin C treatment had any further effect on blood pressure. There were no differences in heart rate among the 4 groups (data not shown). In either ApoE-KO mice or ApoE-KO/eNOS-Tg mice, plasma vitamin C concentrations were not different from those in control C57BL/6 mice (55.2±5.4 µmol/l, n=5). Vitamin C treatment increased its plasma concentrations approximately 1.5-fold in ApoE-KO/eNOS-Tg mice (**Table 1**).

Vascular biopterin concentrations

We next measured aortic BH4 concentrations and the oxidative state of biopterins. In ApoE-KO mice, aortic BH4 concentrations were approximately 4 times higher compared with BH2 concentrations. Aortic BH4 concentrations in ApoE-KO/eNOS-Tg mice were similar compared with ApoE-KO mice (**Figure 1A**). However, over-expression of GCH increased vascular BH4 concentrations by more than 2-fold in ApoE-KO/eNOS-Tg mice. In contrast, vitamin C treatment did not induce significant changes in vascular BH4 concentrations. Over-expression of eNOS reduced the BH4/BH2 ratio in all groups, reflecting increased oxidative loss of BH4 (**Figure 1B**).

eNOS dimerization in lung homogenates

As expected ¹³, transgenic over-expression of eNOS in the endothelium significantly increased eNOS protein levels in the aorta. However, neither GCH over-expression in the endothelium nor vitamin C treatment affected eNOS protein levels in the aorta of ApoE-KO/eNOS-Tg mice (data not shown).

To investigate the effect of GCH over-expression versus vitamin C supplementation on the stability of eNOS dimer, we used low-temperature SDS-PAGE and western blotting to determine the ratio of eNOS dimer to monomer. We used lung homogenates for this assay, since much higher level of eNOS protein could be obtained in the lung compared with in aortas ¹⁴.

In ApoE-KO/eNOS-Tg mice, eNOS dimer to monomer ratio was significantly reduced compared with ApoE-KO mice in lung homogenates (**Figure 2A and 2B**) reflecting an imbalance between eNOS protein and BH4 concentrations. GCH over-expression partially restored the eNOS dimer to monomer ratio in ApoE-KO/eNOS-Tg mice, but vitamin C treatment had no effect.

Superoxide production from the endothelium

In the lucigenin method, eNOS over-expression augmented chemiluminescence signals from aortas compared with ApoE-KO mice. Both concomitant over-expression of GCH and vitamin C treatment reduced the augmented chemiluminescence signals in ApoE-KO/eNOS-Tg mice (**Figure 3**).

Then, superoxide production in the endothelium, and in particular the contribution of uncoupled eNOS to endothelial superoxide production, was evaluated using DHE oxidative fluorescent microtopography. Ethidium fluorescence was detected throughout all layers of the vessel wall (**Figure 4A**). We focused on the vascular superoxide production in endothelial cells by measuring ethidium fluorescence specifically on the luminal side of the internal elastic lamina¹⁶. Endothelial ethidium fluorescence was increased in ApoE-KO/eNOS-Tg mice compared with ApoE-KO mice, and both GCH over-expression and vitamin C treatment decreased its fluorescence (**Figure 4A and 4B**). In particular, vitamin C treatment suppressed fluorescence signals in all layers of the vessel wall. We now probed the contribution of eNOS to net superoxide levels in the endothelium using the NOS inhibitor L-NAME. Although L-NAME had no effect on ethidium fluorescence in ApoE-KO mice, it decreased DHE fluorescence in the ApoE-KO/eNOS-Tg mice, indicating the presence of NOS-mediated superoxide production in the endothelium. In contrast, endothelial ethidium fluorescence was increased by L-NAME in ApoE-KO/eNOS-Tg/GCH-Tg mice, indicating that NOS was a net producer of NO rather than superoxide in this group. Importantly, although basal endothelial ethidium fluorescence was lower in vitamin C-treated ApoE-KO/eNOS-Tg mice than in control ApoE-KO mice or ApoE-KO/eNOS-Tg mice, there was no change following L-NAME incubation, suggesting that the reduction in superoxide levels was due to an anti-oxidative effect of vitamin C rather than a direct effect on eNOS coupling.

Atherosclerotic lesion formation

To evaluate the net effect of these different vascular biochemical changes in the four groups on atherosclerosis progression, we measured atherosclerotic lesion formation at the aortic sinus. In accordance with our previous study ⁶, performed in mice with more severe hypercholesterolemia than that in the present study, histological examination staining revealed that eNOS over-expression markedly accelerated the atherosclerotic lesion formation in both genders (**Figure 5**). Augmented endothelial BH4 levels by GCH over-expression significantly reduced atherosclerotic lesion formation compared with ApoE-KO/eNOS-Tg mice, and indeed restored plaque progression to control ApoE-KO mice levels. In contrast, vitamin C treatment had no effects on accelerated atherosclerotic lesion formation in ApoE-KO/eNOS-Tg mice.

These results indicate that over-expression of eNOS in ApoE-KO mice leads to eNOS uncoupling due to a stoichiometric imbalance between levels of eNOS and BH4. The reduction in vascular NO bioavailability and increased superoxide production are associated with accelerated atherosclerosis. A general reduction in vascular superoxide levels alone, by vitamin C treatment, is insufficient to retard atherosclerosis. In contrast, restoration of eNOS/BH4 stoichiometry and eNOS coupling, by augmented endothelial BH4 biosynthesis, is able to restore disease progression to control ApoE-KO mice levels.

Discussion

In this paper we investigated the mechanisms by which BH4 and vitamin C regulate production of NO or superoxide from eNOS, and how this relates to atherosclerosis progression. To focus on and accentuate the effect of uncoupled eNOS in atherosclerosis progression, we chose to evaluate an ApoE-KO mouse model with eNOS over-expression in the vascular endothelium – previously shown to have accelerated atherosclerosis and eNOS uncoupling⁶. By crossing this mouse with an endothelium-targeted GCH transgenic mouse¹², we compared the effect of specific augmentation of endothelial BH4 synthesis with vitamin C treatment, and report the following novel findings: First, GCH over-expression, but not vitamin C treatment, was able to increase aortic BH4 levels and increase eNOS dimer stability. Second, endothelial superoxide production was higher in eNOS over-expressing ApoE-KO mice compared with controls, and this was at least in part due to superoxide derived from uncoupled eNOS. Compared with vitamin C treatment, GCH over-expression led to a marked reduction in eNOS-derived superoxide production. Third, only a specific increase in endothelial BH4 synthesis by GCH over-expression was able to inhibit the accelerated atherosclerosis progression seen in eNOS over-expressing mice; vitamin C treatment had no effect on accelerated atherosclerosis in ApoE-KO/eNOS-Tg mice, despite a general reduction in vascular oxidative stress.

We previously demonstrated that eNOS over-expression in the endothelium of high fat-fed ApoE-KO mice caused greater atherosclerotic lesion formation, in association with increased endothelial superoxide production mediated by uncoupled eNOS⁶. In the present study, we now show that the acceleration of atherogenesis due to uncoupled eNOS also occurs in ApoE-KO mice with less severe hypercholesterolemia. These and other reports suggest that the relationship between eNOS and BH4 availability is a critical determinant of eNOS enzymatic function in both health and disease states.

Vitamin C may be important in maintaining BH4 levels in the setting of vascular oxidative stress. Vitamin C supplementation improves endothelial dysfunction in smokers, in subjects with hypercholesterolemia¹⁷, and in patients with diabetes mellitus¹⁸, or coronary artery disease¹⁹. In addition, chronic dietary supplementation with vitamin C increased the BH4/BH2 ratio and NO bioavailability in the aortas of ApoE-KO mice⁸; and there is a dose-dependent effect of vitamin C on BH4 levels and NO synthesis in cultured endothelial cells²⁰. Although the mechanism for this

effect is not fully resolved, recent data indicate that vitamin C may act by reducing the BH3 radical to regenerate BH4²¹.

The reported effects of long-term vitamin C treatment on atherosclerotic lesion formation in mice have been variable, likely due to differences in the mouse models and experimental conditions used. d'Uscio et al observed that vitamin C improved endothelial function and suppressed atherosclerotic lesion formation in ApoE-KO mice fed a high-fat Western-type diet⁸, whereas Nakata et al showed that vitamin C did not affect atherosclerotic plaque area but reduced plaque collagen content in ApoE-KO mice that were further genetically modified to be unable to synthesize vitamin C²². We now report findings in the ApoE-KO/eNOS-Tg mice fed a standard (non-high fat) chow. We used the ApoE-KO/eNOS-Tg mice model in order to specifically evaluate aspects of eNOS uncoupling, since we know from previous studies that eNOS over-expression leads to eNOS uncoupling in both normal animals and in the ApoE-KO mice^{6,11}. Furthermore, we used 500 mg/kg body weight/day vitamin C supplementation, a dose based on previous studies²², whereas d'Uscio et al used 1%/kg chow/day, implying that the administered dose of vitamin C in that protocol was approximately 3-5 times higher. In the study of d'Uscio et al, vitamin C treatment resulted in the reduction of vascular BH2 concentrations without changing BH4 concentrations, suggesting that vitamin C inhibited BH4 oxidation. It is possible that, in the setting of marked hypercholesterolemia, their model was associated with more oxidation of vascular BH4 than we observed in our study, and the higher dose of vitamin C inhibited BH4 oxidation and increased BH4/BH2 ratio in vessels.

It remains uncertain whether the ratio of BH4 to the oxidized biopterin BH2 is as important as the absolute BH4 concentrations in determining eNOS enzymatic activity *in vivo*. Biochemical studies indicate that BH2, which has no eNOS co-factor activity, can compete with BH4 for eNOS binding²³, and experiments in the DOCA-salt model of mouse hypertension suggest that the ratio of BH4/BH2 may be a determinant of NOS uncoupling²⁴. In contrast, in the streptozotocin mouse model of diabetes, the absolute concentration of endothelial BH4 appeared to be more important than the BH4/BH2 ratio in NOS regulation¹⁶. In the present study, the BH4/BH2 ratio did not change either in mice with GCH over-expression or in those treated by vitamin C, suggesting that in our model absolute BH4 levels rather than the BH4/BH2 ratio determine eNOS coupling.

As the limitation of our study, we used DHE staining to evaluate superoxide production

from the endothelium. Ethidium, the oxidation product of DHE, intercalates with DNA. The ethidium signals may, therefore, be influenced by cellular chromatin density, and the HPLC-based technique is recommended to omit such a problem in the use of DHE. In the present study, however, it was important to differentiate the signals only from the endothelium, which is the site of eNOS uncoupling, from those from other vascular cells. We also measured superoxide from whole aortic tissue by the lucigenin method and confirmed the augmented superoxide production in eNOS over-expressed mice, which was decreased by BH4 treatment. In addition, as we mentioned already, we cannot rule out the possibility that, in the presence of high oxidative stress, higher dose of vitamin C might reduce atherosclerotic lesion formation by inhibiting BH4 oxidation.

In conclusion, our findings demonstrate that increased synthesis of BH4 by GCH over-expression in the endothelium was able to specifically restore eNOS uncoupling, and resulted in the inhibition of atherosclerotic lesion formation. In contrast, a general reduction in vascular superoxide levels by oral vitamin C treatment had no effect on eNOS coupling and did not inhibit atherosclerosis. These data support the concept that strategies to increase eNOS expression without a concomitant augmentation of endothelial BH4 concentrations may be detrimental in the setting of atherosclerotic disease²⁵, and may offer further mechanistic data to explain the failure of non-specific anti-oxidant therapies to reduce vascular disease progression in large clinical trials.

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Figure Legends

Figure 1: Vascular biopterin concentrations.

A. Vascular BH4 concentrations in aorta homogenates were measured by HPLC. GCH over-expression significantly increased vascular BH4 levels compared with other groups. **B.** Vascular BH2 levels were measured and the ratio of BH4/BH2 was evaluated. (* : $p < 0.05$ versus ApoE-KO/eNOS-Tg/GCH-Tg mice, †: $p < 0.01$ versus ApoE-KO mice, $n = 8$ per each group.).

Figure 2: eNOS dimer formation in lung homogenates

A. Representative immunoblots for total eNOS protein (upper panel) and those of low-temperature SDS-PAGE immunoblotting for eNOS monomer and dimer formation (lower panel) in lung homogenates. **B.** Bands were analyzed and quantified by densitometry and the eNOS dimer/monomer ratio was evaluated. (* : $p < 0.01$ versus ApoE-KO mice, † : $p < 0.05$ versus ApoE-KO/eNOS-Tg mice, $n = 6$ per each group.)

Figure 3: Superoxide production from aorta.

Aortic rings were incubated with the Cu-Zn superoxide dismutase inhibitor and vascular superoxide levels were measured by chemiluminescence with 10 μ M lucigenin as described in Methods. The counts by a luminometer were corrected by vessel dry weights. (* : $p < 0.05$ versus ApoE-KO/eNOS-Tg mice, $n = 6$ per each group)

Figure 4: Dihydroethidium staining of aortic endothelial cells.

A. Representative sections (x 200) with dihydroethidium staining from each group are shown (a : ApoE-KO mice, b : ApoE-KO/eNOS-Tg mice, c : ApoE-KO/eNOS-Tg/GCH-Tg mice, d : Vitamin C treated ApoE-KO/eNOS-Tg mice). White bar represents 20 μ m and elastic laminae exhibit green autofluorescence. White arrows indicate specific endothelial cell ethidium fluorescence. **B.** Specific endothelial cell ethidium fluorescence was measured on the luminal side of the internal elastic lamina only and expressed in arbitrary units. White bars indicate values in the absence of L-NAME and black bars show those in the presence of L-NAME incubation. (* : $p < 0.05$ versus ApoE-KO mice, † : $p < 0.001$ versus ApoE-KO/eNOS-Tg mice, ‡: $p < 0.01$ versus, no pre-treated ApoE-KO/eNOS-Tg/GCH-Tg mice, § : $p < 0.05$ versus ApoE-KO mice, $n = 6-8$ per each group.)

Figure 5: Atherosclerotic lesion formation at the aortic sinus.

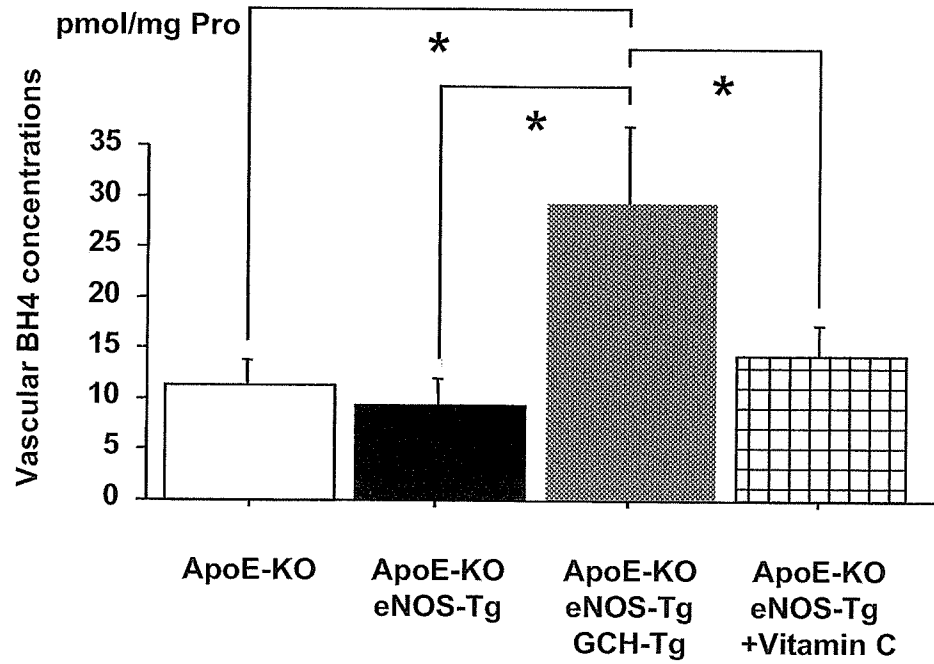
A. Representative figures (x 40) of Sudan III stained atherosclerotic lesion at the aortic sinus (a : ApoE-KO mice, b : ApoE-KO/eNOS-Tg mice, c : ApoE-KO/eNOS-Tg/GCH-Tg mice, d : Vitamin C treated ApoE-KO/eNOS-Tg mice). A black bar indicates 500 μ m. **B.** Each plot represents the total lesion area in each mouse and the mean per group is indicated by a horizontal line. (* : $p < 0.0001$ versus ApoE-KO mice, † : $p < 0.005$ versus ApoE-KO/eNOS-Tg mice, ‡ : $p < 0.0005$ versus ApoE-KO/eNOS-Tg mice, § : $p < 0.00001$ versus ApoE-KO/eNOS-Tg/GCH-Tg mice, ||: $p < 0.001$ versus ApoE-KO mice, n=10 per group for each sex.)

Table 1 Body weight, Plasma lipid contents, Vitamin C levels and Blood pressure

	ApoE-KO	ApoE-KO eNOS-Tg	ApoE-KO eNOS-Tg GCH-Tg	ApoE-KO eNOS-Tg +Vitamin C
Female body weight (g)	21.8±0.5	22.1±1.3	22.5±0.4	21.9±0.9
Male body weight (g)	28.7±0.6	27.5±0.7	29.2±0.7	27.1±0.7
Total cholesterol (mmol/L)	12.4±0.7	12.3±0.7	12.2±0.9	12.7±1.4
Triglyceride (mmol/L)	0.45±0.04	0.44±0.08	0.46±0.07	0.47±0.02
HDL-cholesterol (mmol/L)	0.26±0.03	0.25±0.02	0.28±0.03	0.30±0.04
Vitamin C (µmol/L)	56.3±4.0	54.5±8.0	52.8±6.8	80.7±6.3 *
Systolic BP (mmHg)	108.8±1.3	102.0±1.4 †	103.5±1.6 ‡	103.1±1.5 ‡

Data were shown as means±SEM (* : p<0.05 versus ApoE-KO mice, † : p<0.01 versus ApoE-KO mice, ‡ : p<0.05 versus ApoE-KO mice, n=10 per each group).

1A



1B

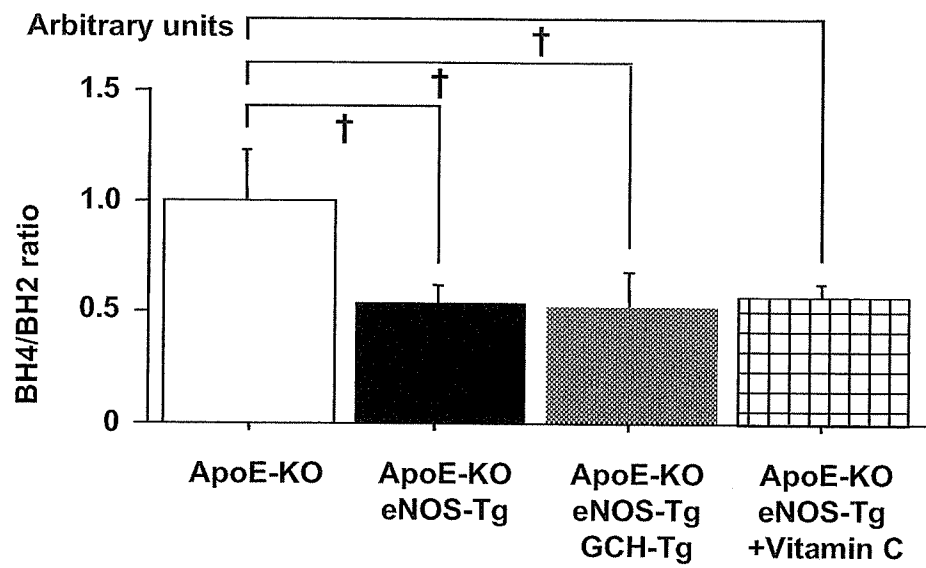
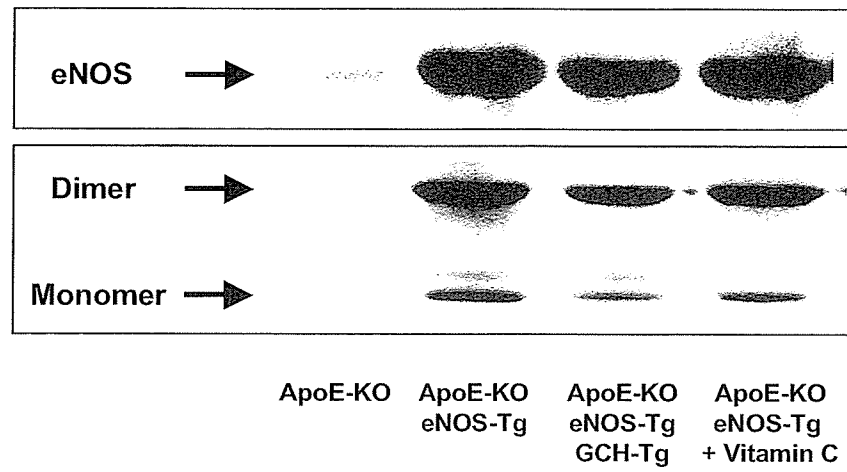


Figure 1

2A



2B

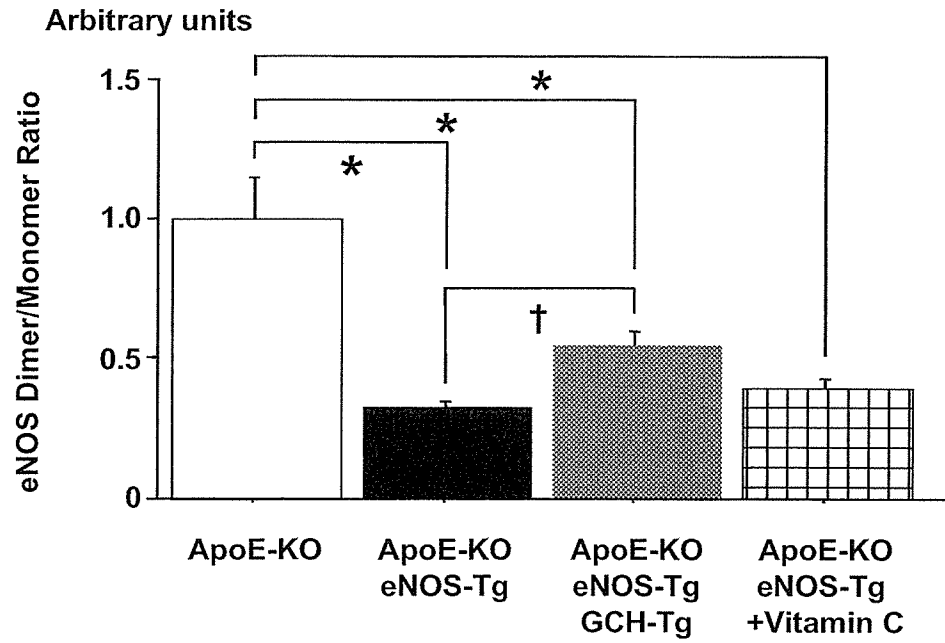


Figure 2

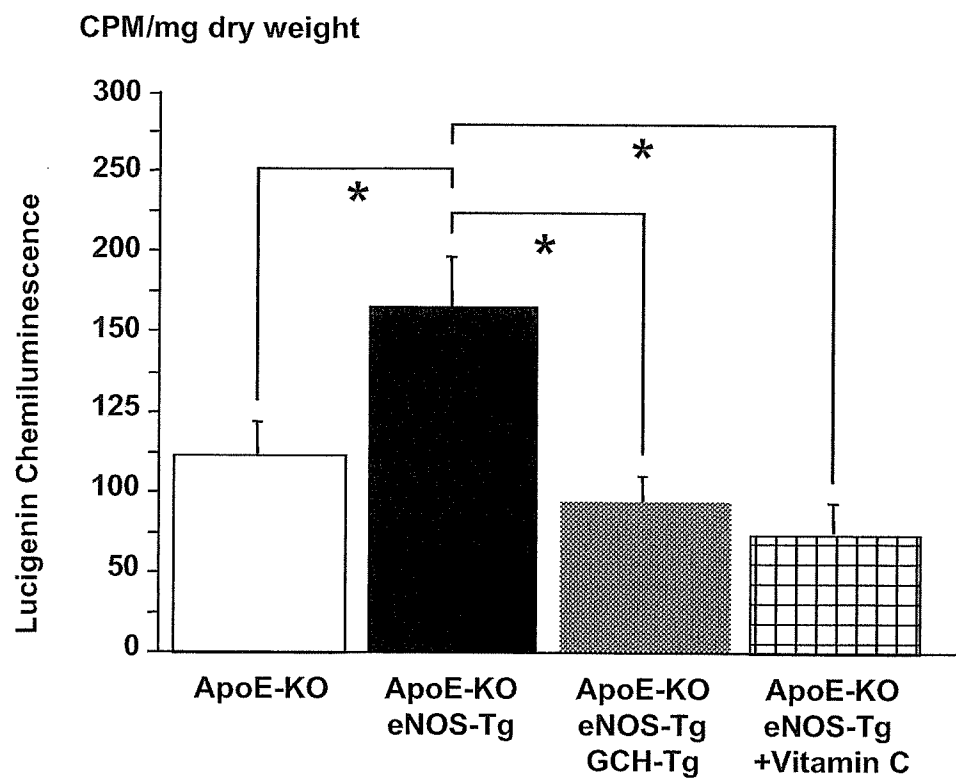
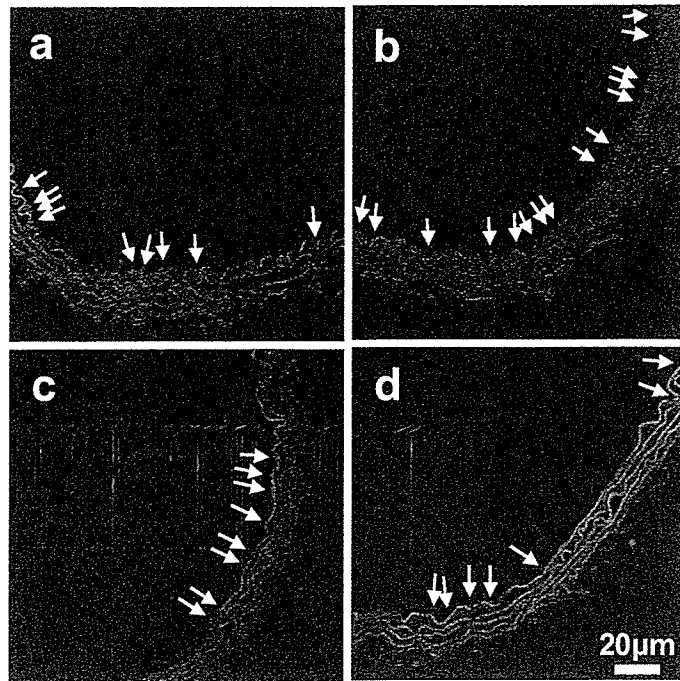


Figure 3

4A



4B

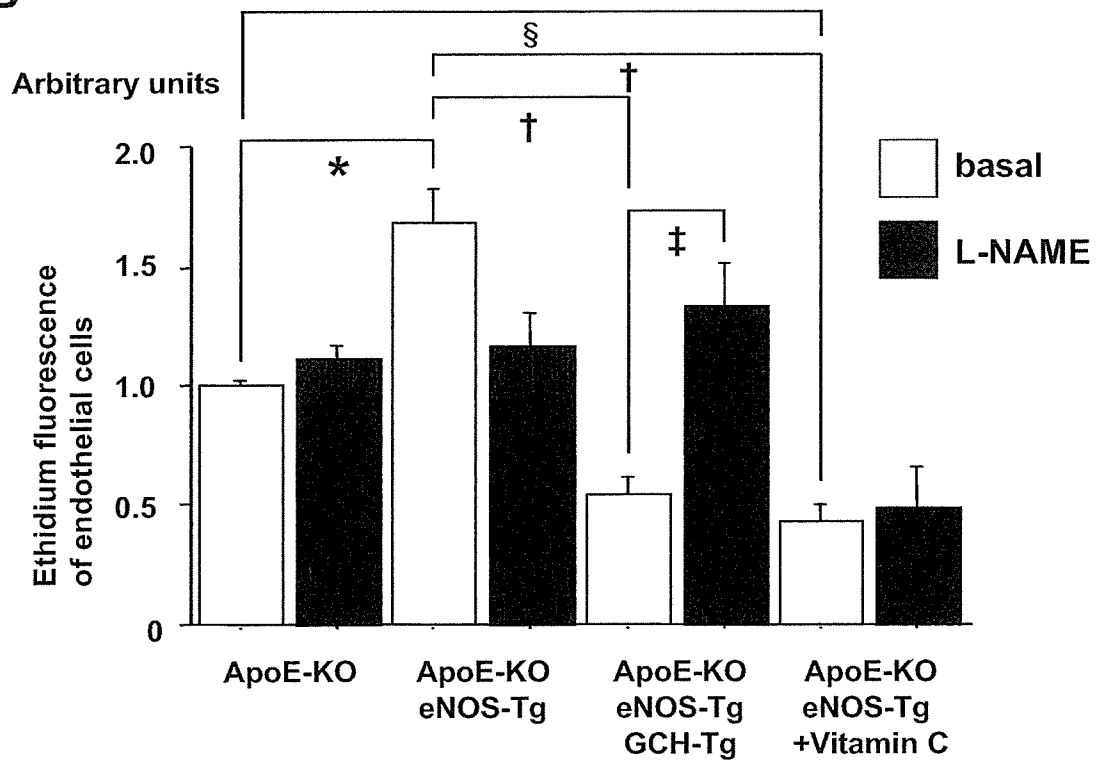


Figure 4