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].

ATVB/2007/142182 Downloaded from http://submit-atvb.ahajournals.org on March 4, 2007

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 lamina [16]. 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

ATVB/2007/142182 Downloaded from http://submit-atvb.ahajournals.org on March 4, 2007

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.

ATVB/2007/142182 Downloaded from http://submit-atvb.ahajournals.org on March 4, 2007

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 uncoupling [6]. By crossing this mouse with an endothelium-targeted GCH transgenic mouse [12], 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 eNOS [6]. 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 hypercholesterolemia [17], and in patients with diabetes mellitus [18], or coronary artery disease [19]. In addition, chronic dietary supplementation with vitamin C increased the BH4/BH2 ratio and NO bioavailability in the aortas of ApoE-KO mice [8]; and there is a dose-dependent effect of vitamin C on BH4 levels and NO synthesis in cultured endothelial cells [20]. Although the mechanism for this

ATVB/2007/142182 Downloaded from http://submit-atvb.ahajournals.org on March 4, 2007

effect is not fully resolved, recent data indicate that vitamin C may act by reducing the BH3 radical to regenerate BH4 [21].

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 diet [8], 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 C [22]. 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 studies [22], 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 binding [23], and experiments in the DOCA-salt model of mouse hypertension suggest that the ratio of BH4/BH2 may be a determinant of NOS uncoupling [24]. 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 regulation [16]. 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

ATVB/2007/142182 Downloaded from http://submit-atvb.ahajournals.org on March 4, 2007

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 disease [25], 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.

ATVB/2007/142182 Downloaded from http://submit-atvb.ahajournals.org on March 4, 2007

References

1. Lloyd-Jones DM, Bloch KD. The vascular biology of nitric oxide and its role in atherogenesis. *Annual Review of Medicine*. 1996;47:365-375.
2. Knowles JW, Reddick RL, Jennette JC, Shesely EG, Smithies O, Maeda N. Enhanced atherosclerosis and kidney dysfunction in eNOS(-/-)Apoe(-/-) mice are ameliorated by enalapril treatment. *J Clin Invest*. 2000;105:451-458.
3. Kuhlencordt PJ, Gyurko R, Han F, Scherrer-Crosbie M, Aretz TH, Hajjar R, Picard MH, Huang PL. Accelerated atherosclerosis, aortic aneurysm formation, and ischemic heart disease in apolipoprotein E/endothelial nitric oxide synthase double-knockout mice. *Circulation*. 2001;104:448-454.
4. Kanazawa K, Kawashima S, Mikami S, Miwa Y, Hirata K, Suematsu M, Hayashi Y, Itoh H, Yokoyama M. Endothelial constitutve type nitric oxide synthase protein and mRNA increased in rabbit atherosclerotic aorta despite impaired endothelium-dependent vascular relaxation. *Am J Pathol* 1996;148:1949-1956.
5. Bouloumie A, Bauersachs J, Linz W, Scholkens BA, Wiemer G, Fleming I, Busse R. Endothelial dysfunction coincides with an enhanced nitric oxide synthase expression and superoxide anion production. *Hypertension*. 1997;30:934-941.
6. Ozaki M, Kawashima S, Yamashita T, Hirase T, Namiki M, Inoue N, Hirata Ki, Yasui H, Sakurai H, Yoshida Y, Masada M, Yokoyama M. Overexpression of endothelial nitric oxide synthase accelerates atherosclerotic lesion formation in apoE-deficient mice. *J Clin Invest*. 2002;110:331-340.
7. Pou S, Pou WS, Bredt DS, Snyder SH, Rosen GM. Generation of superoxide by purified brain nitric oxide synthase. *J Biol Chem* 1992; 267:24173-24176
8. d'Uscio LV, Milstien S, Richardson D, Smith L, Katusic ZS. Long-term vitamin C treatment increases vascular tetrahydrobiopterin levels and nitric oxide synthase activity. *Circ Res*. 2003;92:88-95.
9. Duarte TL, Lunec J. Review: When is an antioxidant not an antioxidant? A review of novel actions and reactions of vitamin C. *Free Radic Res*. 2005;39:671-686.
10. Waters DD, Alderman EL, Hsia J, Howard BV, Cobb FR, Rogers WJ, Ouyang P, Thompson P, Tardif JC, Higginson L, Bittner V, Steffes M, Gordon DJ, Proschan M, Younes N, Verter JI. Effects of hormone replacement therapy and antioxidant vitamin supplements on coronary atherosclerosis in postmenopausal women :a randomized controlled trial. *JAMA* 2002:288:2432-2440
11. Bendall JK, Alp NJ, Warrick N, Cai S, Adlam D, Rockett K,Yokoyama M, Kawashima S, Channon KM. Stoichiometric relationships between endothelial tetrahydrobiopterin, eNOS activity and eNOS coupling in vivo: Insights from transgenic mice with endothelial-targeted GTPCH and eNOS over-expression. *Circ Res*. 2005;97:864-871.
12. Alp NJ, McAteer MA, Khoo J, Choudhury RP, Channon KM. Increased endothelial tetrahydrobiopterin synthesis by targeted transgenic GTP-cyclohydrolase I overexpression reduces endothelial dysfunction and atherosclerosis in ApoE-knockout

ATVB/2007/142182 Downloaded from http://submit-atvb.ahajournals.org on March 4, 2007

mice. *Arterioscler Thromb Vasc Biol.* 2004;24:445-450.

13. Tani Y, Ohno T. Analysis of 6R- and 6S-tetrahydrobiopterin and other pterins by reversed-phase ion-pair liquid-chromatography with fluorimetric detection by post-column sodium nitrite oxidation. *J Chromatogr.* 1993;617:249-255.
14. Ohashi Y, Kawashima S, Hirata K, Yamashita T, Ishida T, Inoue N, Sakoda T, Kurihara H, Yazaki Y, Yokoyama M. Hypotension and reduced nitric oxide- elicited vasorelaxation in transgenic mice overexpressing endothelial nitric oxide synthase. *J Clin Invest.* 1998;102:2061-2071.
15. Zou MH, Shi C, Cohen RA. Oxidation of the zinc-thiolate complex and uncoupling of endothelial nitric oxide synthase by peroxynitrite. *J Clin Invest.* 2002;109:817-826.
16. Alp NJ, Mussa S, Khoo J, Cai S, Guzik T, Jefferson A, Goh N, Rockett KA, Channon KM. Tetrahydrobiopterin-dependent preservation of nitric oxide-mediated endothelial function in diabetes by targeted transgenic GTP-cyclohydrolase I overexpression. *J Clin Invest.* 2003;112:725-735.
17. Ting HH, Timimi FK, Haley EA, Roddy MA, Ganz P, Creager MA. Vitamin C improves endothelium-dependent vasodilation in forearm resistance vessels of humans with hypercholesterolemia. *Circulation.* 1997;95:2617-2622.
18. Ting HH, Timimi FK, Boles KS, Creager SJ, Ganz P, Creager MA. Vitamin C improves endothelium-dependent vasodilation in patients with non-insulin-dependent diabetes mellitus. *J Clin Invest.* 1996;97:22-28.
19. Gokce N, Keaney JF, Jr., Frei B, Holbrook M, Olesiak M, Zachariah BJ, Leeuwenburgh C, Heinecke JW, Vita JA. Long-term ascorbic acid administration reverses endothelial vasomotor dysfunction in patients with coronary artery disease. *Circulation.* 1999;99:3234-3240.
20. Huang A, Vita JA, Venema RC, Keaney JF Jr. Ascorbic acid enhances endothelial nitric oxide synthase activity by increasing intracellular tetrahydrobiopterin. *J Biol Chem.* 2000; 275:17399-17406.
21. Patel KB, Stratford MR, Wardman P, Everett SA. Oxidation of tetrahydrobiopterin by biological radicals and scavenging of the trihydrobiopterin radical by ascorbate. *Free Radic Biol Med.* 2002;32:203-211.
22. Nakata Y, Maeda N. Vulnerable atherosclerotic plaque morphology in apolipoprotein E-deficient mice unable to make ascorbic Acid. *Circulation.* 2002;105:1485-1490.
23. Vasquez-Vivar J, Martasek P, Whitsett J, Joseph J, Kalyanaraman B. The ratio between tetrahydrobiopterin and oxidized tetrahydrobiopterin analogues controls superoxide release from endothelial nitric oxide synthase: an EPR spin trapping study. *Biochem J.* 2002;362:733-739.

24 Landmesser U, Dikalov S, Price SR, McCann L, Fukai T, Holland SM, Mitch WE, Harrison DG. Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. *J Clin Invest.* 2003;111:1201-1209.

25 Kawashima S, Yokoyama M. Dysfunction of endothelial nitric oxide synthase and atherosclerosis. *Arterioscler Thromb Vasc Biol.* 2004;24:998-1005.

ATVB/2007/142182 Downloaded from http://submit-atvb.ahajournals.org on March 4, 2007

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 μM 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 μm 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.)

ATVB/2007/142182 Downloaded from http://submit-atvb.ahajournals.org on March 4, 2007

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 μm. **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.)

ATVB/2007/142182 Downloaded from http://submit-atvb.ahajournals.org on March 4, 2007

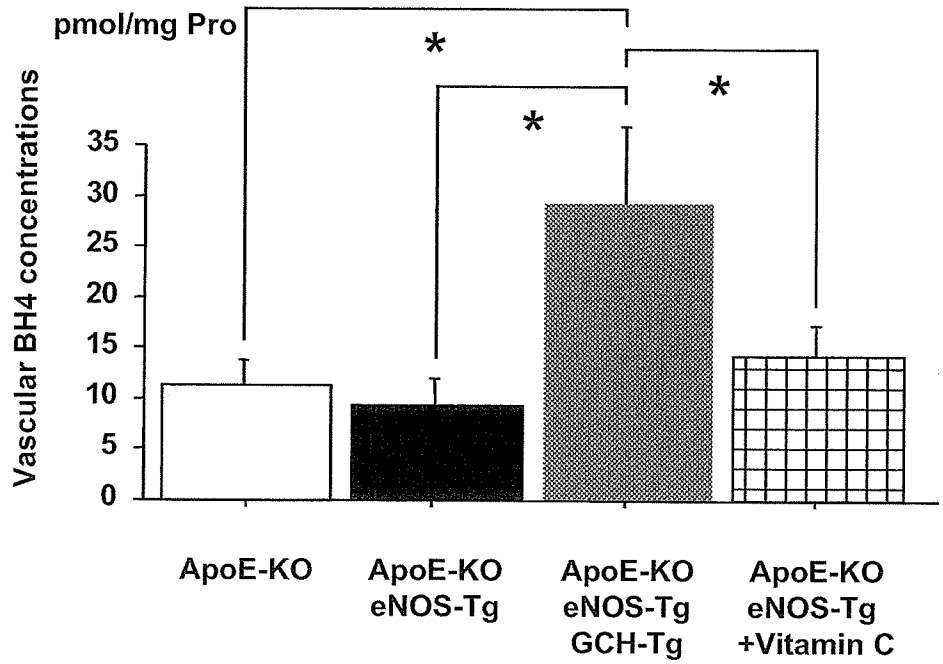
Table 1 Body weight, Plasma lipid contents, Vitamin C levels and Blood pressure

	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).

ATVB/2007/142182 Downloaded from http://submit-atvb.ahajournals.org on March 4, 2007

1A



1B

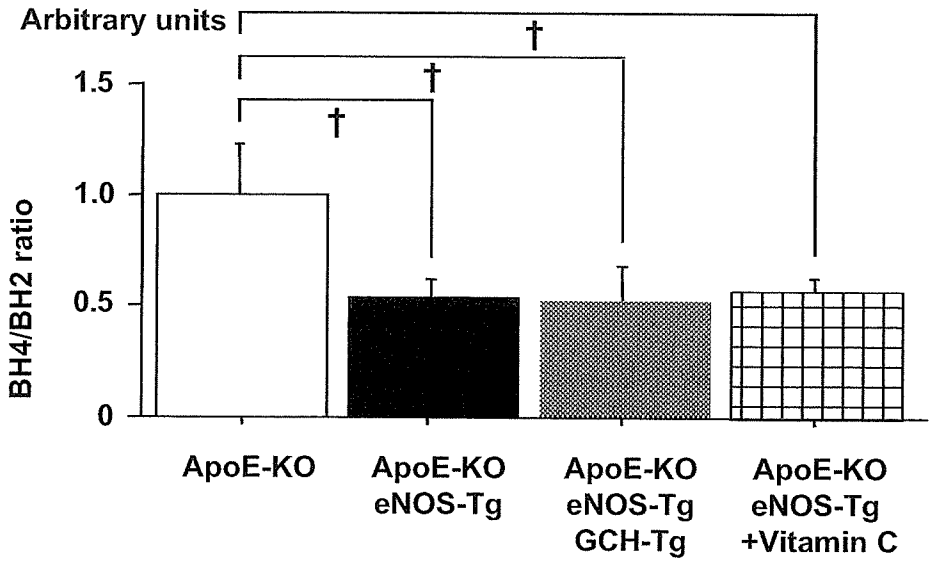
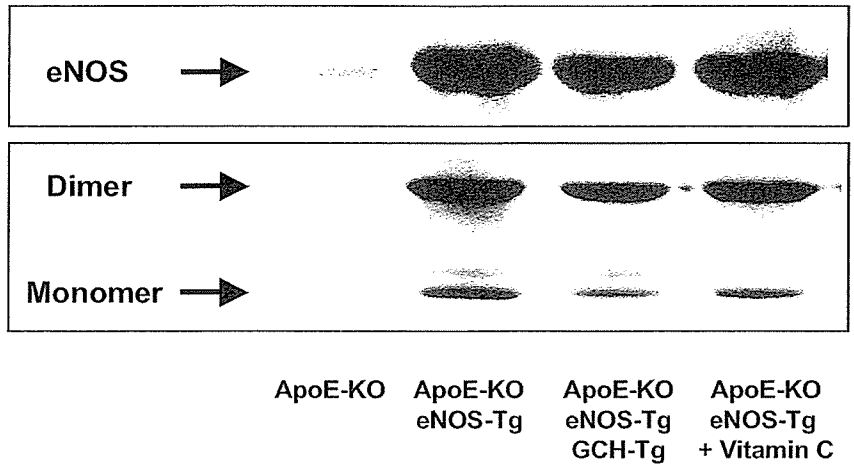


Figure 1

ATVB/2007/142182 Downloaded from http://submit-atvb.ahajournals.org on March 4, 2007

2A



2B

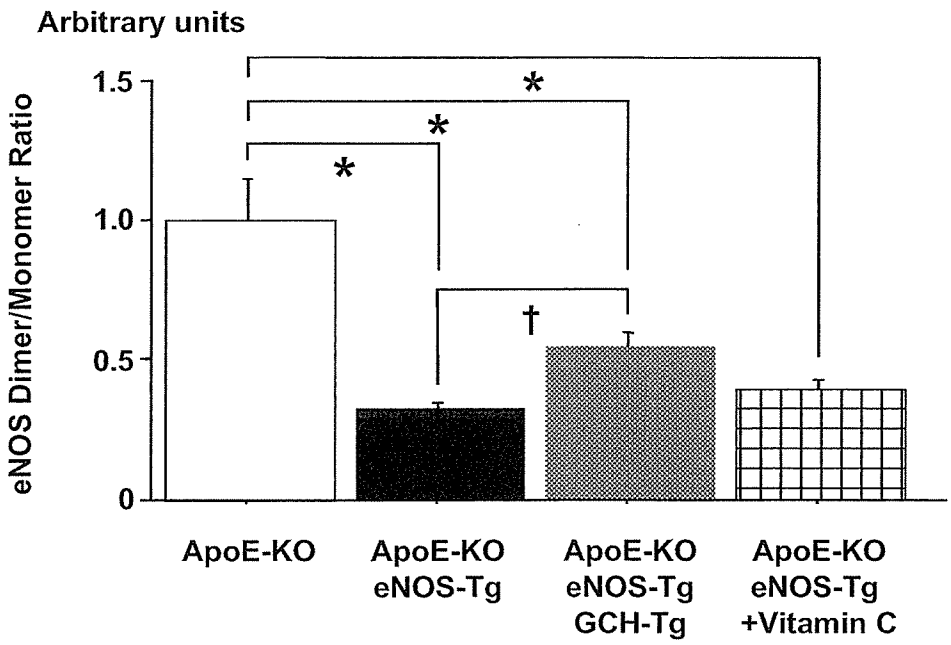


Figure 2

ATVB/2007/142182 Downloaded from http://submit-atvb.ahajournals.org on March 4, 2007

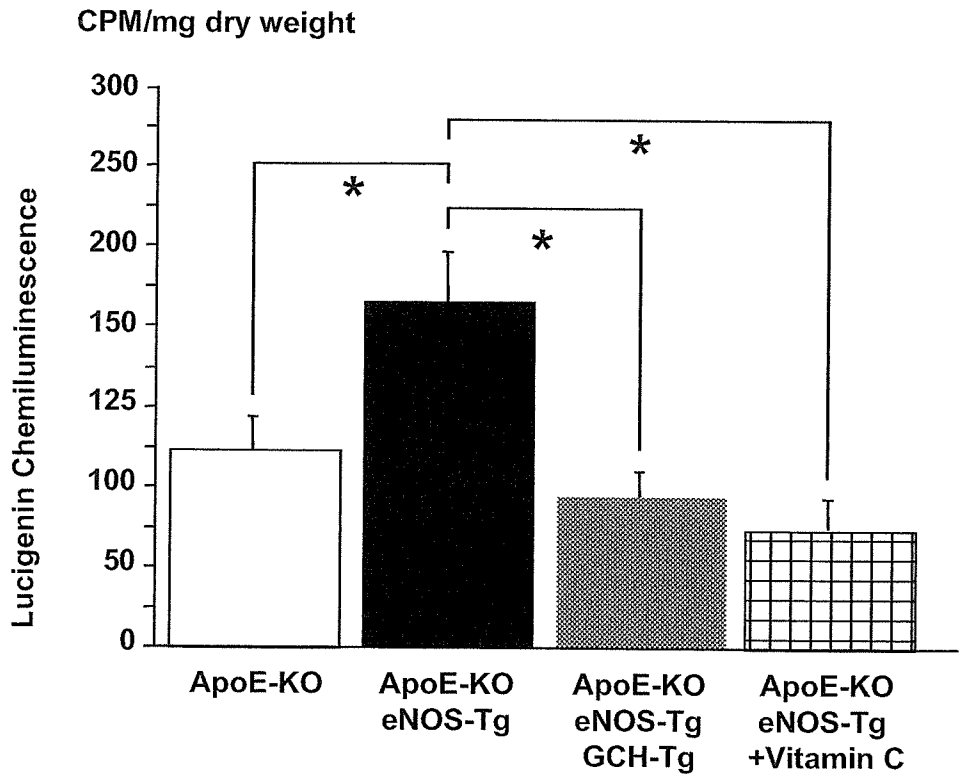
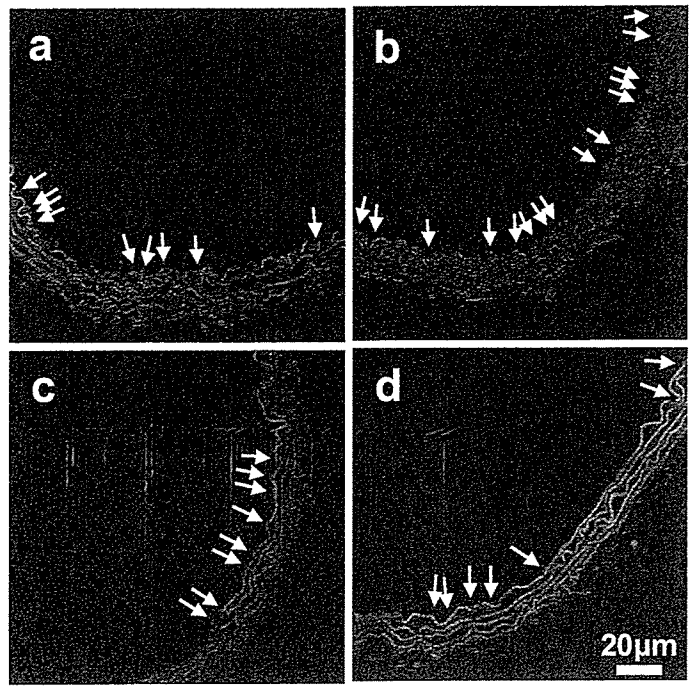


Figure 3

ATVB/2007/142182 Downloaded from http://submit-atvb.ahajournals.org on March 4, 2007

4A



4B

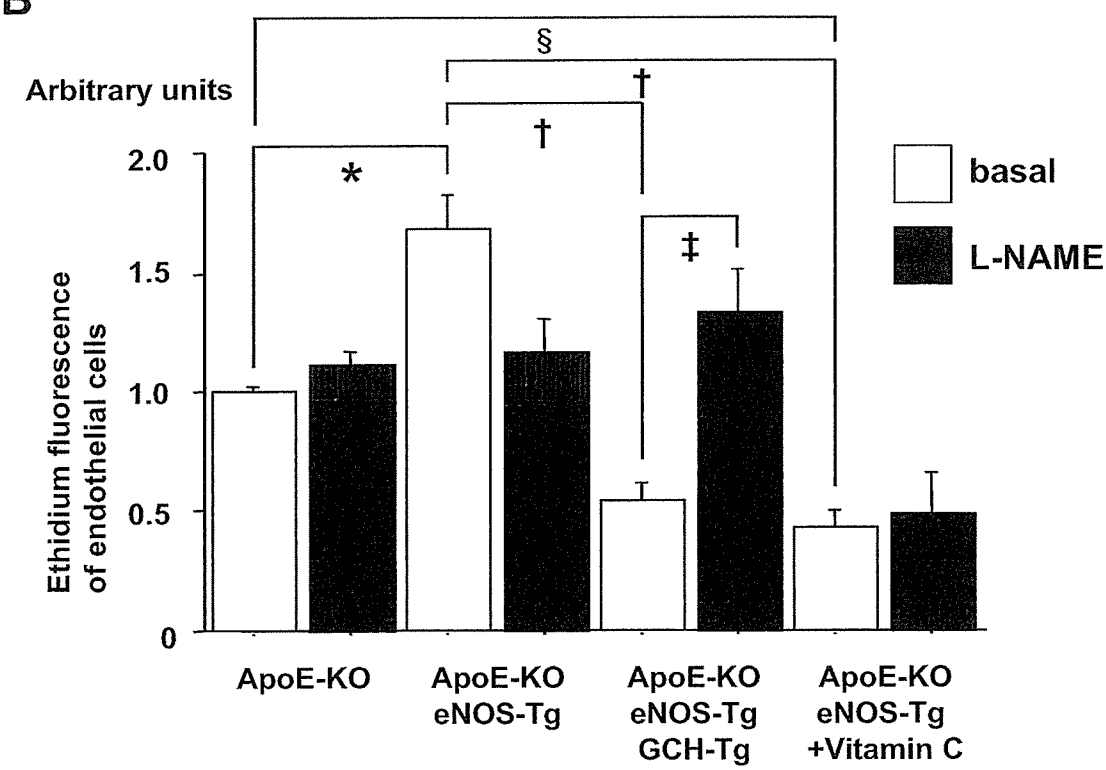
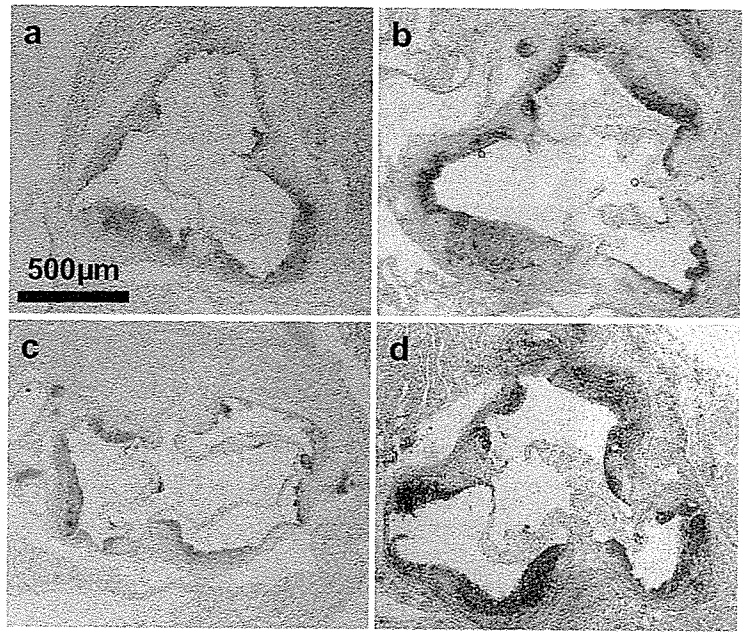


Figure 4

ATVB/2007/142182 Downloaded from http://submit-atvb.ahajournals.org on March 4, 2007

5A



5B

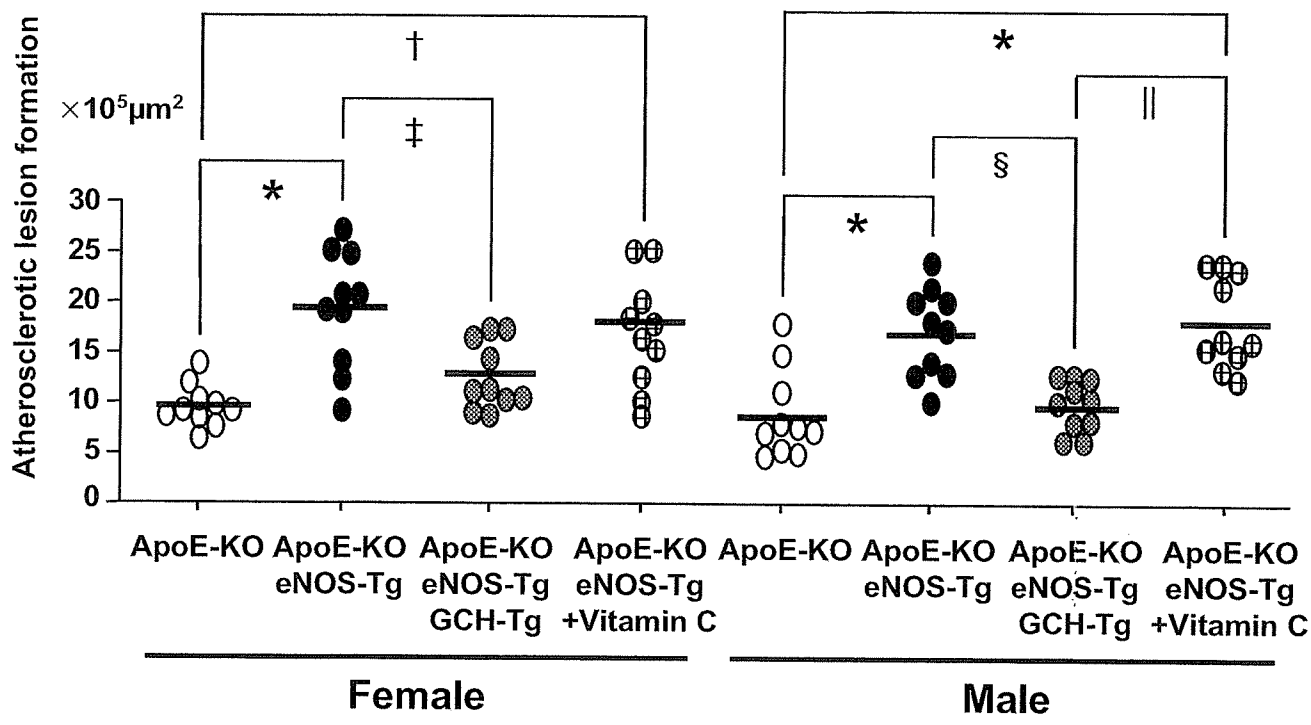


Figure 5

ATVB/2007/142182 Downloaded from http://submit-atvb.ahajournals.org on March 4, 2007